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UNIVERSITY OF CALIFORNIA

SANTA CRUZ

Investigations of Halogenated Constituents Isolated from Marine Sponges Associated with Cyanobacterial Symbionts

A dissertation submitted in partial satisfaction
of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

CHEMISTRY

by

William David Clark

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The Dissertation of William David Clark
is approved:

Professor Phillip Crews, Chair

Professor W. Todd Wipke

Professor Bakthan Singaram

Dean of Graduate Studies

Abstract

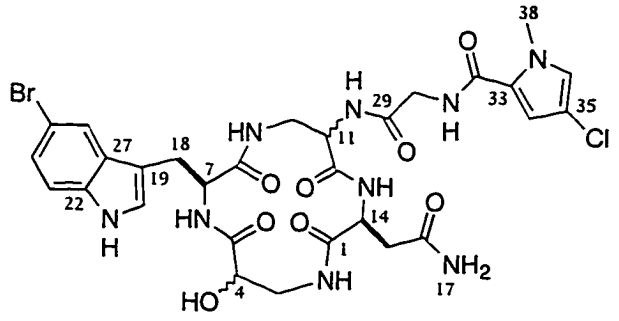
The work of this dissertation is the culmination of five years of research in the field of marine natural products chemistry. It involves the investigation of halogenated constituents isolated from Indo-Pacific marine sponges associated with cyanobacterial symbionts. The primary goal in the UCSC research group is to seek out novel and interesting secondary metabolites produced by marine organisms, specifically marine sponges, isolate and elucidate their chemical structures and determine their biological activity against cancer.

An unusual cytotoxic halogenated hexapeptide, cyclocinamide A, was isolated as a minor constituent from the marine sponge *Psammocinia* (Thorectidae: Dictyoceratida) collected in Papua New Guinea. The structure elucidation of cyclocinamide A was carried out using data derived from a variety of one-dimensional and two-dimensional ^{1}H and ^{13}C NMR techniques. Cyclocinamide A exhibited potent *in vitro* selective activity against Colon-38 tumor cells.

During the evaluation of >60 collections of the Indo-Pacific marine sponge *Dysidea* (Dysideidae: Dictyoceratida) seven new halogenated compounds were characterized. Three new chlorinated peptides continue a theme represented by the known polychlorinated tetrapeptides dysidenin and isodysidenin. One collection yielded the diastereomeric metabolites dysidenins C and D. The absolute stereochemistry of dysidenin C was determined by X-ray crystallography as 2*S*, 5*R*, 7*S*, 13*S*. Another specimen yielded the tetrachloro peptide 9, 11-didechlorodysidenin. Brominated dioxins, tetrabromodioxin and tribromodioxin, were identified from another specimen. They represent the first ever naturally-occurring dioxins isolated from *Dysidea*. Two ketide

amino acids introduce a new subclass of chlorinated peptides represented by herbamides A and B.

A chemical and morphological analysis was performed on forty-three specimens of the Indo-Pacific sponge *Dysidea*. To assist in the characterization of the species a polybrominated diphenyl ether or polychlorinated peptide profile was identified by using electrospray ionization mass and ^{1}H-NMR spectra of the dichloromethane fractions of the specimens. Taxonomic analysis revealed five distinct *Dysidea herbacea*-like morphological types; all carrying endosymbiotic cyanobacteria (*Oscillatoria*-like) and each showing direct correlation with their chemical profiles. The degree of bromination or chlorination of the polybrominated diphenyl ethers or the polychlorinated peptides, respectively, directly correlated with the morphological grouping.



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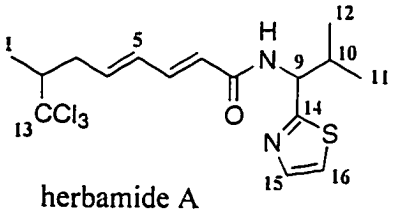

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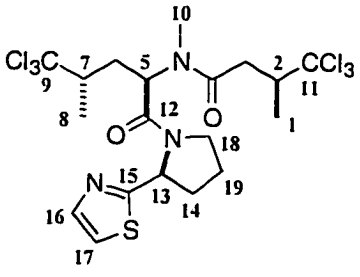

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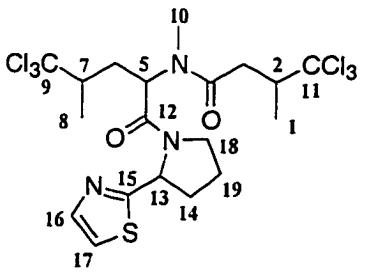

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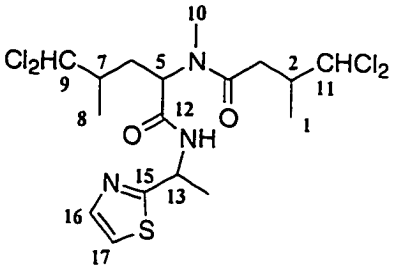

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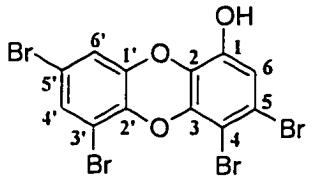

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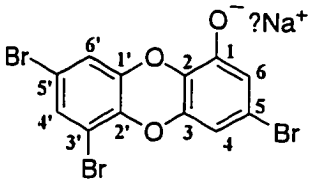

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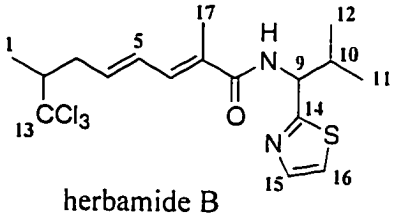

herbamide B

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To my loving wife Penny and daughter Christine

Acknowledgments

During the five years of my graduate work many people have been instrumental in my success. First and foremost I would like to thank my wife Penny who has always been there for me and is to this day my strongest supporter. She has made many sacrifices that go beyond description. She is a loving mother to our daughter Christine and a very special woman. I love her dearly. Without her support, none of this would have been possible. At the beginning of my second year we were blessed with a beautiful little girl, Christine, who is now 3 1/2 years old. She has been a wonderful inspiration during these times. Whenever I was frustrated with my research or things were not going well, she was always there to remind me not to take it so seriously, enjoy myself and have fun. For these reasons I dedicate this dissertation to the two people that are closest to my heart, Penny and Christine.

I would like to thank all of my family, Penny's family, specifically Gene, Sue, Colleen and Holly, and our numerous friends who over the past few years have lent incredible moral as well as financial support to both Penny and me. I would especially like to thank my Mom and Dad for their tremendous loving and moral support over my entire life. They have watched me go through many trying times and understand better than anyone what an incredible achievement this truely is for me.

Since I have been a part of the UCSC marine natural products research group I have encountered many individuals at the University who have contributed to my education in one way or another. I have enjoyed the numerous interactions I have had with the faculty of this Chemistry Department. It is an unique environment where everyone is approachable and willing to answer your questions. I would especially like to thank Professor Phillip Crews, my research advisor, whose enthusiasm for organic

chemistry lead me to a career in this field rather than marine biology. I would like to thank Phil for his educational and financial support over the last five years. Phil has been the primary catalyst in obtaining the necessary equipment, facilities and instrumentation to perform my research successfully as well as for others in the Chemistry Department in general. He is a valued member of the faculty and should be commended for his efforts.

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I would also like to thank all of the post doctoral people I have had the pleasure to work with over the years; Barb Peters, Jim Todd, Khisal Alvi, Jaime Rodriguez, Marcel Jaspars, Yang Sheng Wanggui and Leanne Murray. Their experience has been very valuable in contributing to my education. I have especially enjoyed those Monday morning discussions with Leanne over coffee about the trials and tribulations of our favorite people; Sheridan, Delen, G'Kar, Londo, Siskel, Daz, Worf, Janeway, Tuvok, Chakotay and so on.

I have enjoyed working with many of my fellow colleagues in the research group who are on the same quest to obtain those three letters or have already achieved this goal and moved on. One memorable event during this time was the wig and erotic dessert party hosted by Blaine Harrison. Due to this encounter I don't think I can hear the song "Mustang Sally" in quite the same way anymore. Also quite enjoyable is to have Blaine

explain the merits of the "worthless UCSC shuttle system". Another source of constant amusement has been to watch Blaine and Sam Sperry, the surfing duo, try to rile our colleagues into a frenzy. This begins with a hovering motion around the individual; their unsuspecting prey unaware of the assault about to be delivered upon them until it is too late. This leads to eyeballs bulging, tempers flaring or flushed cheeks and somebody leaving the room in haste. They obviously derive some sort of sadistic pleasure from this action. My thanks also go to Leif Abrell, who derives sadistic pleasure as well, for my indoctrination into the world of "music that I will never buy in a million years" club. There has always been a clear choice in listening pleasure after this experience. On a final note, I have enjoyed these graduate years and will relish them for a long time. It has been difficult on many occasions, but there is no substitute for hard work and perseverance. By proceeding in this manner you can acheive any goal you desire. In the words of Winston Churchill "Never give up".

List of Abbreviations

Ac	acetate
APT	attached proton test
BPO	bromoperoxidase
BuOH	butanol
C38	colon-38 mouse tumor cell
calcd.	calculated
CPO	chloroperoxidase
COSY	correlation spectroscopy
d	doulblet
dd	double doublet
DEPT	distortionless enhancement by polarization transfer
DMF	dimethylformamide
DMSO	dimethylsulfoxide
EP	eluting power
ESIMS	electrospray ionization mass spectroscopy
EtOH	ethanol
HMBC	heteronuclear multiple bond correlation
HMQC	heteronuclear multiple quantum coherence
HPLC	high performance liquid chromatography
HREIMS	high resolution electron impact mass spectroscopy
HRFABMS	high resolution fast atom bombardment mass spectroscopy
IC_{50}	50% inhibition concentration
IMPDH	ionsine monophosphate
IPO	iodoperoxidase
IR	infrared
L1210	leukemia tumor cell line
LC_{100}	lethal concentration at 100 %
LRFABMS	low resolution fast atom bombardment mass spectroscopy
m	multiplet
MeOH	methanol
MHz	megahertz
mL	milliliter

mmu	millimass units
MeCN	acetonitrile
NCI	National Cancer Institute
ng	nanogram
nOe	nuclear Overhauser effect
NMR	nuclear magnetic resonance
PNG	Papua New Guinea
ppm	parts per million
SS	solvent strength
s	singlet
t	triplet
THF	tetrahydrofuran
TLC	thin-layer chromatography
UV	ultraviolet
µg	microgram

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Chapter 1-Introduction

Marine Sponge-Papua New Guinea Photo Courtesy of Jay Burreson, Ph.D.

1. Background

The work of this dissertation is the culmination of five years of research in the field of marine natural products chemistry. The primary goal in the UCSC marine natural products research group is to seek out novel and interesting secondary metabolites produced by marine organisms, specifically marine sponges and fungi, isolate and elucidate their chemical structures and determine their biological activity against cancer. This is achieved in a bioassay guided isolation scheme whereby the biological activity of constituent(s) is tracked by determining their toxicity to brine shrimp and activity in mechanism-based and cell-based assays.

As suggested by the title, the focus was on the *Investigations of Halogenated Constituents Isolated from Marine Sponges (Porifera: Demospongiae: Dictyoceratida) Associated with Cyanobacterial Symbionts*. Before a detailed discussion of the work can begin, the background must be established to visualize its placement in the field. I will begin with a discussion about the presence of halogens in the marine environment followed by a discussion of the process of biohalogenation and the biosynthesis of halogenated metabolites of marine organisms in general. The marine environment is replete with microorganisms that play a role in their association with marine sponges. It is currently believed that many secondary metabolites isolated from marine sponges are in fact of microbial origin. Therefore, I will discuss the presence of these microorganisms, specifically cyanobacteria, in the marine environment and their symbiotic relationship with sponges. Next, I will look at the chemical history of halogenated constituents isolated over the years from marine sponges of the order Dictyoceratida. Finally, the present studies will be introduced and their placement in the field established to complete the picture.

2. Halides in the Marine Environment

Our earth is abundant with the naturally occurring halides; chloride, bromide, iodide and fluoride. Consequently, large numbers of naturally occurring organohalogen compounds are found in nature. Virtually every type of organism from marine and terrestrial plants to bacteria and fungi to insects, marine animals and even mammals are capable of producing metabolites that contain halides.[1] This is even more prevalent in the marine environment because of the high concentrations of halides (Cl^-, Br^-, I^-) in the oceans available for incorporation into biosynthetic pathways (Table 1.1).

Table 1.1. Distribution of Halides in the Environment.[1]

Halide	Oceans (mg/L)	Sedimentary Rocks (mg/kg)	Fungi (mg/kg)	Wood Pulp (mg/kg)	Plants (mg/kg)
Cl^-	19,000	10-320	-	70-2100	200-10,000
Br^-	65	1.6-3	100	-	-
I^-	0.05	0.3	-	-	-
F^-	1.4	270-740	-	-	-

For an excellent and comprehensive survey of naturally occurring organohalogen compounds from *all* sources, one should consult Gribble's review (reference 1). The primary source for chloride, fluoride, and possibly bromide is through geothermal processes such as volcanic eruptions. For example, the eruption of El Chichon alone in 1982 produced 9% (0.04×10^6 tons) of the world's HCl! Bromide and iodide were identified in the algae *Asparagopsis armata, Falkenbergia doubleti* and *Bonnemaisonia asparagoides* as early as 1926, long before the characterization of any organohalogen compounds containing these halides was undertaken. Fluorine in the form of potassium fluorosilicate was found incorporated in the sponge *Halichondria moorei*. Therefore, it is

not surprising that marine organisms, over the eons, have adapted to the production of organohalogen compounds.

3. Biosynthesis of Halogenated Metabolites by Marine Organisms

Marine organisms have been reported to produce a wide variety of halogenated metabolites. This includes everything from simple halohydrocarbons such as bromoform, chloroform, dibromomethane and methyl iodide to more complex compounds like halogenated terpenes, steroids and peptides.[1,2] The main questions to ask are how and why this occurs?

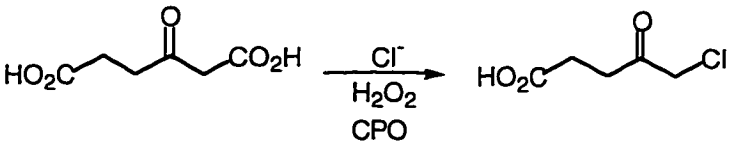
3.1. Biohalogenation

The process of biohalogenation answers the question as to how halogenated secondary metabolites are produced in marine organisms. This is accomplished by the haloperoxidases: chloroperoxidase (CPO), bromoperoxidase (BPO) and iodoperoxidase (IPO).[1,3] Haloperoxidases are enzymes that catalyze the oxidation of chloride, bromide or iodide with hydrogen peroxide resulting in the halogenation of an organic substrate (Scheme 1). As a general rule the peroxidase activity is based on the most electronegative halide that is able to be oxidized by H_2O_2 catalyzed by the enzyme. Therefore,

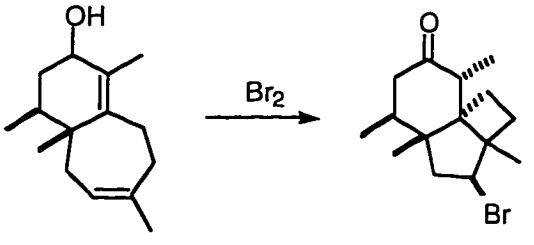
$$\text{Org-H} + X^- + H_2O_2 + H^+ \longrightarrow \text{Org-X} + 2H_2O$$

Scheme 1

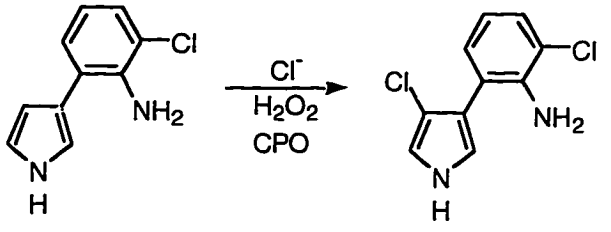
chloroperoxidase catalyzes the oxidation of chloride, bromide and iodide; bromoperoxidase catalyzes the oxidation of bromide and iodide and iodoperoxidase catalyzes only iodide. This is shown more specifically in Schemes 2-6 for some functionalities found in the marine environment.[1,4-8]



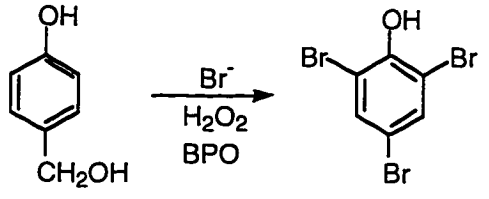
Scheme 2[4]



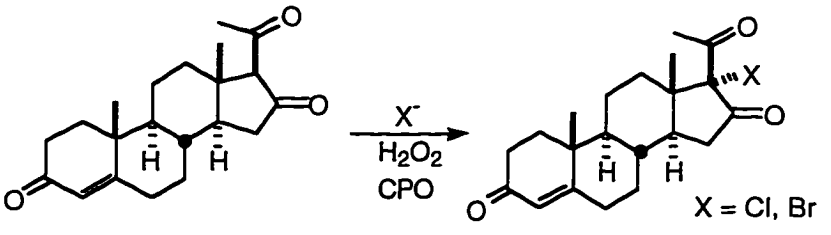
Scheme 3[5]



Scheme 4[6]



Scheme 5[7]



Scheme 6[8]

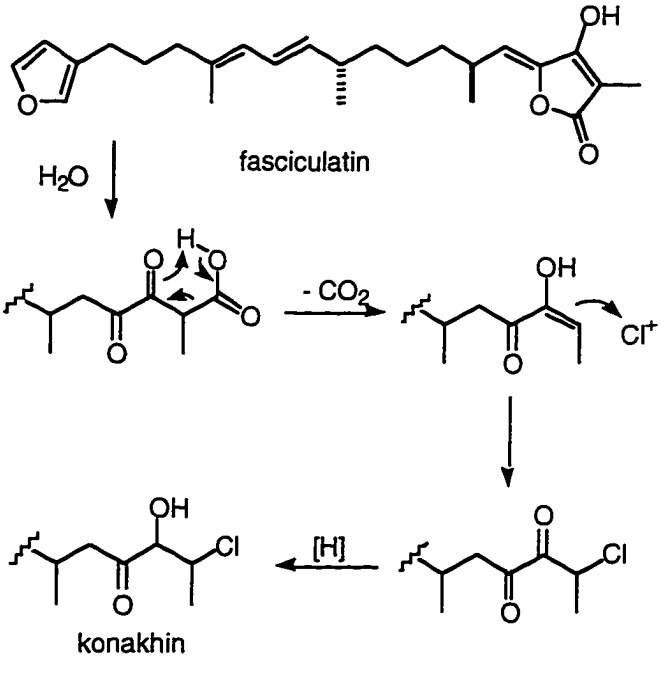
Haloperoxidases have been identified in just about every class of marine organism and the activity of bromoperoxidase and iodoperoxidase in marine algae has been well-documented (references within).[1,3] As for chloroperoxidase, little is known about its activity in marine organisms. It has primarily been studied in bacteria and fungi. Recently, a CPO was isolated from a marine worm[9] and a vanadium bromoperoxidase and iron-heme bromoperoxidase were shown to exhibit chloroperoxidase activity.[10,11] This raises an interesting question. How are the plethora of chlorinated metabolites being produced in marine organisms and specifically in sponges? It is possible that marine bacteria and fungi are the culprits due to the abundance of these organisms found on sponges, but more studies are needed to answer the question definitively.

3.2. Biosynthesis of Halogenated Compounds

The next question to ask is why do marine organisms produce secondary metabolites of such great diversity at all? They appear to serve no biological function in the organism. The most popular belief is that marine organisms produce secondary metabolites as a mechanism of defense to ward off predators. This is especially true for sessile marine invertebrates such as sponges which have no other means of defense against predation.[12] In more general terms it has been postulated that natural products are produced in response to stimuli which maintains the organism's survival fitness.[13]

Additionally, natural products are normally complex structures that require great amounts of metabolic energy in their construction and would have been weeded out through natural selection had they not served a purpose.[14] Consequently, secondary metabolites from marine organisms, many of them halogenated, are of pharmaceutical interest due to their widely ranging biological activities which include antifungal, antibacterial, antineoplastic, antiinflammatory, antiparasitic and antiviral.[2,15,16]

The chemistry of marine sponges of the Dictyoceratida order consists mainly of sesqui- and sesterterpenes and has been thoroughly examined for many years.[2,16,17] The biosynthesis of terpenes has also been well studied.[18] However, studies are limited when it comes to halogenated metabolites of this order. Biomimetic synthesis studies have been carried out where the bromination of terpenes from algae was examined.[19,20,21] Recently, a biogenetic route was proposed for the synthesis of konakhin, a chlorinated degraded sesterterpene, from fasciculatin (Scheme 7).[22]



Scheme 7

It was envisioned that decarboxylation of fasciculatin, in the carboxylic form, would give the enol which in turn would undergo haloperoxidase catalyzed chlorination to give the diketone. Finally, reduction of the ketone would give konakhin.

Another example of a biosynthetic study of a halognated marine natural product from Dictyoceratida sponges was of the chlorinated peptide 13-demethylisodysidenin from *Dysidea herbacea*.[23] In this preliminary study they measured incorporation of [1-^{14}C]-leucine or [U-^{14}C]-valine in intact sponges maintained in aquaria. However, the results were inconclusive and further studies are underway. Garson proposed that 13-demethylisodysidenin was a tetrapeptide with the following possible biosynthesis (Figure 1). The full biosynthetic pathway is still unknown and the issue of biohalogenation of these compounds still remains to be addressed.

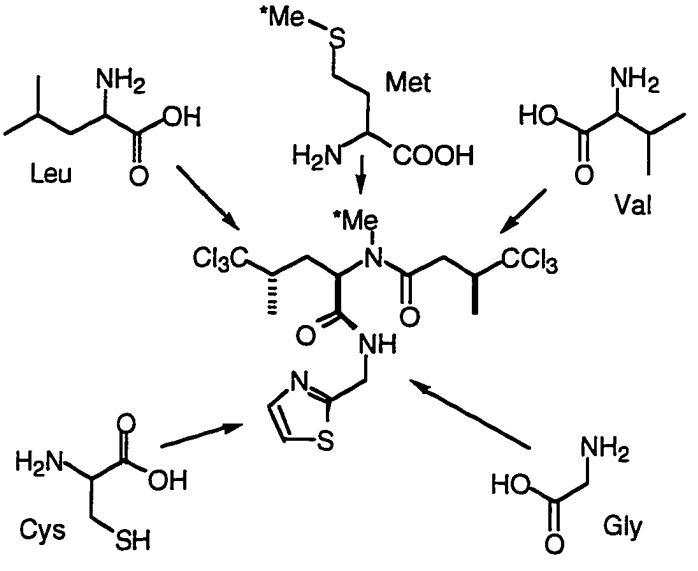


Figure 1

4. Symbiotic Cyanobacteria in the Marine Environment

Cyanobacteria (blue-green algae) are photosynthetic microorganisms that have a long history on Earth. Their fossils have been identified in sediments from the Pre-Cambrian period, nearly three billion years old and were probably the first organisms to release oxygen into the early atmosphere.[24,25] They are considered prokaryotic organisms because they lack a true nucleus and are the most likely evolutionary link between bacteria and plants. Their morphology ranges from unicellular to filamentous and they are found in a variety of habitats as well as in symbiotic relationship with many organisms (Table 1.2).[26]

Table 1.2. Classification of Symbiotic Cyanobacteria.

Section	Characteristics	Symbiotic cyanobacterial genera	Symbiosis
I	Unicellular	*Aphanocapsa*	Marine sponges
		Gloeocapsa, Gloeothece, Synechocystis	Lichens
		Aphanocapsa/Gloeocapsa	Hair of polar bears
		A thin-walled unicellular cyanobacterium	Diatom *(Rhopalodia)*
		Cyanelles	Glaucophytes and *Paulinella*
		Hyella	Lichen
		Pleurocapsa minor	Bacteria
II	Filamentous	*Phormidium, Oscillatoria*	Marine sponges
		Anabaena	*Azolla*
		Nostoc	Lichens, bryophytes, cycads and *Gunnera*
		Scytonema, Calothrix	Lichens
		Richelia intracellularis	Diatom *(Rhizosolenia*
		Fischerella	Lichens

Symbiotic cyanobacteria have been found in association with 38 genera of marine sponges belonging to the Calcarea and Demospongiae classes and live, intracellularly and

intercellularly, throughout the sponge tissue or on its surface.[27,28] The role of cyanobacteria in symbioses is not completely understood and is the subject of current research. However, it has been speculated that they serve three functions. First, they may protect the host sponge from high light intensity.[29] Secondly, they are photosynthetically active and transfer 5 to 12% of the fixed carbon, probably as glycerol, to the host sponge and finally, in two sponges, *Theonella swinhoei* and *Siphonochalina tabernacula*, they have exhibited the ability to fix N_2.[30,31] In another study further support for the metabolic integration between a symbiotic unicellular cyanobacterium *Aphanocapsa* and the marine sponges *Chondrilla nucula* and *Petrosia ficiformis* was examined. They measured the amount of primary metabolites (sugars, proteins, etc.) produced in *C. nucula*, containing cyanobacteria, grown in dark caves.[32] The lack of sunlight saw the depletion of these metabolites.

5. The Sponge/Symbiont Association

Symbiotic cyanobacteria in marine sponges are known to exist in two morphological forms, unicellular and filamentous, and are characterized as the *Aphanocapsa* and *Phormidium* or *Oscillatoria* genera respectively. The unicellular cyanobacterium *Aphanocapsa* has been the subject of much biological study since the late 1970's and into the 80's.[33,34,35] This is probably due to its abundant nature in many genera of sponges. On the other hand, the biological study of filamentous cyanobacteria in marine sponges has been more limited. One classic example is the biological examination of the symbiotic association between the filamentous cyanobacteria *Oscillatoria spongeliae* and the Dictyoceratida sponge *Dysidea herbacea*. It was found to occupy 30-40% of the sponge cell volume and was recently isolated from the sponge tissue in order

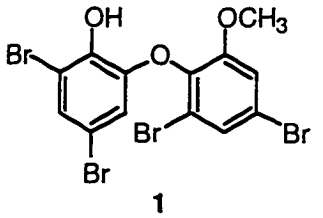
to better understand its symbiotic relationship with *Dysidea*.[36,37] Upon closer examination it was found to be of the genus *Oscillatoria* instead of *Phormidium* due to its morphological differences.[36] The chemotaxonomy of symbiotic cyanobacteria is still poorly understood and is the subject of current biological study.[38,39] I will now examine the chemistry of some sponges associated with these cyanobacterial symbionts as well as sponges associated with non-photosynthetic bacteria for comparison.

5.1. Chemistry of Bacterial Symbionts Isolated from Marine Sponges

Before I examine the chemistry of sponges containing photosynthetic cyanobacteria it is important to understand that some of the metabolites isolated from marine sponges could possibly be due to non-photosynthetic bacteria that are also abundant on the host sponge.[40,41] Therefore, I will look at the few examples that exist of Dictyoceratida sponges whereby bacterial symbionts were separated from the sponge host and their chemistry examined.

5.1.1. Brominated Diphenyl Ether from a Vibrio sp.

The source of the chemistry of the marine sponge *Dysidea herbacea* has been the subject of debate and speculation for many years. Three different classes of secondary metabolites have been isolated from the sponge; brominated diphenyl ethers, chlorinated peptides and furanosesquiterpenes. Each class is distinctly different raising the question as to the source of the components. In 1991, Elyakov *et al* reported a tetrabrominated diphenyl ether (**1**) by gas chromatography-mass spectrometry (GC-MS) in the broth extracts of a cultured bacteria from the sponge.[42,43] The bacteria was identified as a



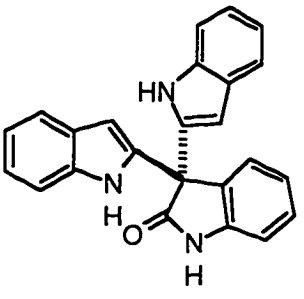
1

Vibrio sp, fairly common in the marine environment. There is debate over the validity of this work for a couple of reasons. First, the yield obtained was extremely small by comparison to extracts from the sponge host itself. Bromophenols are normally found in quantities of 2-10% by dry weight of the sponge.[44]

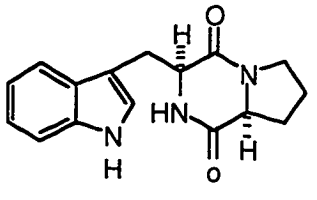
More recently, it was shown by Faulkner that the brominated diphenyl ethers were concentrated in the filamentous cyanobacterium *Oscillatoria spongeliae*.[45] The cyanobacterial cells were separated from the sponge cells by flow cytometry and analyzed for their chemical content. The brominated diphenyl ethers were isolated from the cyanobacterial cells and not the sponge cells suggesting that *Oscillatoria* is responsible for their production. This data is in obvious conflict with the previous results which suggested a non-photosynthetic bacteria was responsible for the production of the bromophenols. One issue not addressed by either researcher is the fact that bacteria are known to live on the surface of cyanobacteria in symbiotic relationship as well.[28,46] It is possible in this case that a *Vibrio* sp. and *Oscillatoria spongeliae* are in fact the symbiotic pair to be considered responsible for bromophenol production. This theory would still satisfy the results of both studies. In the first study separation of the bacteria *Vibrio* sp. from its cyanobacterial host may have been the reason for the low production levels and the process of flow cytometry would not take into account that non-photosynthetic bacteria were living on *Oscillatoria*. This theory is feasible in light of the data available, but would require more study to either prove or disprove it.

5.1.2. Indole Trimer from a Vibrio sp.

The Okinawan marine sponge *Hyrtios altum* has been the source of several extremely cytotoxic macrolides named altohyrtins A, B, and C and 5-desacetylaltohyrtin A (discussed later). These compounds were isolated in such small yield that it prompted the researchers to search for a microorganism as the potential source of the metabolites. Additionally, several other compounds from a *Cinachyra* sp. and a *Spongia* sp. had similar macrolide structures. During the culture of a bacterium, identified as a *Vibrio* sp., from the marine sponge *Hyrtios altum* the new antibiotic trisindoline (**2a**) was isolated along with the known diketopiperazine brevianamide F (**2b**).[47]



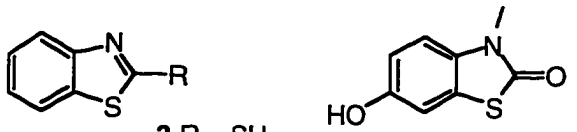
2a



2b

5.1.3. Benzothiazoles from a Micrococcus sp.

The marine bacterium *Micrococcus* sp. has consistently been isolated from the sponge *Tedania ignis* from the Caribbean. After examination of fermentation culture extracts, four benzothiazoles (**3-6**) were isolated.[48] This is the first time these compounds have been reported from the marine environment. Benzothiazoles are rarely seen as natural products. There was no reported biological activity.



3 R = SH
4 R = CH_3
5 R = OH

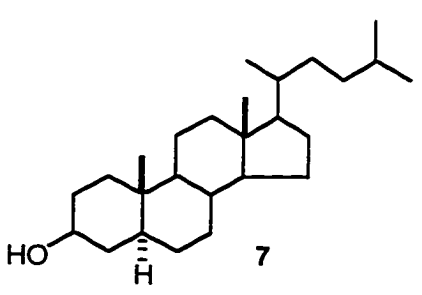
6

5.2. Chemistry of Cyanobacterial Symbionts Isolated from Marine Sponges

As mentioned earlier, the chemistry of the marine sponge *Dysidea herbacea* has been explored extensively and is the only example of a Dictyoceratida sponge containing cyanobacterial symbionts whose chemistry has been evaluated. The study determining the source of the brominated diphenyl ethers has already been discussed. Two other studies have been conducted in relationship to identifying the source of the secondary metabolites of this sponge.

5.2.1. Sterols from Dysidea herbacea and Terpios zeteki

In 1979, Djerassi *et al* reported the isolation of steroidal mixtures of the Australian sponge *Dysidea herbacea* and the Hawaiian sponge *Terpios zeteki*.[49] Two new steroids (**7**) and (**8**) were isolated from the sponges. The presence of these new steroids along with the other known steriods isolated suggested that they were typical of the type isolated from cyanobacteria. Steroid (**8**) comprised ~75% of the sterol mixture of *Dysidea herbacea*. It is interesting to note that the symbiont is known to occupy 40% of the sponge volume in *Dysidea*. This may explain the high percentage of (**8**) in the sterol mixture.



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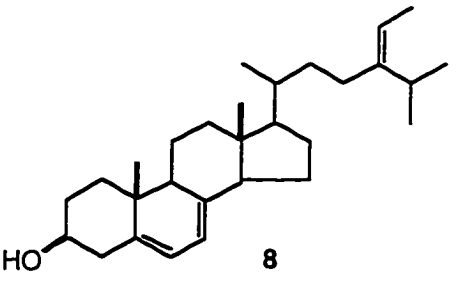

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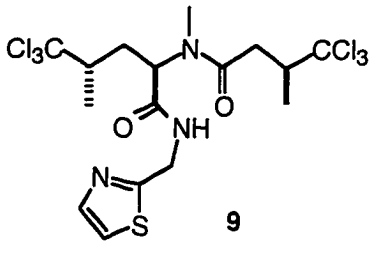
5.2.2. Halogenated Metabolites from Dysidea herbacea

As discussed before the brominated diphenyl ether (**10**) was isolated from the cyanobacterial cells of *Dysidea herbacea* by Faulkner. In an similar attempt to determine the source of the chlorinated peptides isolated from *D. herbacea* a study was undertaken where the cyanobacterium *Oscillatoria spongeliae* was separated from the sponge cells by flow cytometry.[50,51] During this study the chlorinated peptide 13-demethylisodysidenin (**9**) was isolated from the cyanobacterial cells and the sponge cells were found to produce only furanosesquiterpenes as was speculated (e.g. the known (+)-(4a*S*, 6*R*, 8a*S*)-6-hydroxyfurodysinin isolated during my dissertation work).



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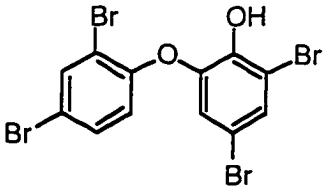

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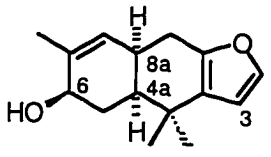


(+)-(4a*S*, 6*R*, 8a*S*)-6-hydroxyfurodysinin

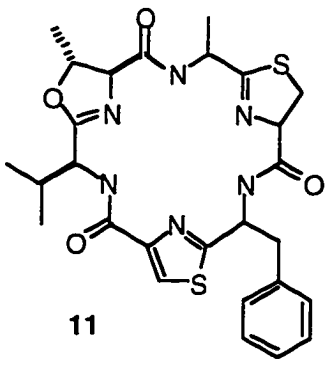
It should be noted that the brominated diphenyl ethers and the chlorinated peptides have never been isolated from the same sponge host. One possible explanation is that there are two chemotypes of *Oscillatoria*. It is known that only one algae endosymbiont occupies the sponge at a time.[36] Therefore, more studies will be needed in order to determine the nature of the symbionts.

5.3. Further Examples for Speculation

Although the symbiotic relationship between *D. herbacea* and *O. spongeliae* has been well explored, there are other examples which suggest symbiotic cyanobacteria are the sources of secondary metabolites in marine organisms. Here are two more examples from *D. herbacea* and two from marine tunicates.

5.3.1. Thiazole and Oxazole Peptides from Terrestrial Cyanobacteria and Marine Tunicates

Marine tunicates of the genus *Lissoclinum* are prolific sources of cytotoxic cyclic peptides.[2] These peptides are unusual and characterized by amino acids which contain thiazole and oxazole functionalities. There is circumstantial evidence that suggests these secondary metabolites are being produced by symbiotic *Prochloron* spp., prokaryotic algae that resemble cyanobacteria. Morphologically speaking they are very similar. Some recent studies identified cyclic peptides (**11**), (**12**) and (**13**) from marine tunicates of the genus *Lissoclinum*.[52,53,54] Interestingly, Moore *et al* isolated (**12**) from a terrestrial cyanobacteria identified as *Westiellopsis prolifica* in a mud sample on the island of Oahu, Hawaii. Similarly in 1995, cyclic peptide (**14**) was isolated from another terrestrial



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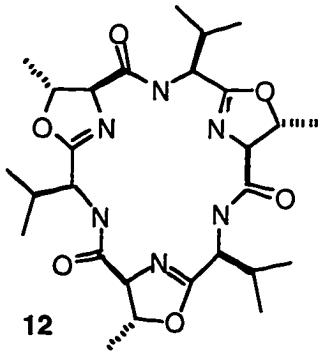

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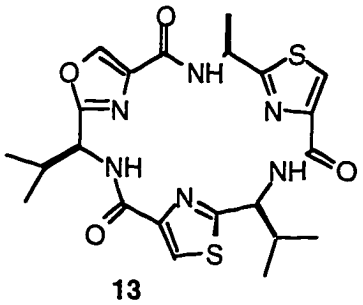

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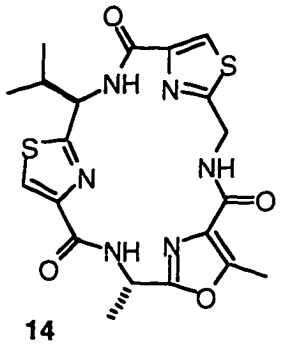

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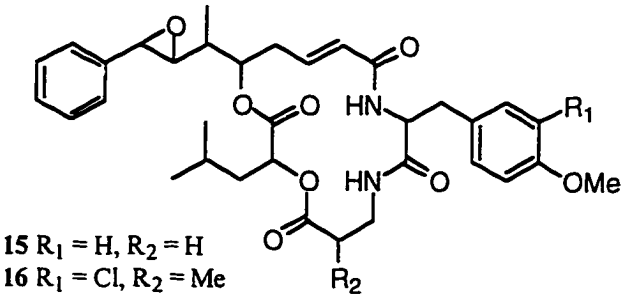
cyanophyte of the genus *Nostoc*.[56] It is feasible from the constituents isolated that algae endosymbionts are possible candidates for their production.

5.3.2. Depsipeptides from Terrestrial Cyanobacteria and the Marine Sponge Dysidea arenaria

In 1990, a Merck group isolated the first of a series of depsipeptides, later named the cryptophycins (**16**) by Moore at University of Hawaii, from a cultured terrestrial cyanobacterium of *Nostoc* sp.[57] Later in 1994, Kitagawa *et al* isolated the potent cytotoxic depsipeptide arenastatin A (**15**) from the Okinawan marine sponge *Dysidea arenaria*.[58] The only difference between the two compounds is that in cryptophycin the hydrogen at R_2 has been replaced with a methyl group and the *O*-methyltyrosine has

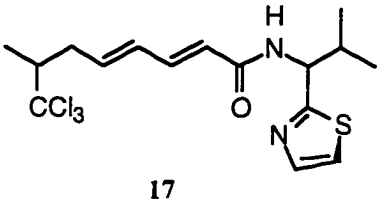
been chlorinated. Interestingly, there have been no reports of the species *arenaria* from *Dysidea* containing symbiotic cyanobacteria.



15 $R_1 = H, R_2 = H$
16 $R_1 = Cl, R_2 = Me$

5.3.3. Ketide Amino Acids from Free-Floating Cyanobacteria and the Marine Sponge Dysidea herbacea

In 1995, Clark and Crews reported on the isolation of a new class of chlorinated peptides from *Dysidea herbacea*, ketide amino acids represented by herbamide A (**17**).[59] The isolation and structure elucidation of (**17**) is the subject of Chapter 3 in this dissertation. The known chlorinated peptide dysidenin was also isolated from the same sponge host. This discovery introduced another subclass of metabolites that may be metabolites of *Oscillatoria spongeliae*. The following year Gerwick reported the isolation of a similar ketide amino acid, barbamide (**18**), from the free-floating Caribbean cyanobacterium *Lyngbya majuscula* (family Oscillatoriaceae).[60] Barbamide (**18**) exhibited



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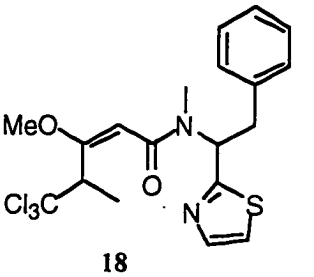

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molluscicidal activity with a $LC_{100} < 100$ μg/mL. Interestingly, herbamide A (**17**) was inactive in the same assay.[60] The amino acid portion of (**17**) can be envisioned to consist of a dipeptide from valine and cysteine and the dipeptide portion of (**18**) from phenylalanine and cysteine. The biogenesis of both of these molecules will be discussed in more detail in Chapter 3.

In conclusion, there is the possibility that symbiotic cyanobacteria play a role in the secondary metabolism of marine organisms, especially in the Dictyoceratida sponge *Dysidea*. Additionally, many of these constituents are halogenated and exhibit some sort of biological activity solidifying their desirability. I will now look at the chemical history of halogenated metabolites from Dictyoceratida sponges and their associated bioactivities in general.

6. Chemical History of Halogenated Metabolites from Marine Sponges of the Order Dictyoceratida

Marine sponges of the order Dictyoceratida are best known for their abundance of sesqui- and sesterterpenes. Approximately 60% of all known sesqui- and sesterterpenes from the Porifera come from this order (information based on literature suggests 20 orders).[16] Halogenated metabolites are also prolific in the Dictyoceratida order compared with the Porifera as a whole. The Dictyoceratida order is responsible for approximately one fifth of all known brominated and chlorinated compounds combined and one fourth of all known chlorinated compounds if considered alone.[16] Iodinated and fluorinated compounds have not been isolated from Dictyoceratida sponges and are rare to marine sponges in general. Table 1.3 below shows the phylogenetic family tree of the Dictyoceratida order.[16] Currently there is debate over the placement of the family Dysideidae in this order. It has been proposed to be in the Dendraceratida order.

Table 1.3. Phylogenetic Family Tree of the Marine Sponge Order Dictyoceratida.

Phylum	Class	Order	Family	Genus	Species
Porifera	Demospongiae	Dictyoceratida	Dysideidae	Dysidea	amblia
					arenaria
					avara
					camera
					chlorea
					cinerea
					etheria
					fragilis
					granulosa
					herbacea
					incrustans
					pallescens
					sp
					spinosula
					tupha
				Euryspongia	rosea
					sp
				Spongionella	gracilis
					sp
			Spongiidae	Carteriospongia	foliascens
					sp
				Hippospongia	cf.
					communis
					gossypina
					metachromia
					sp
				Hyatella	sp
				Lendenfeldia	frondosa
					sp
				Phyllospongia	dendyi
					foliascens
					papyracea
					sp
				Spongia	agaricina
					arabica
					fragilis
					hispida
					idia
					lacustris
					nitens
					oceania
					officinalis
					zimocca
					sp
			Thorectidae	Aplysinopsis	reticulata
					sp
				Cacospongia	cf.
					mollior
					scalaris
					sp
				Collospongia	auris
				Dactylospongia	elegans

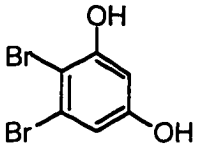
Phylum	Class	Order	Family	Genus	Species
Porifera	Demospongiae	Dictyoceratida	Thorectidae	Dactylospongia	sp
				Fasciospongia	cavernosa
					sp
				Fenestraspongia	sp
				Hyrtios	altum
					arenosa
					erecta
					erectus
					eubamma
					sp
				Ircinia	campana
					dendroides
					fasciculata
					felix
					foetida
					muscarum
					oros
					pipetta
					ramosa
					spinosula
					strobiliana
					variabilis
					wistarii
					sp
				Leiosella	idia
				Luffariella	geometrica
					variabilis
					sp
				Psammocinia	rugosa
					sp
				Sarcotragus	spinulosus
					sp
				Smenospongia	aurea
					echina
					sp
				Strepsichordaia	lendenfeldi
				Thorecta	choanoides
					horridus
					vasiforis
					sp
				Thorectandra	excavatus
			(no family)	Aphrocallistes	vastus
				Coscinoderma	sp
				Heteronema	erecta
				Polyfibrospongia	maynardii

6.1. Brominated Metabolites

There are a total of 44 brominated compounds from Dictyoceratida of which 27 are polybrominated diphenyl ethers from the sponge *Dysidea herbacea* in the Dysideidae family. The remaining brominated compounds are found throughout the Thorectidae and Spongiidae families. They consist of terpenes and indole derivatives.

6.1.1. Diphenyl Ethers from Dysidea herbacea

Over the years a series of brominated diphenyl ether metabolites (**19-45**) have been isolated from the marine sponges *Dysidea herbacea* and *Phyllospongia foliascens* from the Indo-Pacific (Table 1.4).[61-70] The number of bromine substituents varies from two up to six. Recently, the apparent precursors to these compounds, (**19**) and (**20**), were also isolated.[61] As already discussed these constituents appear to be associated with the cyanobacterial symbiont *Oscillatoria spongeliae* and occupy 2-10% by dry weight of the sponge.



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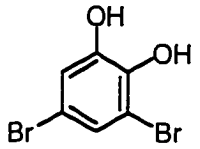

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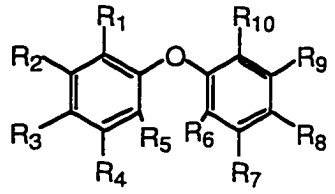
In one report it was suggested that some bromophenols were isolated from the marine sponge *Phyllospongia foliascens*, but this was probably incorrect due to the fact that both *Phyllospongia* and *Dysidea* are morphologically similar and can easily be misidentified.[64] I will address the chemotaxonomy problem of *Dysidea* in greater detail

Table 1.4. Known Brominated Diphenyl Ethers from the Sponge *Dysidea herbacea.*



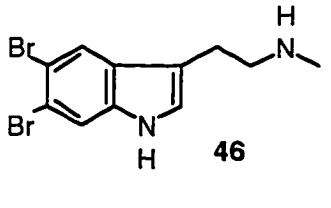
Cmp. #	R_1	R_2	R_3	R_4	R_5	R_6	R_7	R_8	R_9	R_{10}	Ref.
21	H	H	Br	H	H	Br	H	H	H	OH	62
22	H	H	Br	H	H	Br	H	Br	H	OH	62
23	OH	H	H	Br	H	H	Br	H	Br	OH	63
24	OH	H	Br	H	H	H	Br	H	Br	OMe	63
25	Br	H	Br	H	H	H	Br	H	Br	OH	64
26	Br	H	Br	H	H	Br	H	Br	H	OH	65
27	Br	H	Br	H	H	H	H	Br	Br	OH	66
28	Br	H	Br	H	H	Br	H	Br	H	OMe	67
29	OH	H	Br	H	Br	H	Br	H	Br	OH	63
30	OMe	H	Br	H	Br	H	Br	H	Br	OH	61
31	OH	H	Br	H	Br	H	Br	H	Br	OMe	68
32	Br	H	Br	H	H	Br	Cl	Br	H	OH	65
33	OMe	H	Br	H	Br	H	Br	H	Br	OMe	64
34	Br	H	H	H	H	Br	Br	Br	Br	OH	69
35	Br	H	Br	H	H	H	Br	Br	Br	OH	64
36	Br	H	Br	H	H	Br	Br	Br	H	OH	62
37	OH	H	Br	Br	Br	H	Br	H	Br	OH	61
38	OH	Br	Br	H	Br	H	Br	H	Br	OH	68
39	OH	H	Br	Br	Br	Br	H	Br	H	OH	61
40	OH	H	Br	Br	Br	H	Br	H	Br	OMe	61
41	OMe	Br	Br	H	Br	Br	H	Br	H	OH	63
42	OMe	Br	Br	H	Br	H	Br	H	Br	OH	68
43	OMe	H	Br	Br	Br	H	Br	H	Br	OMe	68
44	OH	Br	Br	Br	Br	H	H	Br	H	Br	70
45	OH	Br	Br	Br	Br	H	Br	H	Br	OH	68

in Chapter 5. In another report one bromophenol isolated was the only example which contained a chlorine substituent, a rarity for these constituents.[65] Interestingly, another bromophenol was isolated from a marine sponge collected on the littoral of Mozambique while all others came from the Indo-Pacific region.[70]

Early on it was discovered that these compounds were able to inhibit the growth of both Gram-negative and Gram-positive bacteria and consequently have been the subject of total syntheses.[71] In a collaboration between the UCSC group, Oklahoma group and a pharmaceutical group it was reported that many of these brominated diphenyl ethers inhibited ionsine monophosphate dehydrogenase (IMPDH), guanosine monophosphate synthetase, and 15-lipoxygenase enzymes.[61] They were also tested in two other enzyme assays, protein tyrosine kinase pp60 and matrix metalloprotease, but no activity was observed.

6.1.2. Tryptophan Derivatives from Smenospongia, Aplysina, and Polyfibrospongia

Another family of antimicrobial brominated metabolites have been isolated from three different genera and appear to be tryptophan derived. The first of these was obtained in 1973 from the aqueous ethanol extract of the Caribbean sponge *Polyfibrospongia maynardii*, dibromotryptamines (**46**) and (**47**).[72] Both inhibited Gram-positive and Gram-negative bacteria *in vitro*, but were inactive when administered subcutaneously or orally against standard bacterial infections.



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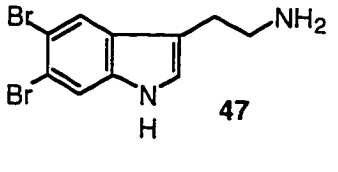

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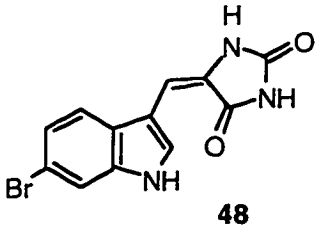

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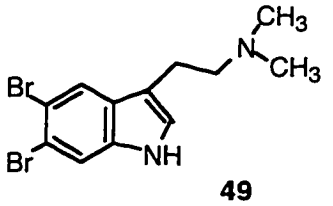

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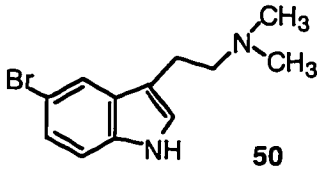
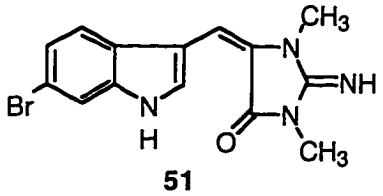


50

In 1980 Faulkner *et al* identified three more antimicrobial bromoindoles, (**48**), (**49**) and (**50**), from the closely related Caribbean sponges *Smenospongia aurea* and *echina*.[73] The crude extracts were found to inhibit the growth of *Staphylococcus aureus* and *Candida albicans*. Upon hydrogenation of both (**49**) and (**50**) *N*, *N*-dimethyltryptamine was obtained. The placement of the bromine atoms at C-5 in (**50**) and C-6 in (**48**) was determined by comparison to the aromatic proton spectra of 5-bromo-indole-3-carboxaldehyde and 6-bromo-indole-3-carboxaldehyde. The proton shifts at C-4, C-5 and C-7 in (**48**) and C-4, C-6 and C-7 in (**50**) were characteristically different (δ 7.70, 7.13 and 7.25 in dmso-*d6* vs. δ 7.77, 7.26 and 7.63 acetone-*d6* respectively). However, this comparison was based on only one example. Proton chemical shifts can be subject to solvent and concentration effects and care should be taken if the comparison is to only one example. Proton data alone are not sufficient. This understanding became important in the structure elucidation of cyclocinamide A (Chapter 5).

The sponge *Smenospongia aurea* was reclassified from *Aplysina aurea* and it was suggested that *Polyfibrospongia echina* should be reclassified as *Smenospongia echina*. Additionally, it was proposed that *Polyfibrospongia maynardii* may in fact be a *Smenospongia* sponge. Faulkner had expected to find the brominated indoles in all

samples of *S. aurea* and *S. echina* examined in the hopes of using these metabolites as chemotaxonomic markers. However, this was not the case. This can best be rationalized by comparison to the previous work. Van Lear *et al* had isolated (**46**) and (**47**) from the *aqueous* ethanol extract whereas Faulkner *et al* isolated their bromoindoles from pure ethanol. It is clear that these metabolites are quite polar in nature and therefore it is feasible that they weren't isolated from some sponge samples due to their extraction procedure. This is further supported by the low yield of (**48**) in one sample and the fact that it precipitated from the aqueous phase after solvent extraction.



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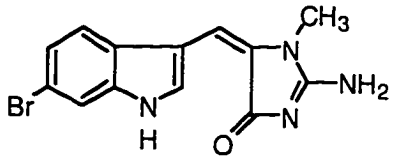


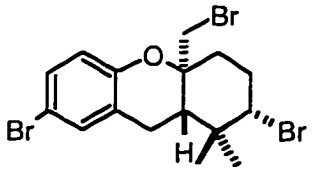
52

Later, in 1985 Rinehart, Jr. *et al* reported the isolation of 5-bromo- and 5,6-dibromotryptamines (**49**) and (**50**) along with the new brominated tryptophan derived components (**51**) and (**52**).[74] These constituents also exhibited antimicrobial activity and were found from morphologically similar sponges of the *Aplysina* and *Smenospongia* genera. It was determined that the *Aplysina* species identified in the field as containing brominated tryptamine constituents should be reclassified as coming from a *Smenospongia* species and that these metabolites are a possible fingerprint for the family Thorectidae in the Dictyoceratida order of sponges.

6.1.3. Meroterpenoids from Cacospongia

In 1989 and 1990 a series of polybrominated meroterpenoids, (**53-61**), were

isolated from a grey, encrusting *Cacospongia* sponge in New South Wales.[75,76] The presence of these metabolites in a sponge is interesting for two reasons. First, these are some of the first reported brominated terpene compounds from a marine sponge. The other report of a brominated terpene from a sponge was from a *Mycale* sp. in the Mediterranean.[77] Secondly, they closely resemble a series of polybrominated



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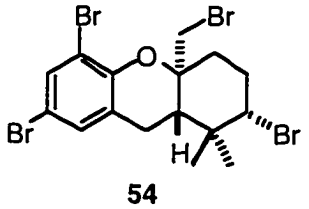

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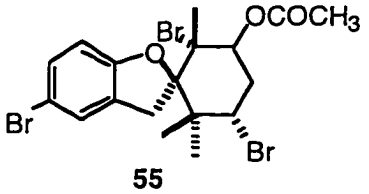

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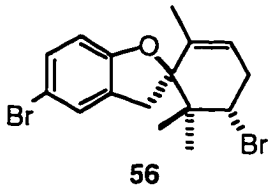

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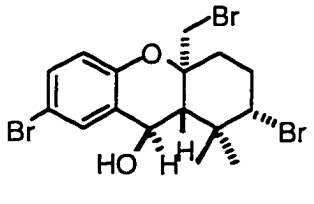

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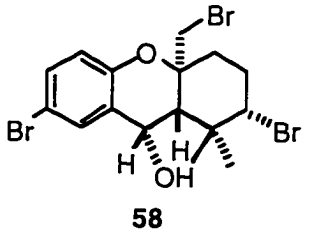

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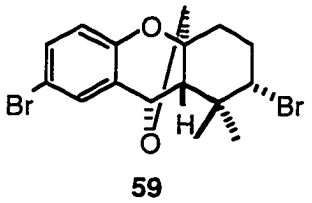

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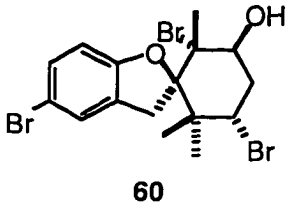

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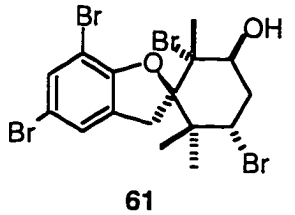
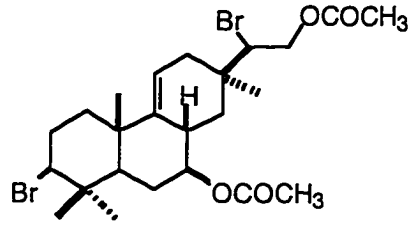


61

meroterpenoids isolated from the tropical green alga *Cymopolia barbata*. Due to the plethora of terpenes present in Dictyoceratida sponges, it is surprising that there are not more examples. The presence of symbionts was ruled out as the source for these brominated constituents by careful collection and electron microscopy analysis[77] and therefore are believed to be true sponge metabolites. As for their function, it was speculated that they may play a structural role since they were devoid of biological activity.

6.1.4. Sesquiterpene from Spongia zimocca

Another example of a brominated terpene isolated from a Dictyoceratida sponge is the dibromosesquiterpene (**62**) identified from the Mediterranean sponge *Spongia zimocca*.[78] It is very similar to compounds from red algae of the genus *Laurencia*. It is interesting to note that algae produce terpenoid compounds similar to those found in Dictyoceratida sponges, but they are usually halogenated. This is probably due to the abundance of known haloperoxidases present in algae that can catalyze the oxidation halides with hydrogen peroxide to promote halogenation in these compounds.



62

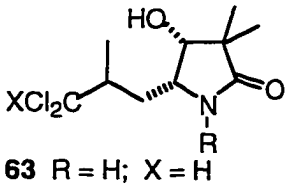
6.2. Chlorinated Metabolites

The number of chlorinated metabolites in marine sponges of the Dictyoceratida order is less numerous than the brominated metabolites. In fact, there are only 25 known compounds containing chlorine of which 21 have been isolated from the sponge *Dysidea herbacea*.

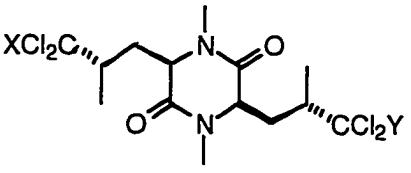
6.2.1. Peptides from Dysidea herbacea

For twenty years now the marine sponge *Dysidea herbacea* has been a prolific source of unusual metabolites, the brominated diphenyl ethers already mentioned and another class of compounds that are peptidic in nature and characterized by polychlorination. The other class of known metabolites from this sponge are furanosesquiterpenes. As was discussed earlier the chlorinated peptides were found associated with the cyanobacterial symbiont *Oscillatoria spongeliae* and the terpenes with the sponge cells. They range in their degree of chlorination from two chlorine atoms up to six chlorine atoms and are normally identified by a characteristic trichloromethyl group. The chlorinated peptides can be divided into five different groups; pyrrolidone containing peptides represented by dysinin (**70**), dysidamides A (**65**), B (**64**) and C (**63**) and dysideapyrrolidone (**72**); diketopiperazines (**66-69**); tripeptide dysideathiazoles (**73-77**); tetrapeptide dysidenins (**78-83**); and the *N* - acyl amino ester herbaceamide (**71**).[79-88]

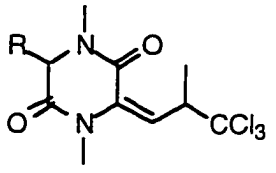
The tetrapeptide dysidenins (**80-83**) have been the subject of some discrepencies in the literature. In the report on the isolation of dysidenin (**83**) a mixture of penta- and hexabromo diphenyl ethers were also identified as isolates from the sponge as minor constituents.[80] This is unusual and the only report of this occurring. The fact that

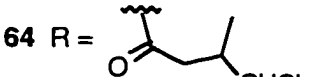


63 R = H; X = H

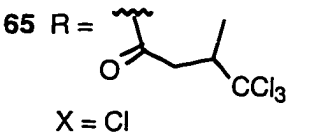


	X	Y
66	H	H
67	Cl	Cl





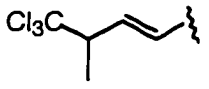
64 R =



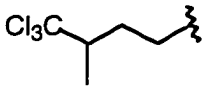
65 R =

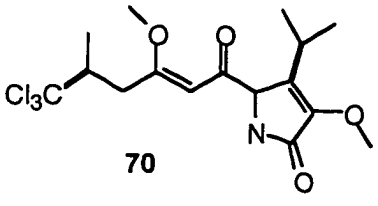
X = Cl

68 R =



69 R =





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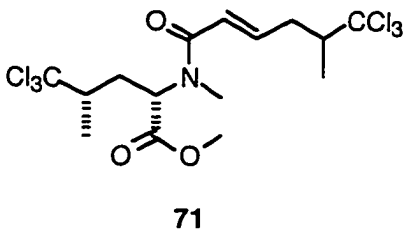

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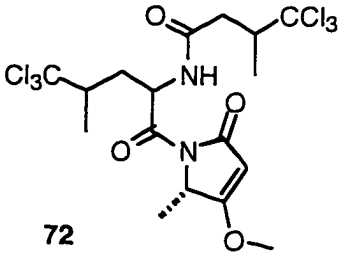

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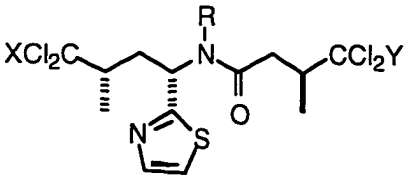
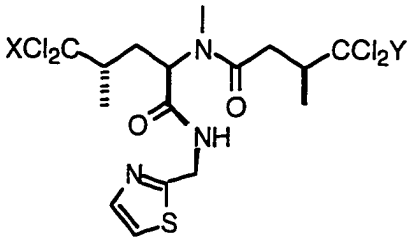
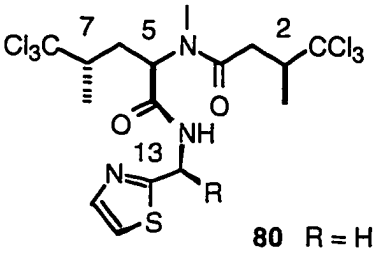


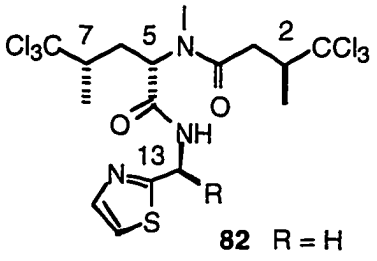
	R	X	Y
73	Me	H	H
74	Me	Cl	H
75	H	Cl	H
76	H	Cl	Cl
77	Me	Cl	Cl



	X	Y
78	H	Cl
79	Cl	H



80 R = H
81 R = Me



82 R = H
83 R = Me

bromophenols are usually isolated in large amounts suggests that there were mixed samples of "bromo" and "chloro" forms[23] of *Dysidea herbacea* extracted. This is understandable considering their morphological similarity.

Another problem associated with the tetrapeptide dysidenins (**80-83**) has been in the assignment of the absolute stereochemistry. In 1981 the absolute configuration of isodysidenin (**81**) was determined by X-ray analysis of an iodomethylated derivative as being 2*R*, 5*S*, 7*R*, 13*R*.[81] Later in 1980 Tursch's group determined the absolute stereochemistry of dysidenin (**80**) as being 2*R*, 5*R*, 7*R*, 13*R* by comparison of ^{1}H-NMR data, optical rotation and acid hydrolysis products between dysidenin and isodysidenin.[89] This meant that dysidenin and isodysidenin were C-5 epimers of one another. One problem that occurred during the hydrolysis of both compounds was the partial epimerization at C-5. Nevertheless, Tursch was able to sort this out and obtain the results reported above.

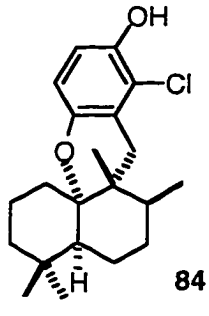
In 1983 Ireland's group reported on the problems associated with the acid hydrolysis of thiazole amino acids and the fact that they undergo extensive racemization.[90] This inspired them to later investigate Tursch's work due to the presence of thiazole amino acids in dysidenin and isodysidenin. Ireland believed that due to the small optical rotation of the derivatized thiazole amino acid hydrolysates of both dysidenin and isodysidenin and their previous experiences with thiazole amino acids that the stereochemistry should be reinvestigated. They found that the configuration at C-13 in both dysidenin and isodysidenin was in fact *S* and not *R* and therefore revised the stereochemistries to 2*S*, 5*S*, 7*S*, 13*S* and 2*S*, 5*R*, 7*S*, 13*S* for dysidenin and isodysidenin, respectively.[91] This was a serendipitous event because the problem was not with the acid hydrolysis work Tursch carried out, but with the assignment of the stereochemistry in the original X-ray data of isodysidenin. Tursch would have obtained the same results

during acid hydrolysis whether the configuration was *S* or *R* at C-13. If the X-ray data had been interpreted correctly, he would have obtained the correct absolute configuration at all centers regardless of any racemization occurring at C-13 of the thiazole amino acid. In 1985, the absolute configuration of dysidenin and isodysidenin was confirmed by the total synthesis of (-)-13-demethylisodysidenin (**80**) and (+)-13-demethyldysidenin (**81**) showing that the dysidenins were of *S* configuration at C-2 and C-7 and *R* and *S* at C-5 in the natural products.[92]

The chlorinated peptides from *D. herbacea* exhibited no antimicrobial activity against *Escherichia coli*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Stapyhlococcus aureus* and *Candida albicans* unlike the brominated diphenyl ethers.[88] However, they were strongly deterrent in fish-feeding experiments. It is not known at this time if the chlorinated peptides serve any other function, other than a fish deterrent, as a product of the symbiont.

6.2.2. Sesquiterpene from Smenospongia aurea

Fattorusso *et al* recently reported on the isolation of a chlorinated sesquiterpene (**84**) from the Caribbean sponge *S. aurea*.[93] It represents a distinct class of metabolites; a C_6-shikimate functionality attached to a sesquiterpene. Other metabolites in this family are avarol and avarone from the sponge *Dysidea avara* and the puupehenones from the sponge *Hyrtios* spp. to be discussed later.[94,95] It was shown to have antibiotic activity.



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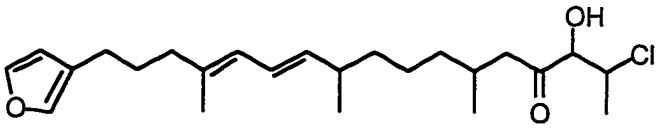
6.2.3. Sesterterpenes from an Unidentified Sponge and Ircinia oros

In 1990 and '91 a group of chlorinated acyclic furanosesterterpenes (**85-87**) were characterized from an unidentified Dictyoceratida sponge off Konakhe near Dakar (**85**) and the North Adriatic sponge *Ircinia oros* (**86, 87**).[22,96] They represent the only



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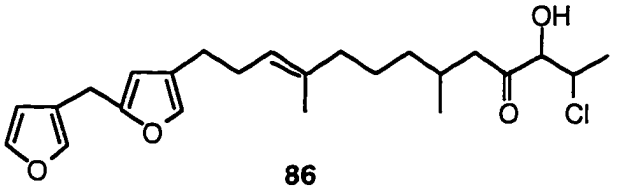

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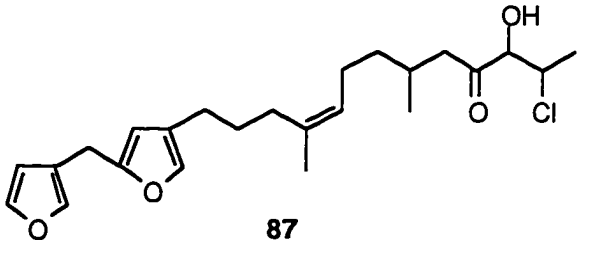


87

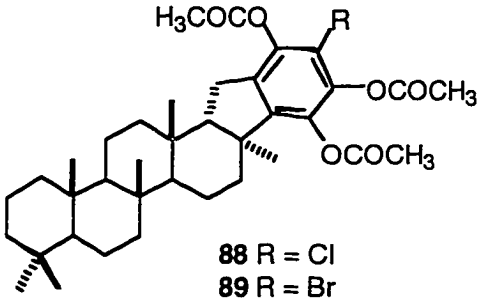
examples of acyclic sesterterpenes that are halogenated from Dictyoceratida sponges. As discussed earlier the chlorination of these compounds can be envisioned to occur as a result of degradation of the carboxylic acid form of the terpene. The crude extract containing compounds (**86**) and (**87**) exhibited high activity in the brine shrimp assay at LD_{50} = 14 μg/ml. However, the bioactivity was attributed to the non-chlorinated sesterterpenes ircinin 1 and 2, other metabolites of the sponge.[97]

6.3. Mixed Halogenated Metabolites

There are a number of halogenated metabolites that are derived from a mixed biogenesis and can contain either chlorine or bromine or both halogens at the same time. They involve the compound classes sesqui- and sesterterpenes and macrolides.

6.3.1. Sesterterpenoids from Dysidea pallescens

In 1987 two halogenated (chloro and bromo) scalarane sesterterpenes (**88**), (**89**) attached to a hydroxyhydroquinone moiety were isolated from the Mediterranean sponge *Dysidea pallescens*.[98] X-ray diffraction analysis of the brominated sesterterpene allowed

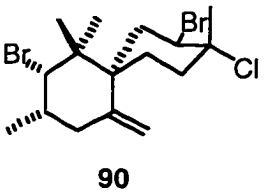


88 R = Cl
89 R = Br

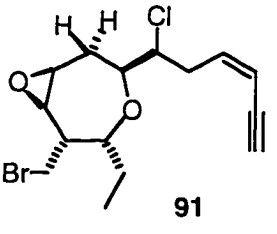
the authors to determine the absolute stereochemistry of both (**88**) and (**89**) as well as the non-halogenated version by comparison to the NMR data of these constituents. The treatment of the non-halogenated sesterterpene with chlorine and bromine gave the natural halogenated constituents. No biological activity was reported for these metabolites.

6.3.2. Chamigrene-Type Sesquiterpenes and C_{15} Acetogenins from Spongia zimocca

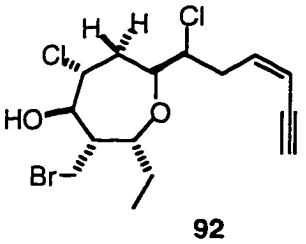
In the early 1990's, a series of mixed halogenated sesquiterpenes (**90-93**) was isolated from the Mediterranean sponge *Spongia zimocca*.[99,100] Compound (**90**) represents the first example of a β-chamigrene-type sesquiterpene from a marine sponge. Previously, an abundance of these types of compounds were identified only with the red algae *Laurencia* and a mollusk of the genus *Aplysia* that feeds on the algae. Metabolites (**91-93**)



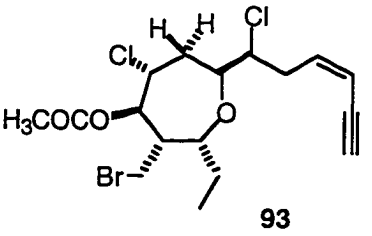
90



91



92



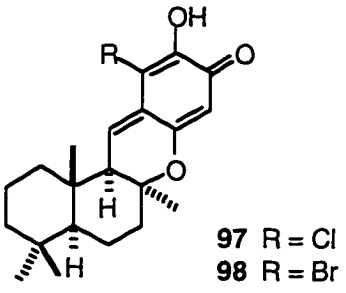
93

are also the first examples of acetogenins with a halogenated C_{15} chain from a sponge and were also known from red algae and mollusks. The sponges containing these compounds were found growing in an area densely populated with the algae and it was speculated that the metabolites were transferred to the sponge from the algae *via* filter-feeding.[100] Technically speaking these compounds are from red algae and shouldn't be called sponge metabolites because they are not biogenetically produced by the sponge. There was no

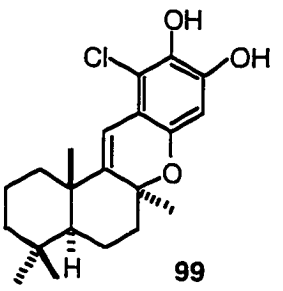
reported biological activity for either type of constituent. However, chemically speaking compound (**90**) and others like it are interesting from a conformational point of view and have been the subject of conformational studies.[101]

6.3.3. Sesquiterpene Quinones from Hyrtios spp.

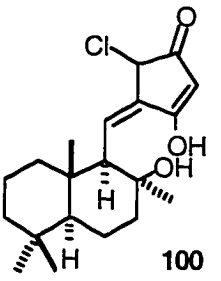
A group of mixed halogenated metabolites were isolated from the Hawaiian sponge *Hyrtios* spp. In 1979 the chloro (**97**) and bromo (**98**) derivatives of puupehenone were identified.[106] Much later, 1995, two additional halogenated metabolites of the same structure class were isolated from the marine sponge; 21-chloropuupehenol (**99**) and molokinenone (**100**).[95] All constituents consist of a sesquiterpene attached to a C_6-shikimate functionality like compound (**84**) already discussed. Compound (**100**) is a ring-contracted variation on this theme. Compound (**99**) decomposed before any biological activity could be measured and (**100**) had insignificant activity compared to the non-halogenated metabolites in this family.



97 R = Cl
98 R = Br



99

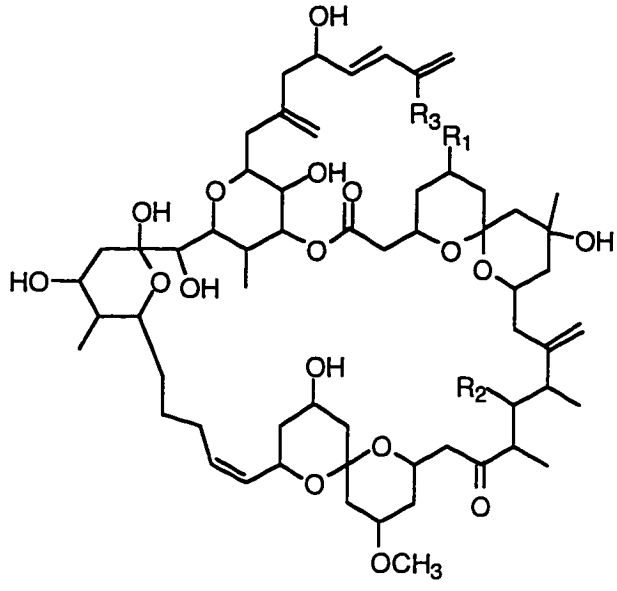


100

6.3.3. Macrocyclic Lactones from Spongia sp. and Hyrtios altum

In 1993 two groups reported the isolation and characterization of the same cytotoxic macrocyclic lactone compounds. Kitagawa *et al* identified their metabolites as

altohyrtins A (**94**), B (**96**), C (**97**) and 5-desacetyl-altohyrtin A (**95**) from the marine sponge *Hyrtios altum*.[102,103] At the same time Pettit *et al* reported the isolation of spongistatins 1 (**94**), 2 (**97**) and 3 (**95**) from a *Spongia* sp.[104,105] Both groups reported potent cytotoxicity against cancer cell lines. Altohyrtin A exhibited extremely potent activity against KB (IC_{50} = 0.01 ng/ml) and L1210 (IC_{50} = 0.1 ng/ml) cell lines.



94 R_1 =OAc; R_2 = OAc; R_3 = Cl
95 R_1 =OH; R_2 =OAc; R_3 = Cl
96 R_1 =OAc; R_2 = OAc; R_3 = Br
97 R_1 =OAc; R_2 = OAc; R_3 = H

Altohyrtins B, C and 5-desacetyl-altohyrtin A had very similar activities against KB cell lines in the ng/ml range. The spongistatins were found to be extremely potent in the NCI's panel of 60 human cancer cell lines with melanoma, lung, colon and brain cell lines being especially sensitive. Halogenation, bromine or chlorine, occurs in the terminal double bond of the side chain.

To date there have been 82 brominated and/or chlorinated metabolites isolated from marine sponges of the order Dictyoceratida belonging to six classes of compounds; peptides, sesqui- and sesterterpenes, diphenyl ethers, indoles and macrolides.

7. Present Studies

Now that I have examined the halogenated chemistry of Dictyoceratida sponges, halogens in the marine environment, the process of biohalogenation and the sponge/symbiont association, I can discuss the work of this dissertation in context. The sum of my work consists of the isolation and structure elucidation of eight new halogenated compounds and an extensive chemotaxonomy study. A summary of each chapter follows.

7.1. Chapter 2

The title of Chapter 2 is *Cyclocinamide A, An Unusual Cytotoxic Halogenated Hexapeptide from the Marine Sponge* Psammocinia. During an expedition to Papua New Guinea a new species of sponge was identified due its difference in morphological characteristics. Also notable was the extent of cyanobacteria present on the sponge. For the above mentioned reasons work began on the sponge in the hopes of finding some new chemistry. At first ^{1}H-NMR spectra indicated that the major metabolites present were acyclic furanosesterterpenes and of no interest. However, potent biological activity in a brine shrimp assay and in tumor cell lines at Wayne State told another story. Therefore, a bioassay guided isolation was initiated to search for the minor bioactive components. The result of this was the isolation and structure elucidation of the halogenated and potent hexapeptide cyclocinamide A. This is the first example of a cyclic tetrapeptide from a Dictyoceratida sponge and it has no parallel in marine natural products chemistry in general.

7.2. Chapter 3

The title of Chapter 3 is *A Novel Chlorinated Ketide Amino Acid, Herbamide A, from the Marine Sponge* Dysidea herbacea. This work introduces the first compound in a new series of chlorinated peptides from *Dysidea herbacea*, the ketide amino acid herbamides. The focus is on the isolation and structure elucidation of herbamide A.

7.3. Chapter 4

The title of Chapter 4 is *New Halogenated Constituents from the Indo-Pacific Sponge* Dysidea. This work introduces six new halogenated compounds to the already long list of *Dysidea* compounds. During the work for Chapter 5, electrospray ionization mass spectrometry indicated the presence of new halogenated metabolites in two samples which did not match the masses of known compounds from this sponge. Therefore, isolation and structure elucidation was initiated on these samples to liberate three new chlorinated tetrapeptides and two brominated dioxins introducing new subclasses of these metabolites. The structure elucidation of another ketide amino acid, herbamide B, is also discussed.

7.4. Chapter 5

The title of Chapter 5 is the *Species Differentiation in the Chemotaxonomic Analysis of Foliose Indo-Pacific Sponges of the Genus* Dysidea *(Dysideidae: Dictyoceratida)*. This work involves a chemotaxonomic analysis of an extensive collection of *Dysidea* sponges collected during three expeditions over a two year period from Papua New Guinea and

Indonesia. The results add enlightening new information to the already well-studied sponge by clarifying its difficult taxonomic nature. It identifies new species of *Dysidea* and establishes the fact that all the previously reported chemistry associated with *Dysidea herbacea* is in fact from more than one species. The quantitative approach of using ^{1}H-NMR coupled with electrospray ionization mass spectrometry in a chemotaxonomic analysis is the first of its kind and paves the way for future studies in this area.

7.5. Chapter 6

The last chapter brings the work presented to a close and poses several questions concerning it. It also speculates on future studies that might be carried out on marine sponges with cyanobacteria and the search for more bioactive components from the marine environment.

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Chapter 2-Cyclocinamide A, An Unusual Cytotoxic Halogenated Hexapeptide from the Marine Sponge *Psammocinia*

Marine Sponge-Papua New Guinea Photo Courtesy of Jay Burreson, Ph.D.

Abstract

Marine sponges of the family Thorectidae continue to be rich sources of novel alkaloid constituents. An unusual cytotoxic halogenated hexapeptide, cyclocinamide A (**1**), was isolated as a minor constituent from the marine sponge *Psammocinia* collected in Papua New Guinea. Previously, *Psammocinia* has yielded only acyclic sesterterpene compounds containing both terminal tetronic acid and furan moieties. The structure elucidation of cyclociniamide A (**1**) was carried out using data derived from a variety of one-dimensional and two-dimensional ^{1}H and ^{13}C NMR techniques. The absolute stereochemistry was assigned as *S* at C7 and C14 based on chiral TLC analysis. Cyclocinamide A (**1**) exhibited potent *in vitro* selective activity in Colon-38 tumor cells.

Background

In the Spring of 1993, the UCSC marine natural products research group took an expedition to Papua New Guninea to explore new areas for collection in the Milne Bay province. It was during this trip that the group began to examine sponges in the field by thin layer chromatography (TLC). The reason for this exercise was to identify those sponge specimens containing chlorophylls because this would indicate the presence of symbiotic cyanobacteria. The group was interested in the chemistry of any sponges that showed evidence of cyanobacteria and it was the main goal of my research in this lab.

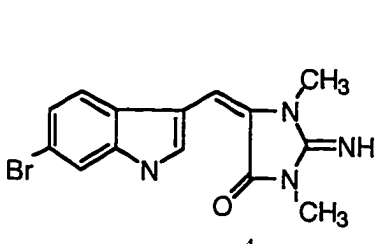
One sponge specimen, collection number 93145, was collected not only for its greenish-purple appearance, but also because the group had never seen it before on previous expeditions. Preliminary taxonomic description in the field by Dr. M. Cristina suggested a new species of *Psammocinia*. The research group had collected another species of this genus during previous trips (coll. no.'s 90135, 91113) which were found to have interesting biological activity. However, the metabolites triggering the activity could never be isolated due to their minute quantities. Therefore, once back in the lab, I began the workup process to try and identify any new chemistry that might be present. This proved disappointing at first because the ^{1}H and ^{13}C NMR spectra indicated the presence of known acyclic sesterterpene compounds. Consequently, the extracts sat on my lab bench and I moved on to something else.

Later on the UCSC research group began to use a bench-top brine shrimp assay to quickly identify those sponge extracts with biological activity rather than wait up to 6 months to get feedback from our collaborators. The group hoped this would speed up the whole process and that we could proceed rapidly to isolate the active components guided by the biological activity of this assay. Therefore, I went back and tested all the sponge

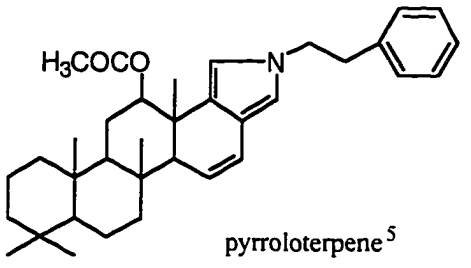
extracts that I had worked on in the brine shrimp assay. The result was the identification of potent activity in the crude extracts of 93145. Shortly thereafter, our collaborators confirmed the same result. This was very exciting and I began to further purify the extracts in a bioassay guided fashion to isolate the active component(s). This lead to the discovery of cyclocinamide A. I will now discuss in detail the isolation and structure elucidation of this novel compound. The manuscript of this work has been submitted to the *Jounal of the American Chemical Society* and was presented at the 213th National Meeting in San Francisco,CA on April 16, 1997.

Introduction

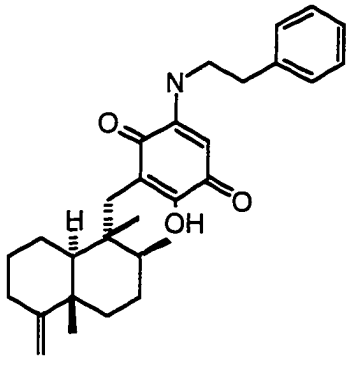
Marine sponges of the family Thorectidae have over the years been the source of numerous compounds of the sesqui- and sesterterpene structure class.[1-3] Additionally, sponges of this family have been found to contain some alkaloid constituents (Figure 2.1); e.g. indole derivatives[4], pyrroloterpenes[5], terpene aminoquinones[6], sphingolipids[7] and manzamine precursors[8], but there have been no reports of peptides.



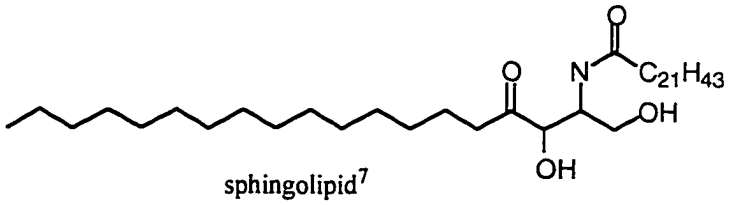
indole derviative[4]



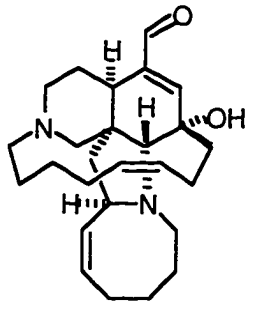
pyrroloterpene[5]



terpene aminoquinone[6]



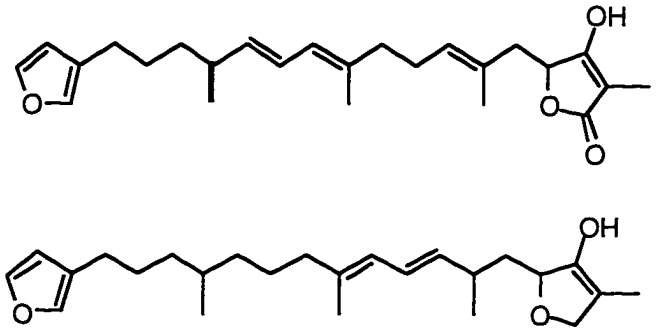
sphingolipid[7]



manzamine precursor[8]

Figure 2.1. Alkaloid Constituents from Thorectidae Family.

Previous literature on the isolation of compounds from the marine sponge genus *Psammocinia* is scarce and the only class of compounds reported are acyclic sesterterpenes containing both terminal tetronic acid and furan moieties.[9,10] During a recent expedition to Papua New Guinea the UCSC marine natural products research group collected an unidentified species of *Psammocinia* (coll. no. 93145) whose crude extracts exhibited potent cytotoxicity to brine shrimp and solid tumor cells at Wayne State. The acyclic sesterterpene-type compounds were inactive in all screens. Therefore, a bioassay guided search for the active component was initiated.



acyclic sesterterpenes[9,10]

Figure 2.2. Acyclic Sesterterpenes from *Psammocinia*.

Collection and Isolation

The sponge exhibited a TLC profile, in the field, containing chlorophylls indicating the presence of cyanobacteria. Each individual measured from 2-3 cm in length and was a greenish-purple color as shown in the underwater and topside photographs (Figures 2.3, 2.4).

Cyclocinamide A was isolated as an amorphous solid in small yield (6.4 mg, 0.005 % of extract) following bioassay guided workup of the crude methanol extract of the sponge. The methanol extract was first partitioned according to our standard procedure[11] (Appendix A) with the subsequent 50% aqueous MeOH solvent partition fraction (FM) being further purified due to its cytotoxicity to brine shrimp[12,13] (Appendix C) and potent activity in tumor cell lines at Wayne State (Scheme 2.1). This was followed with additional chromatography by Sephadex LH20 (50:50, MeOH:CH_2Cl_2) and final purification of the solid tumor selective fraction by HPLC (ODS, 41:7:9:43, MeOH:THF:MeCN:H_2O) to yield pure cyclocinamide A (**1**) ($[\alpha]_D$ = + 29° (*c* 0.10, MeOH)).

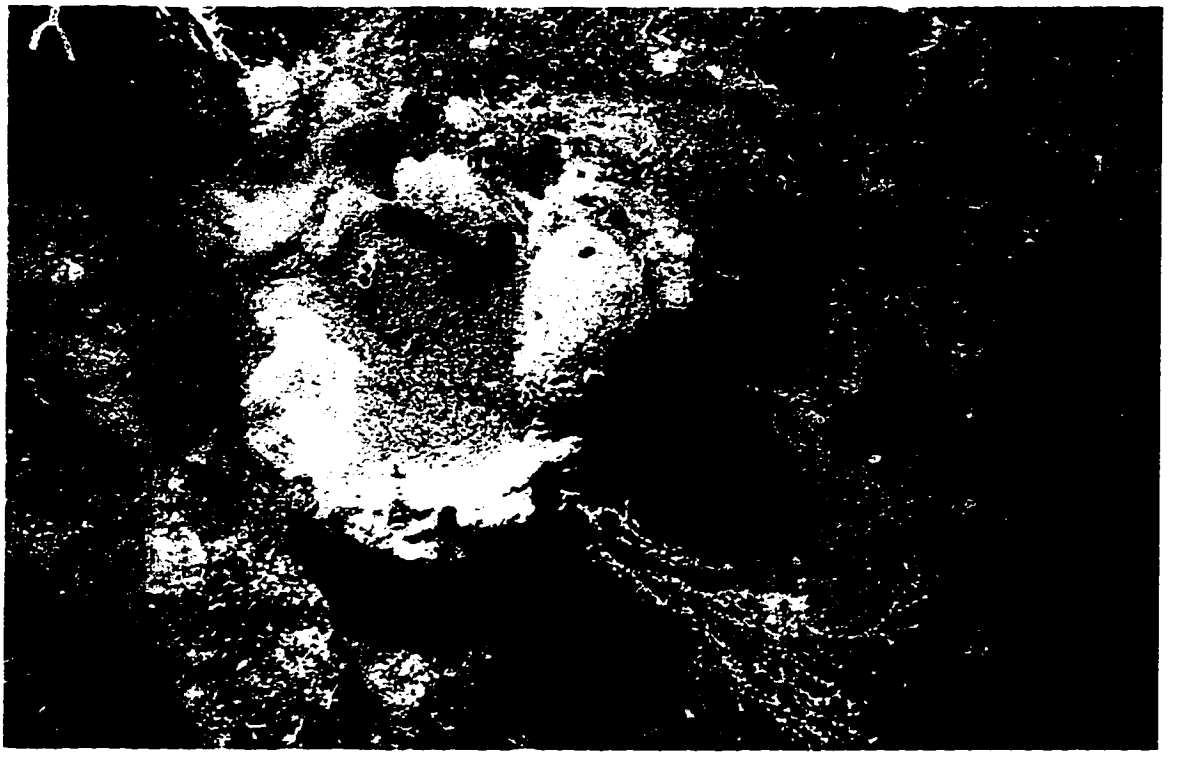


Figure 2.3. Underwater Photograph of 93145.

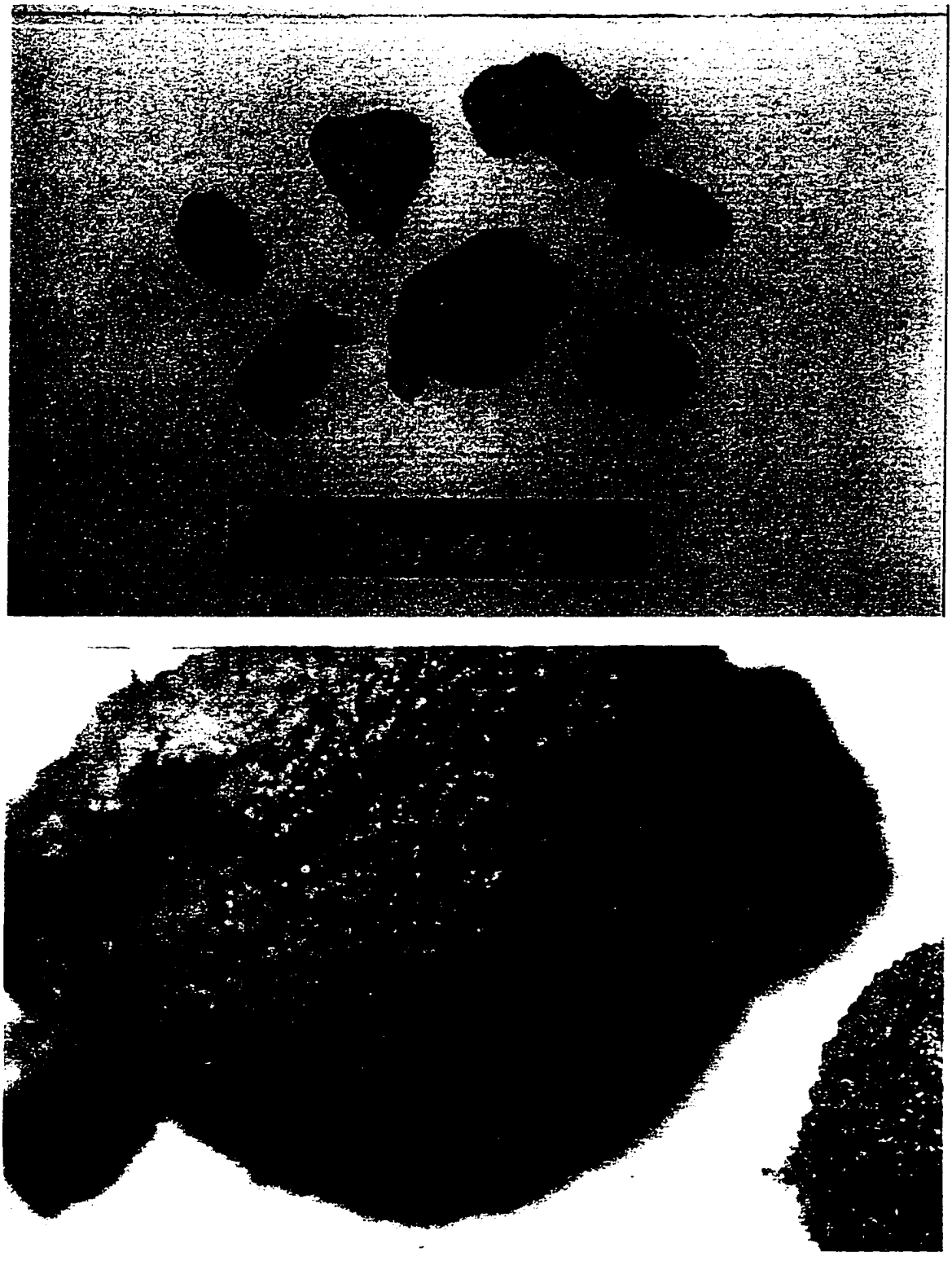
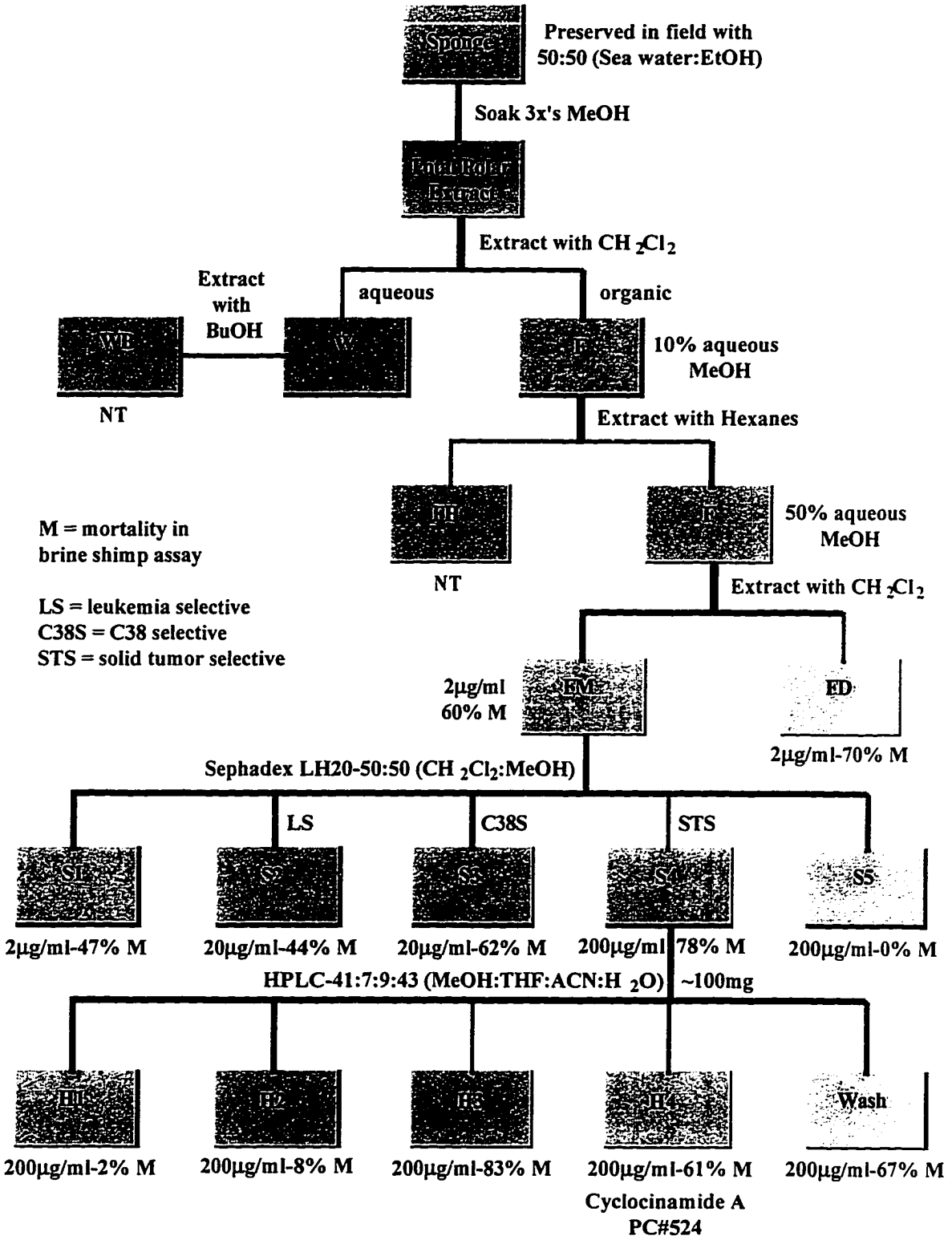


Figure 2.4. Topside and Closeup Photographs of 93145.



Scheme 2.1. Extraction Scheme of 93145.

Structure Elucidation

A molecular formula of $C_{29}H_{33}BrClN_9O_8$ was established by HRFABMS $(MH)^+$ *m/z* 750.1406 (Δ 0.4 mmu of calcd.) with the molecular ion peak exhibiting an isotope distribution pattern characteristic of one bromine and one chlorine atom (Figure 2.12 in Supp. Mat.). Comparison of the ^1H and ^{13}C NMR data (Table 2.1) with the ^{13}C NMR DEPT 135 and the MS data indicated the presence of one methyl, five methylene, ten methine, and thirteen quaternary carbons for a formula of $C_{29}H_{23}$ with the ten remaining protons attached to heteroatoms, nine *N*H's and one OH (Figures 2.7, 2.11 in Supp. Mat.). The molecular formula gave an unsaturation number of seventeen of which seven elements were attributed to carbonyl resonances in the δ 160.9 to 172.7 range (Table 2.1). This information taken along with the amide carbonyl absorption ($\nu_{C=O}$ = 1670 cm^{-1}) and the nine *N*H proton resonances at δ 7.17-11.10 indicated that cyclocinamide A (**1**) was a polypeptide. The UV (λ_{max} 252) data were consistent with extended conjugation characteristic of aromatic systems (Figure 2.13 in Supp. Mat.). This was confirmed by the ^{13}C NMR data and the six proton resonances at δ 6.87-7.67 to give a total of six trisubstituted double bonds. Additionally, the *N*H proton chemical shift at 11.10 was characteristic of the indole ring system *N*H in tryptophan eliminating four of the six trisubstituted double bonds. The two remaining trisubstituted double bonds were attributed to a separate aromatic moiety. Therefore, it was deduced that cyclocinamide A (**1**) contained a total of four rings to satisfy the remaining degrees of unsaturation.

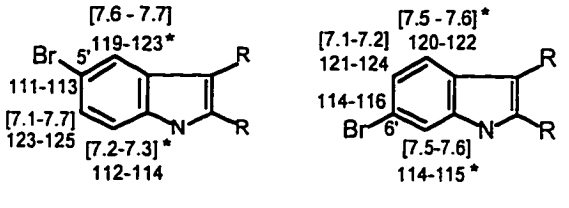
The structure elucidation of substructures (**A-H**) was accomplished after analysis of 1D and 2D (COSY, HMQC, and HMBC (*J* optimized for 9 Hz, 140 Hz decoupled)) ^1H and ^{13}C NMR data (Figure 2.5; Figures 2.14-2.27 in Supp. Mat.). Substructure (**A**) was quickly identified as a tryptophan residue after key COSY and HMBC correlations

Table 2.1. 125/500 MHz NMR data for cyclocinamide A (1) in DMSO-*d6* and CD_3OD.

Position	^{13}C (δ)	^{1}H (*J*, Hz) in DMSO-*d6*	^{1}H (*J*, Hz) in CD_3OD	COSY (H→H)	HMBC* (C→H)	HMBC† (C→H)
1	170.5				15'	2, 15, 15'
2		7.14, NH, bs		3, 3'		
3	42.8	3.36, 1H, ddd, (14, 5, 2)	3.48, dd, (14, 3)	2, 4		
3'		3.50, 1H, ddd, (14, 6, 2)	3.62, dd, (14, 3)			
4	69.7	4.04, 1H, bt, (3)	4.36, t, (3)	3, 3'		
5	170.9				4	6
6		7.90, NH, bd, (9.5)		7		
7	53.3	4.57, 1H, dt, (9.5, 6.5, 6.5)	4.52, t, (6.5)	6, 18, 18'	18, 18'	18, 18'
8	172.7				7	7, 9, 18, 18'
9		8.00, NH, bm		10, 10'		
10	40.3	3.33, 1H, ddd, (14, 6, 4)	3.15, dd, (14, 3)	9, 11		
10'		3.42, 1H, ddd, (14, 6, 4)	4.04, dd, (14, 3)			
11	54.3	4.33, 1H, dt, (7.5, 4, 4)	4.26, t, (3)	10, 10', 28		
12	168.8				13	
13		8.00, NH, bm		14		
14	49.5	4.57, 1H, ddd, (9.5, 7.5, 5)	(under water peak)	13, 15, 15'	15'	15, 15'
15	36.5	2.31, 1H, dd, (15.5, 5)	2.48, dd, (15.5, 6)	14	17	
15'		2.49, 1H, dd, (15.5, 7.5)	2.69, dd, (15.5, 8)			
16	171.9				15, 15'	15, 15'
17		6.80, NH, bs;		17, 17'		
17'		7.28, NH, bs				
18	27.8	2.99, 1H, dd, (15, 7)	3.10, bd, (6.5)	7		
18'		3.03, 1H, dd, (15, 6)				
19	109.6				18, 18', 20, 21	18, 18'
20	125.3	7.17, 1H, s	7.17	21	21	18, 18'
21		11.10, NH, bs		20		
22	134.8				21, 24, 26	26
23	113.4	7.30, 1H, d, (8.5)	7.31, d, (8.5)	24		
24	123.4	7.17, 1H, dd, (8.5, 2)	7.20, dd, (8.5, 2)	23, 26	26	26
25	111.1				23, 26	23, 26
26	120.7	7.67, 1H, d, (2)	7.62, d, (2)	24	24	
27	129.1				20, 21, 23	18, 18', 23
28		8.18, NH, bd, (7.5)		11		
29	169.2				30, 30'	30, 30'
30	42.3	3.78, 1H, dd, (16.5, 4);	3.88, d, (17)	31		
30'		3.84, 1H, dd, (16.5, 5.5)	4.04, d, (17)			
31		8.42, NH, bt, (5.5)		30, 30'		
32	160.9				30'	30, 30', 31
33	124.9				36, 38	36, 38
34	111.8	6.87, 1H, d, (2)	6.83, d, (2)	36	36	36
35	108.6				36	36
36	124.9	7.06, 1H, d, (2)	6.87, d, (2)	34	34	38
37						
38	36.4	3.77, 3H, s	3.80, s			36

*HMBC *J* optimized for 9 Hz, 140 Hz decoupled. †HMBC *J* optimized for 4 Hz, 140 Hz decoupled.

(C19, C20, C22, and C27 → *N*H21) combined with fingerprint ^{13}C NMR chemical shifts at C22, C23, and C27 (δ 134.8, 113.4, and 129.1, respectively) for an indole ring were identified (Table 2.1). A long-range HMBC correlation from C20 to β protons $H_2$18 along with COSY correlations $H_2$18 to α proton H7 (δ 4.57) to *N*H6 attached the indole ring to C7 completing the tryptophan moiety. It was deduced that a halogen substitution on the indole was necessary due to the lack of a downfield proton resonance and the uncharacteristic upfield shift at C25 (δ 111.1). Bromine, rather than chlorine was the most likely candidate based on calculated ^{13}C NMR chemical shift data.[14] Placement of the bromine atom at C25 instead of C24 was deduced by comparison of the ^{1}H NMR chemical shifts, especially at CH23 (δ 113.4 / 7.30 (d) J = 8.5 Hz), C25 (δ 111.1), and CH26 (δ 120.7 / 7.67 (d) J = 2 Hz), to models.[15] The 5'-bromoindole can be distinguished from a 6'-bromoindole using the set of shifts with an * in the models below [^{1}H NMR data in DMSO-d_6 or CD_3OD and ^{13}C NMR data in acetone-d_6, DMSO-d_6 or CD_3OD].



The diamino spin system (**B**) of the 2,3 diamino propionic acid residue (3-amino alanine) was readily assembled by COSY data as was the glycine residue (**C**). Correlations by HMBC from C29 and C32 to diastereotopic α protons $H_2$30 (δ 3.78 & 3.84) completed substructure (**C**). The uncommon *iso*-serine residue (**D**) was recognized as containing an alcohol functionality because of the downfield shift at C4 (δ 69.7) even though the hydroxy proton was not readily visible in the ^{1}H NMR spectra (DMSO-*d6*, 500 MHz). After suppression of the water signal (45°C, DMSO-*d6*, 500 MHz; Figure 2.10 in Supp. Mat.), the hydroxy proton became visible along with the diastereotopic

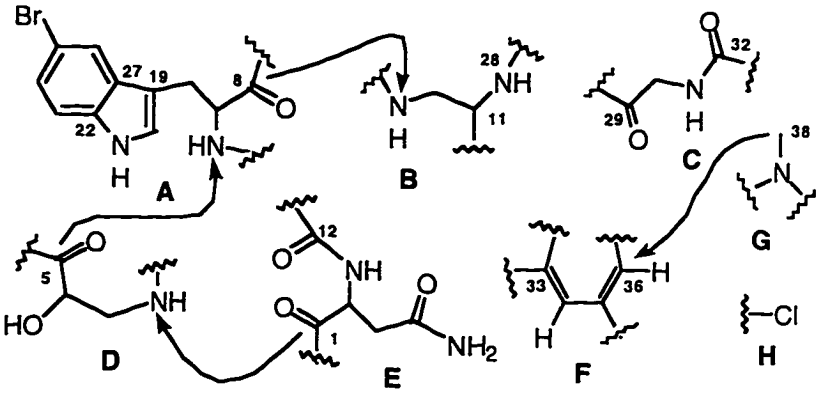


Figure 2.5. Partial Structures (**A-H**) of cyclocinamide A (1). Arrows Indicate Diagnostic HMBC Correlations with *J* Optimized for 4 Hz.

protons at H_23 and H_210 which were partially obstructed. The presence of protons H_23 and H_210 were also confirmed by ^1H NMR and COSY data obtained in CD_3OD/D_2O at 500 MHz (Figures 2.8, 2.18 in Supp. Mat.). Connectivities in this spin system were completed by COSY data and an HMBC correlation from C5 to α proton H4 (δ 4.04).

Substructure (**E**), an extended asparagine residue through C12, was assembled by various COSY and HMBC correlations. Key HMBC data from C12 (δ 168.8) to *N*H13 (δ 8.00) and C1 (δ 170.5), C16 (δ 171.9) to β protons H_215 (δ 2.31 & 2.49) allowed attachment of the three carbonyls to spin system (**E**) (Figure 2.5). Additionally, the diastereotopic NH_217 protons exhibited the characteristic chemical shifts of a formamide functionality (δ 6.80 and 7.28) to complete the asparagine substructure.

Connection of partial structures (**A-H**) was achieved by analysis of additional HMBC and NOESY data coupled with chemical shift information. Key two bond (^2J) correlations that connected substructures (**A**), (**B**), (**D**), and (**E**) were obtained by another HMBC experiment where *J* was optimized for 4 Hz (Figure 2.5; Figures 2.28-2.33 in Supp. Mat.). The asparagine substructure (**E**) was connected to *iso*-serine (**D**) via a HMBC correlation between C1 (δ 170.5) and *N*H2 (δ 7.14). In turn this dipeptide was

connected to the bromo-tryptophan residue (**A**) by HMBC correlation C5 (δ 170.9) to *N*H6 (δ 7.90) and finally the correlation between C8 (δ 172.7) and *N*H9 (δ 8.00) allowed attachment of partial structure (**B**) to the tripeptide residue (**A-D-E**). The final connection between C11 (δ 54.3) and C12 (δ 168.8) to complete the 14-membered ring of cyclocinamide A (**1**) was deduced based on ^{13}C chemical shifts for a peptide structure. Likewise, the amide bond between *N*H28 (δ 8.18) and C29 (δ 169.2) added the glycine substructure (**C**) to the ring on cyclocinamide A (**1**). This attachment was confirmed by strong nOe's from *N*H28 to $H_2$30 (δ 3.78, 3.84) and *N*H31 (δ 8.42) (Figure 2.6; Figures 2.37-2.40 in Supp. Mat.).

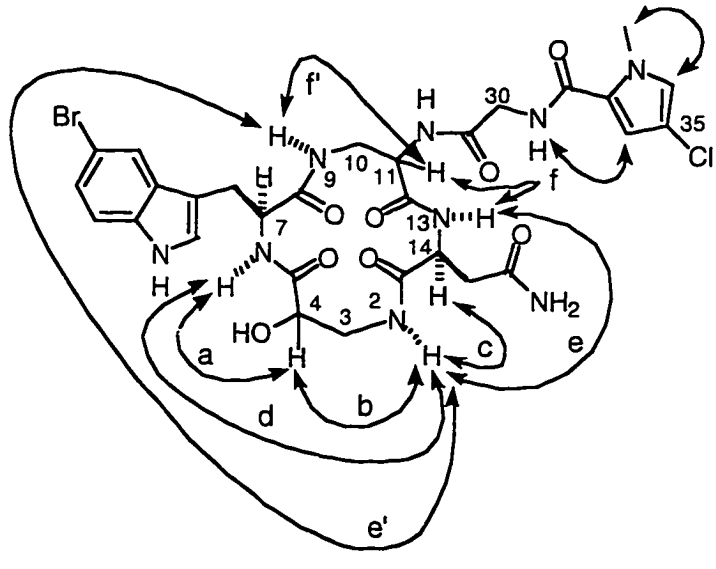
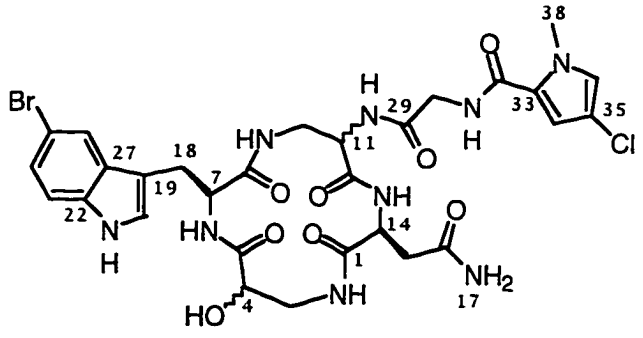


Figure 2.6. Important NOE correlations for cyclocinamide A (**1**) (DMSO-d6, 500 MHz).

The *N*-methyl (**G**) at H38 (δ 3.77) was attached to C36 on (**F**) by HMBC after resolution of C33 and C36 in DMSO-*d6*/Benzene-*d6* (Figures 2.34-2.36 in Supp. Mat.). Completion of the pyrrole ring via attachment of *N*-methyl C38 to C33 and subsequent coupling of C33 to C32 was determined by comparison of ^{13}C NMR chemical shifts to

known *N*-methyl-bromo pyrroles in the literature.[16,17] Additionally, a strong nOe was observed from *N*H31 (δ 8.42) to H34 (δ 6.87) to confirm attachment of the pyrrole to rest of the structure (Figure 2.6) as well as placement of the chlorine atom (**H**) at C35 (δ 108.6) to completed the planar structure of cyclocinamide A (**1**).



cyclocinamide A **1**

The absolute stereochemistries at C7 and C14 were deduced by chiral TLC analysis versus standards.[18] Acid hydrolysis of cyclocinamide A (0.1 mg) was undertaken with 6N HCl in the presence of phenol (3% v/v) to prevent the degradation of the indole ring in tryptophan.[19] After chiral TLC of the hydrolysate against standards, it was deduced that the configuration at C7 and C14 was *S* meaning that the tryptophan and asparagine residues present in cyclociniamide A were L-amino acids (Table 2.2).

Table 2.2. Chiral TLC (thin layer chromatography) Analysis of cyclocinamide A.*

Amino Acid	R_f Value Standard (configuration)	R_f Value Hydrosylate (configuration)	Eluent§
5-Bromo-tryptophan	0.56 (*R*)	-	A
	0.71 (*S*)	0.71 (*S*)	
5-Bromo-tryptophan	0.64 (*R*)	-	B
	0.71 (*S*)	0.71 (*S*)	
Aspartic Acid	0.50 (*R*)	-	A
	0.54 (*S*)	0.54 (*S*)	
Aspartic Acid	0.68 (*R*)	-	B
	0.71 (*S*)	0.71 (*S*)	

* Hydrolysis was performed in a sealed microcapillary tube at 145°C for 3 hours.

§ Development distance was 8-10 cm. Eluent A: 1:1:4 (methanol:water:acetonitrile); eluent B: 4:1:1 (acetone:methanol:water).

The possibility that the six nOe cross-peaks (a, b, c, d, e/e', f/f') and four vicinal $^3J_{3,4}$, $^3J_{10,11}$ couplings observed (Figure 2.6, Table 2.1) could be used to assign the remaining stereocenters at C4 and C11 was pursued. The plan was to compare the distance and dihedral angle constraints against values predicted by computer molecular modeling for the minimium energy conformations of the four possible diastereomers: 4*R*/11*R*, 4*R*/11*S*, 4*S*/11*R*, 4*S*/11*S*. In order to simplify the process, the energy minimization was performed on the ring structure of cyclocinamide A replacing the side chains with methyl substituents. There are several possible conformers of each diastereomer when rotating through the ring each amide bond 180° in different combinations. Due to the overlapping resonances of *N*H9 and *N*H13 some of the nOe's are indistinguishable (e, e', f, f'; Figure 2.6) between the different conformers modeled.

Table 2.3. Comparison of $^3J_{observed}$ vs. $^3J_{calculated}$ for cyclocinamide A diastereomers.

Diastereomer-Conformer Atom #	Coupling for H4→H3, 3' J_{obs} (3.00, 3.00) (J_{calc}, Hz)	Coupling for H11→H10, 10' J_{obs} (3.00, 3.00) (J_{calc}, Hz)	Expected NOE a, b, c, d, e/e', f/f'	Energy (kcal/mol)
4*R*, 11*R*-C8/C12	2.8, 2.5	3.9, 2.0	+, +, +, +, -, -	-23.3
4*R*, 11*R*-C8	2.8, 2.4	3.0, 2.7	+, +, +, +, +, +	-23.1
4*R*, 11*R*-C12	2.9, 2.4	11.8, 3.8	+, +, +, +, +, +	-22.6
4*R*, 11*R*-C1	3.9, 1.5	11.8, 3.2	+, -, -, -, -, +	-22.0
4*R*, 11*R*	3.3, 1.7	11.8, 3.2	+, +, +, +, +, +	-21.7
4*R*, 11*R*-C3	11.0, 4.0	11.7, 3.0	+, +, +, +, +, +	-20.6
4*R*, 11*S*-C8	2.8, 2.5	11.8, 3.4	+, +, +, +, +, +	-23.3
4*R*, 11*S*-C8/C12	3.1, 2.1	11.4, 2.3	+, +, +, +, -, +	-23.0
4*R*, 11*S*-C12	2.9, 2.4	3.1, 2.5	+, +, +, +, +, +	-22.5
4*R*, 11*S*	3.6, 1.8	3.0, 2.6	+, +, +, +, +, -	-20.7
4*S*, 11*R*-C3/C8	3.8, 1.6	2.8, 2.7	+, +, +, +, +, +	-22.7
4*S*, 11*R*-C3	3.8, 1.6	11.7, 2.9	+, +, +, +, +, +	-22.6
4*S*, 11*R*	10.7, 5.1	11.8, 3.5	+, +, +, +, +, +	-22.1
4*S*, 11*R*-C1	10.9, 4.5	11.8, 3.2	-, +, -, -, -, +	-21.5
4*S*, 11*S*-C1/C3/C12	4.1, 1.5	2.8, 2.7	+, -, -, -, +, +	-28.0
4*S*, 11*S*-C3/C12	5.4, 1.0	2.8, 2.8	+, +, +, +, -, +	-26.2
4*S*, 11*S*-C3/C5/C12	4.6, 1.2	3.1, 2.4	+, +, +, -, -, +	-24.8
4*S*, 11*S*	10.8, 4.8	2.8, 2.7	+, -, +, +, +, +	-21.0

The lowest energy conformation for each diastereomer was searched by minimizing several different conformations followed by molecular dynamics and finally by energy minimization again until the energy gradient was less then .0001 Kcal/mol.[20] Several conformers for each diastereomer were modelled as shown in Table 2.3 (Figures 2.41-2.58 in Supp. Mat.). Four or more low energy conformers were found for each diastereomer whose predicted conformation across the C3-C4 bonds and C10-C11 bonds is consistent with that estimated from the ^3J values. The distances estimated for the first three diastereomers, 4*R*/11*R*, 4*R*/11*S*, 4*S*/11*R*, are consistent with the observed nOe data. Similarly interconversion between the two lowest energy forms of the 4*S*/11*S* diastereomer in Table 2.3 results in a similar agreement. Thus, the absolute configurations at C4 and C11 are unassigned but might be suspected as being *S* based on biogenetic arguments.

Discussion

The isolation of cyclocinamide A (**1**) was greatly facilitated by the favorable cytotoxicity profile exhibited by semi-pure extract fractions in the Corbett-Valeriote soft agar disk diffusion assay.[21] For example, a fraction of coll. no. 93145 (FMS4) displayed zone sizes in the primary assay as follows [mg/disk: L1210/C38/M17-Adr/CX1] 100: 400/940/580/400 versus 5-fluorouracil (standard chemotherapeutic agent) [mg/disk: L1210/C38/CX1] 2.5: 40/900/0 which represents significant selective cytotoxicity. A zone differential of 250 (=6.5 mm) units or greater is the basis for denoting an agent as either a solid tumor or a leukemia selective compound. A 1-log dilution produces a 330 unit zone change on average. Thus, a 250 unit differential represents approximately an eight fold change in sensitivity. Purified **1** exhibited the following profile [mg/disk: L1210/C38/M17-Adr/CX1] 50: 0/500/0/0 and it represents robust tumor selectivity against C38. Only 2 mg of **1** was available for follow-up via an *in vivo* experiment. Thus, a single mouse with early stage M-16 (Mammary Adenocarcinoma #16) was given a total dose of 20 mg/kg but the response was no activity and no toxicity. Such experiments will be continued in the future using material being prepared by total synthesis in order to test for anti-tumor activity *in vivo* at the maximum tolerated dose.

Overall, the structure of cyclocinamide A has no parallels among known natural products. Its tetrapeptide core, consisting of a 14 membered ring, along with the dipeptide side chain terminating in the proline derived *N*-methyl chloro pyrrole are both extremely distinctive. A rather distant analogy to the unusual features present in **1** is represented by asterin, a plant derived 16-membered ring pentapeptide with a dichloroproline residue.[22] Aside from diketopiperazines, the list of other small cyclic peptides from marine organisms is very small and includes compounds such as the

nodularins[23]: cyclic pentapeptides from cyanobacteria; and kahalalide D[24]: a cyclic depsipeptide from a mollusk comprised of three amino acids cyclized by a β-hydoxy fatty acid.

Supplementary Material-
cyclocinamide A

Figure 2.7. ^{1}H-NMR Spectra (DMSO-d6, 500 MHz)

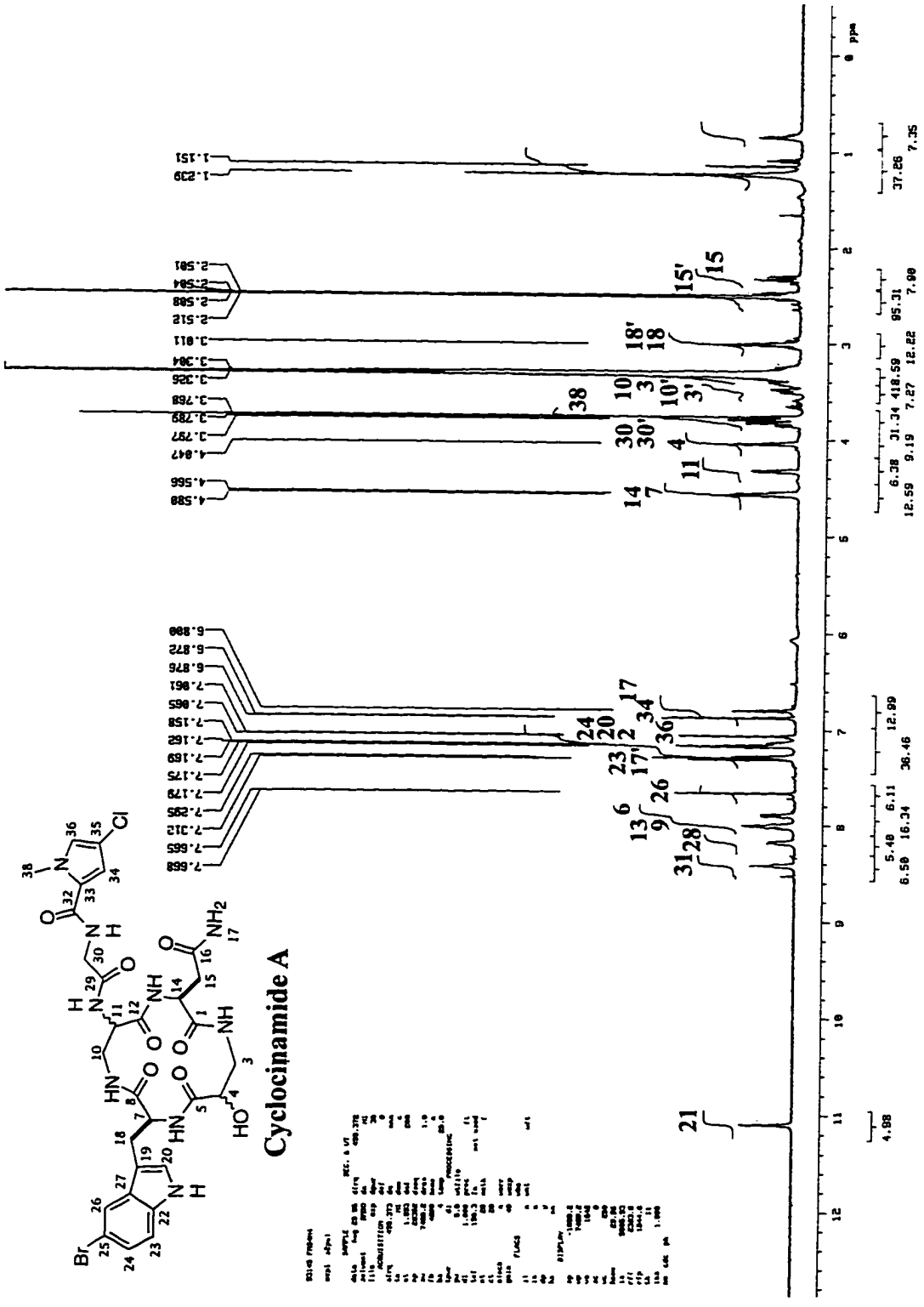


Figure 2.8. ^{1}H-NMR Spectra (CD_3OD, 500 MHz)

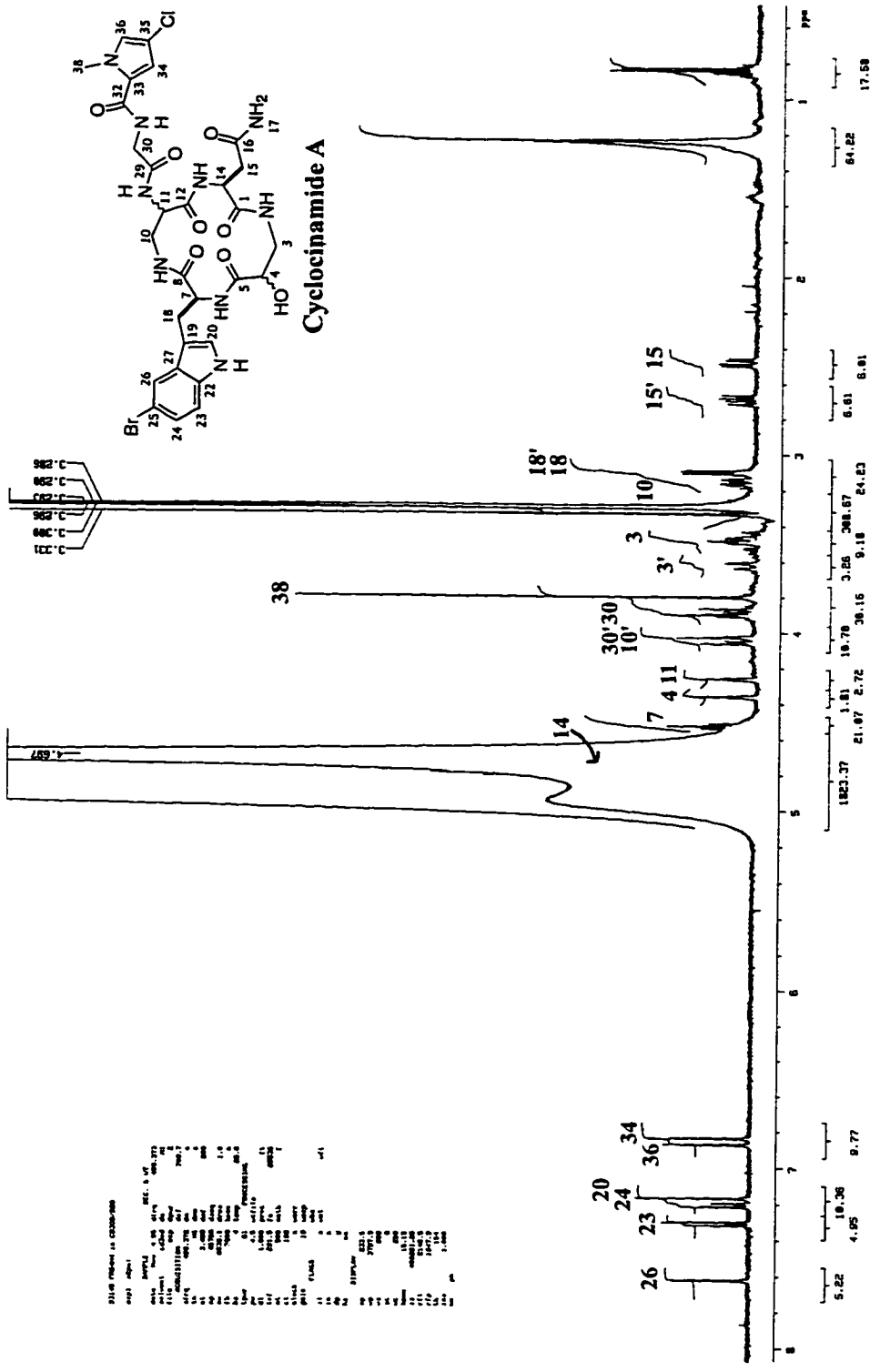


Figure 2.9. ^{1}H-NMR Spectra (DMSO-d6/Benzene-d6, 500 MHz)

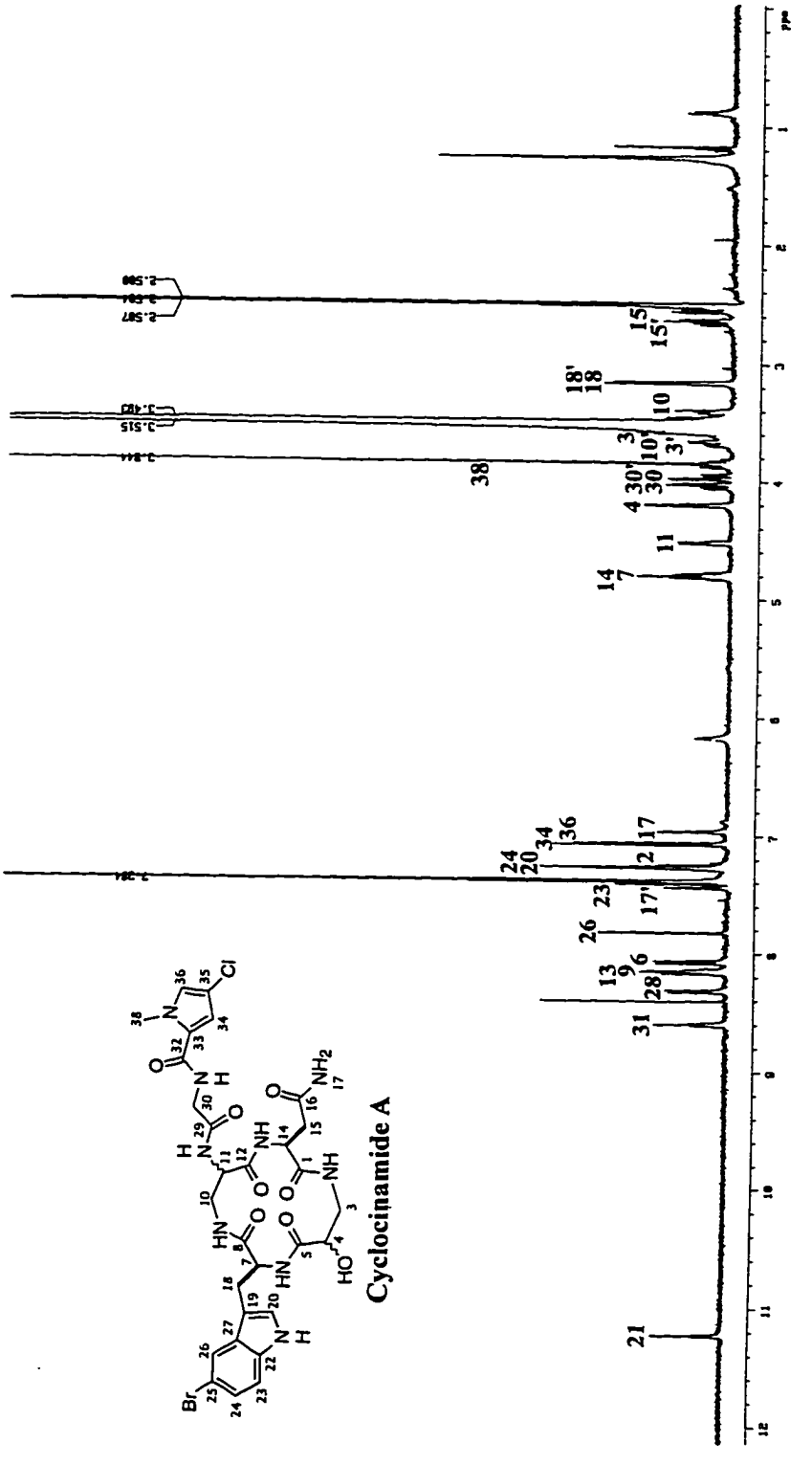


Figure 2.10. ^{1}H-NMR H_2O Suppression Spectra/45°C (DMSO-d6, 500 MHz)

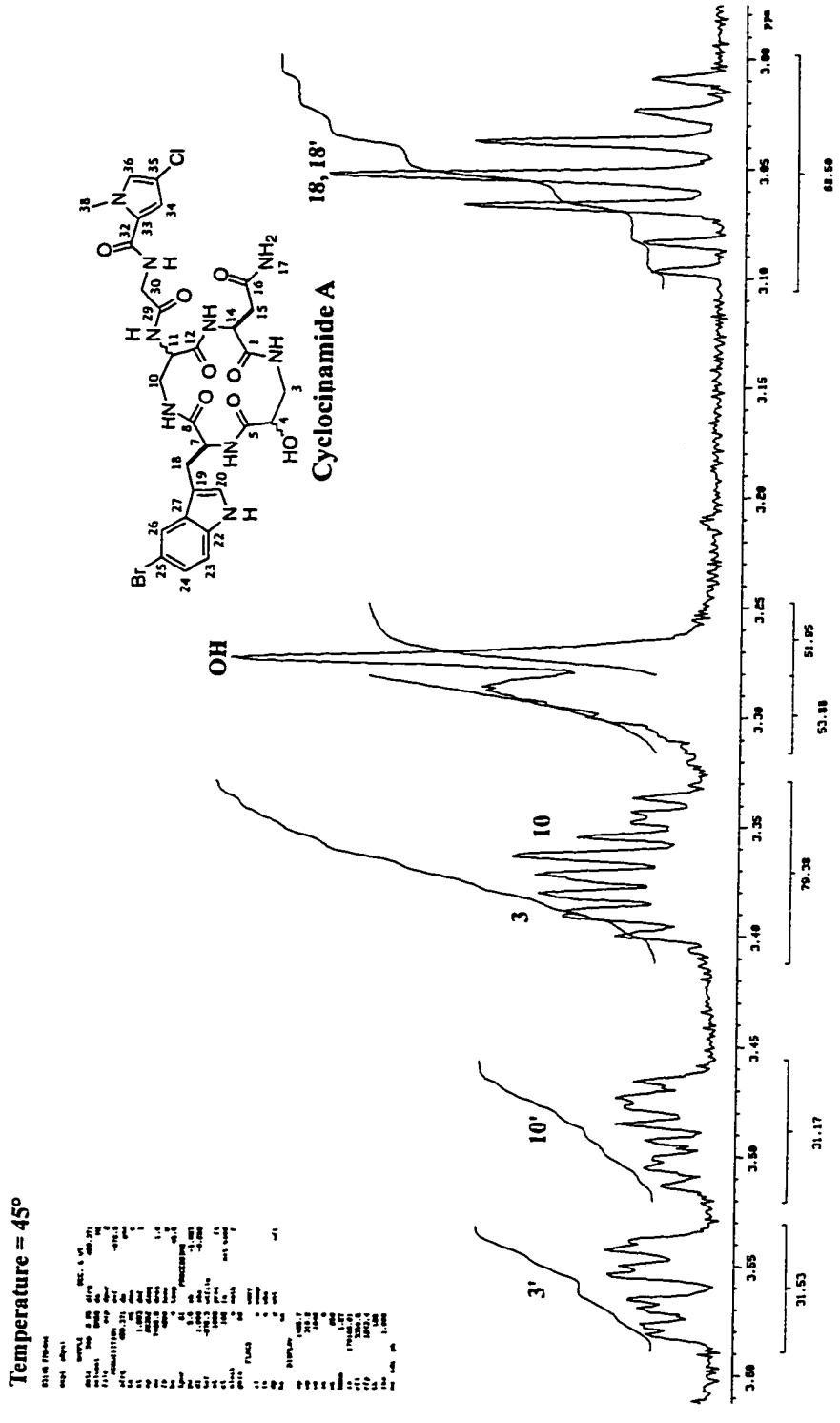


Figure 2.11. ^{13}C/Dept 135-NMR Spectra (DMSO-d6, 125 MHz)

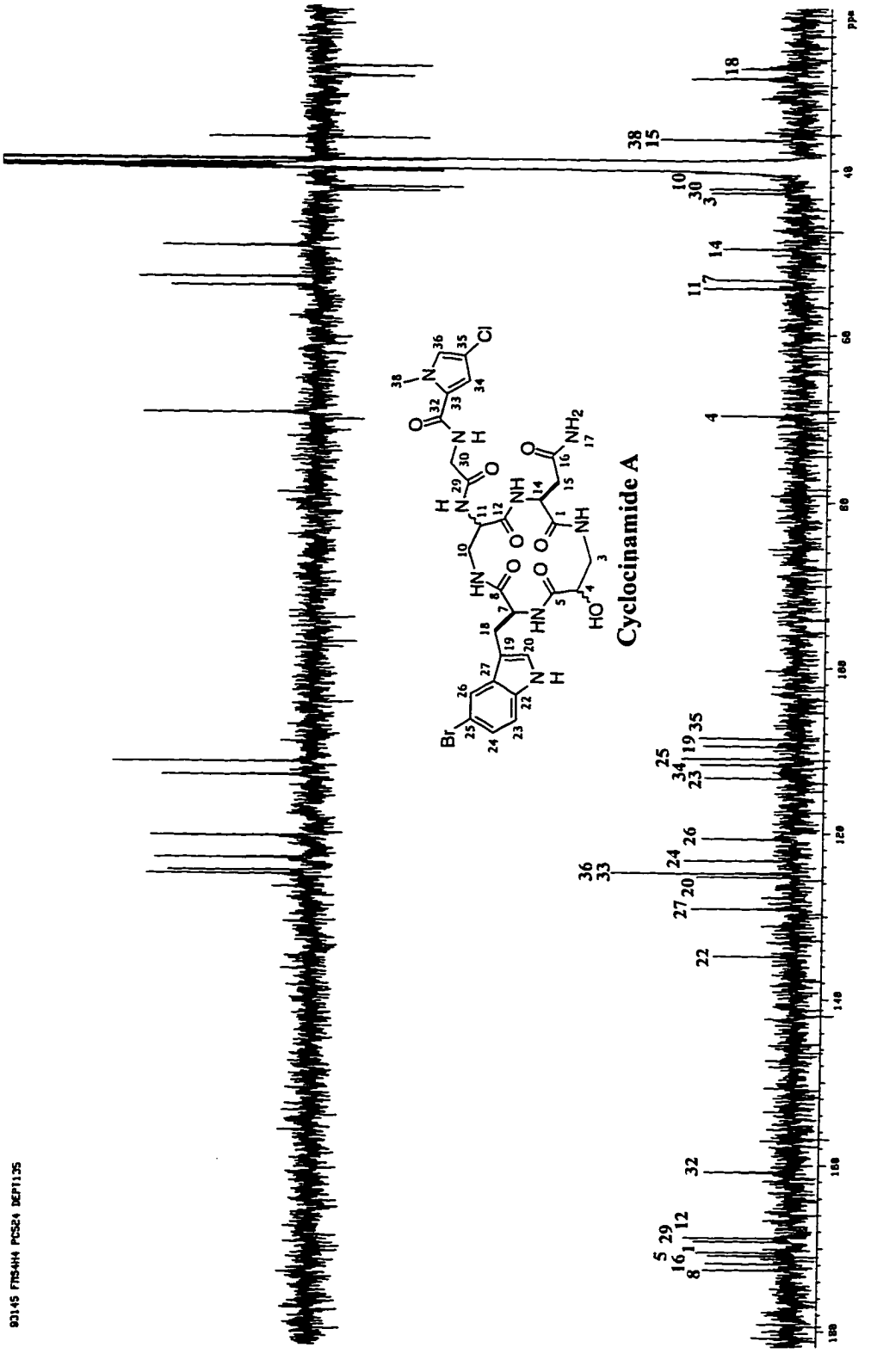


Figure 2.12. LRFAB Mass Spectra

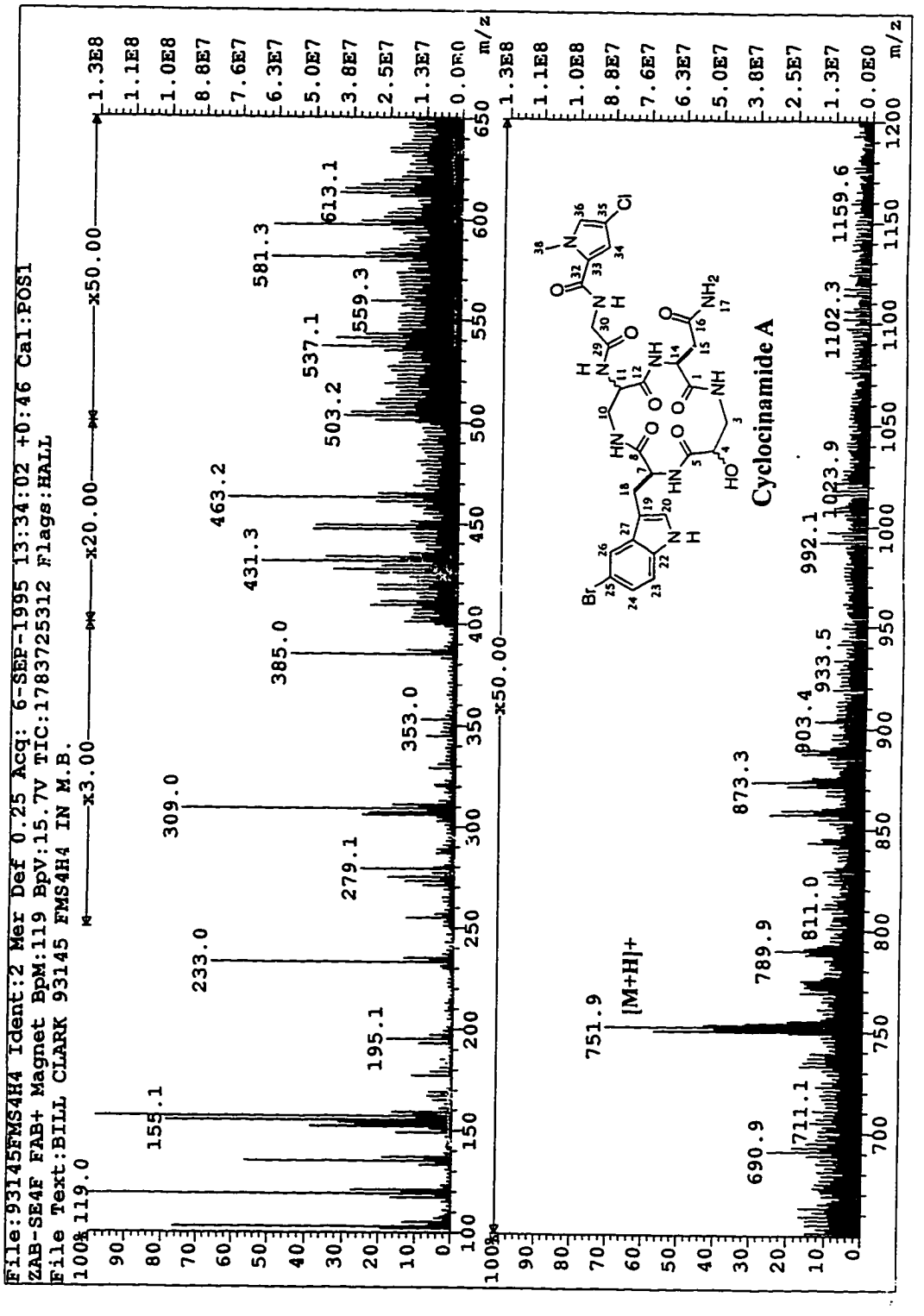


Figure 2.13. UV Spectra (MeOH)

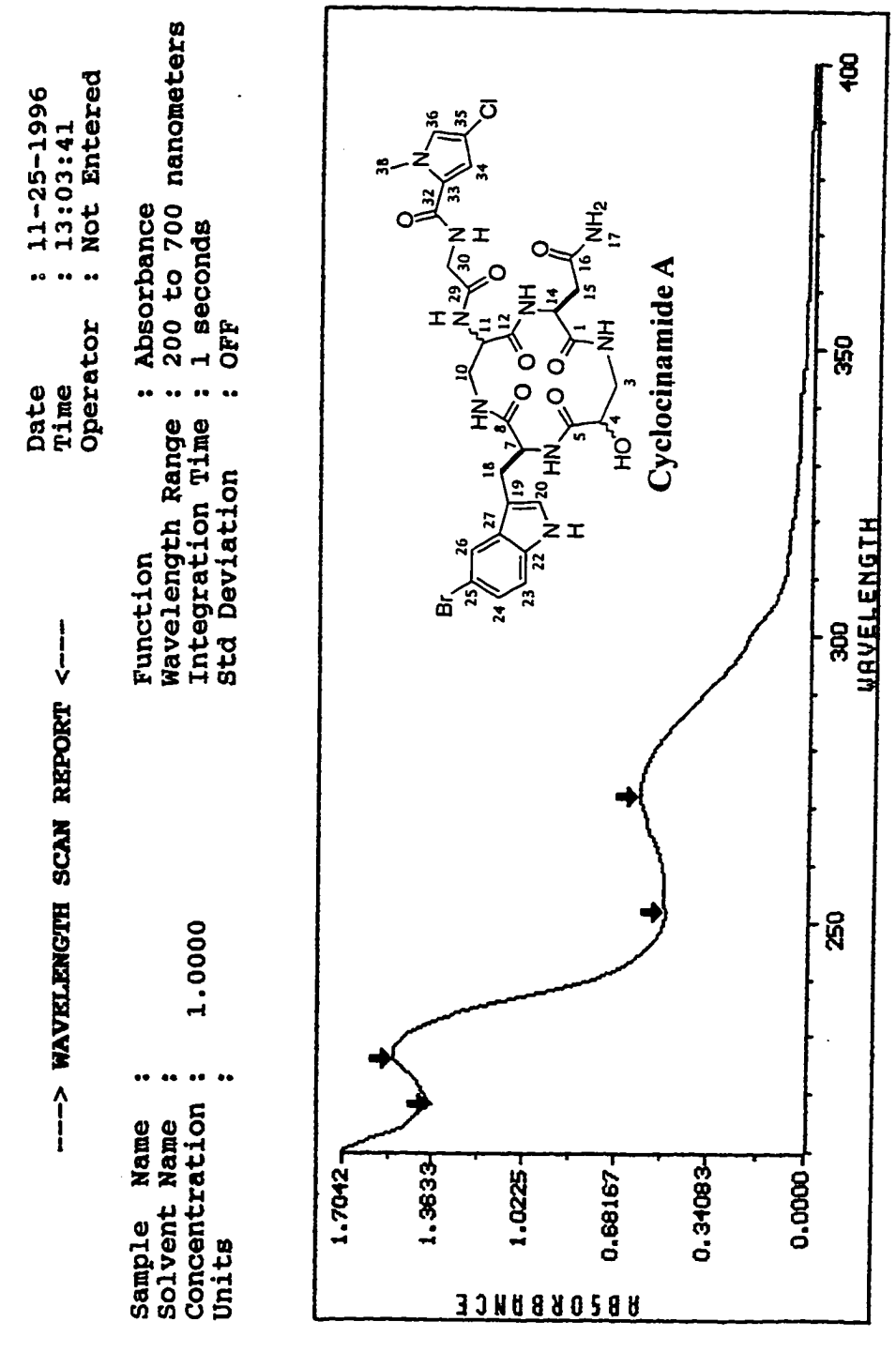


Figure 2.14. COSY Spectra (DMSO-d6, 500 MHz)

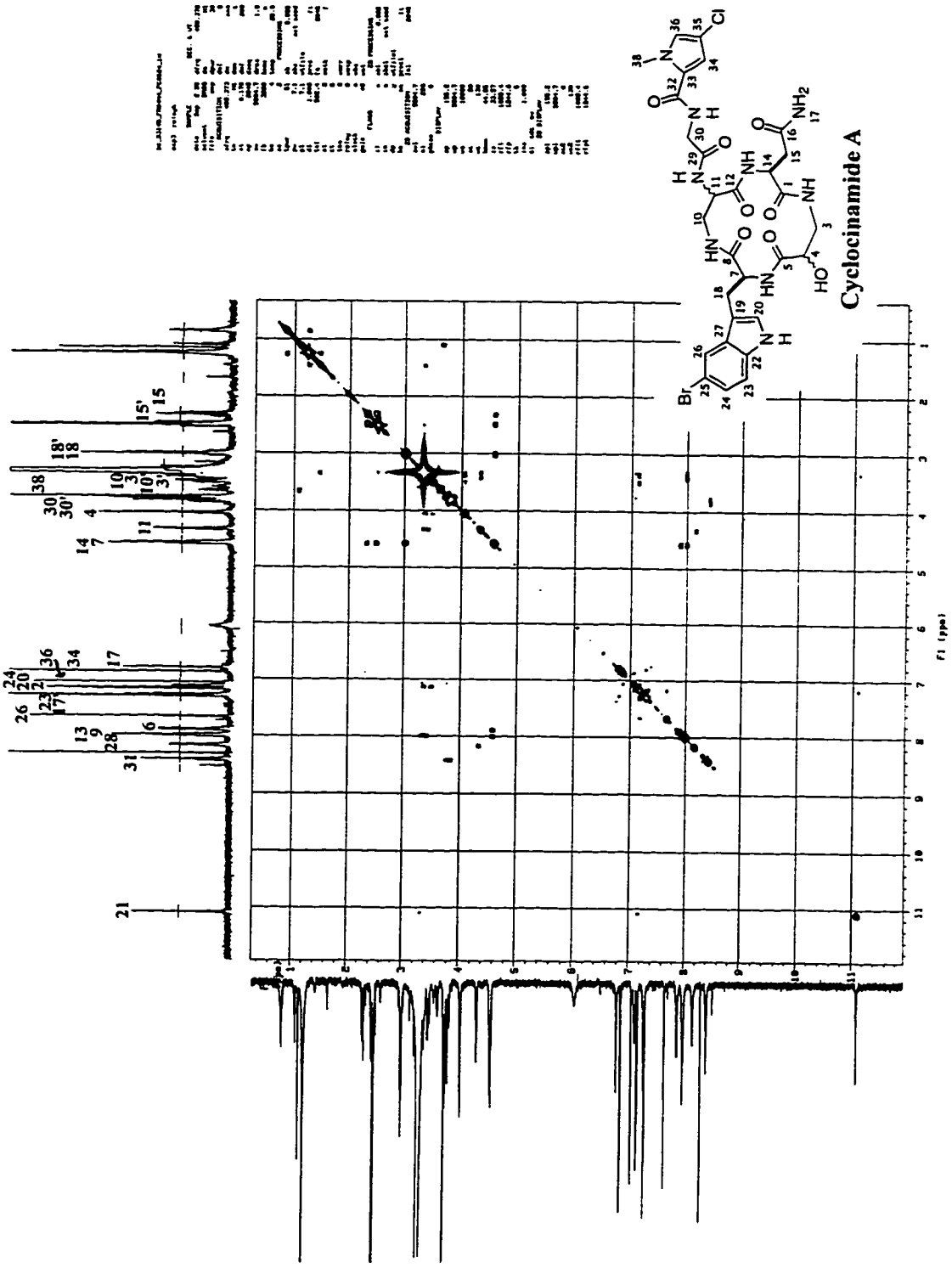


Figure 2.15. COSY Expansion Spectra (DMSO-d6, 500 MHz)

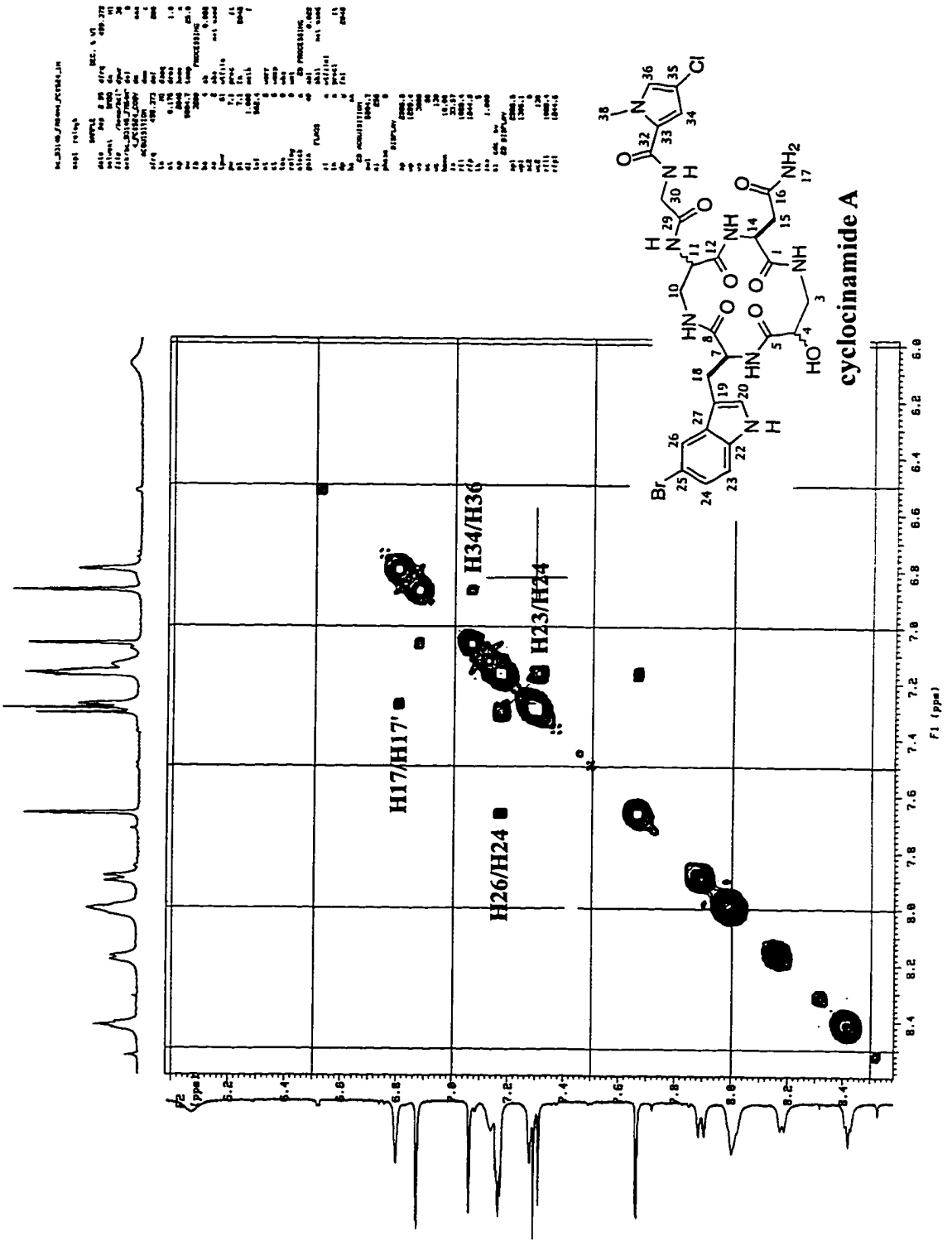


Figure 2.16. COSY Expansion Spectra (DMSO-d6, 500 MHz)

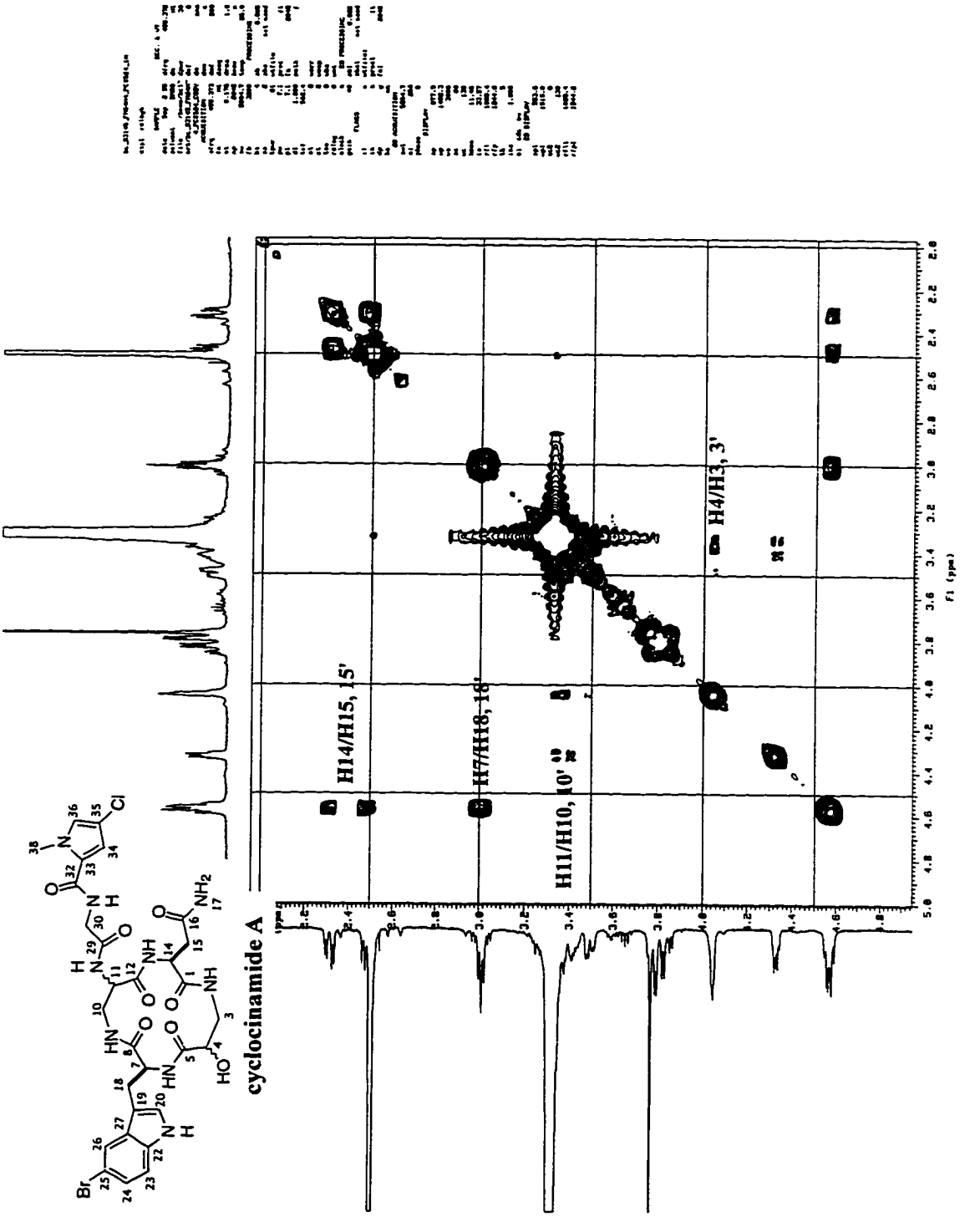


Figure 2.17. COSY Expansion Spectra (DMSO-d6, 500 MHz)

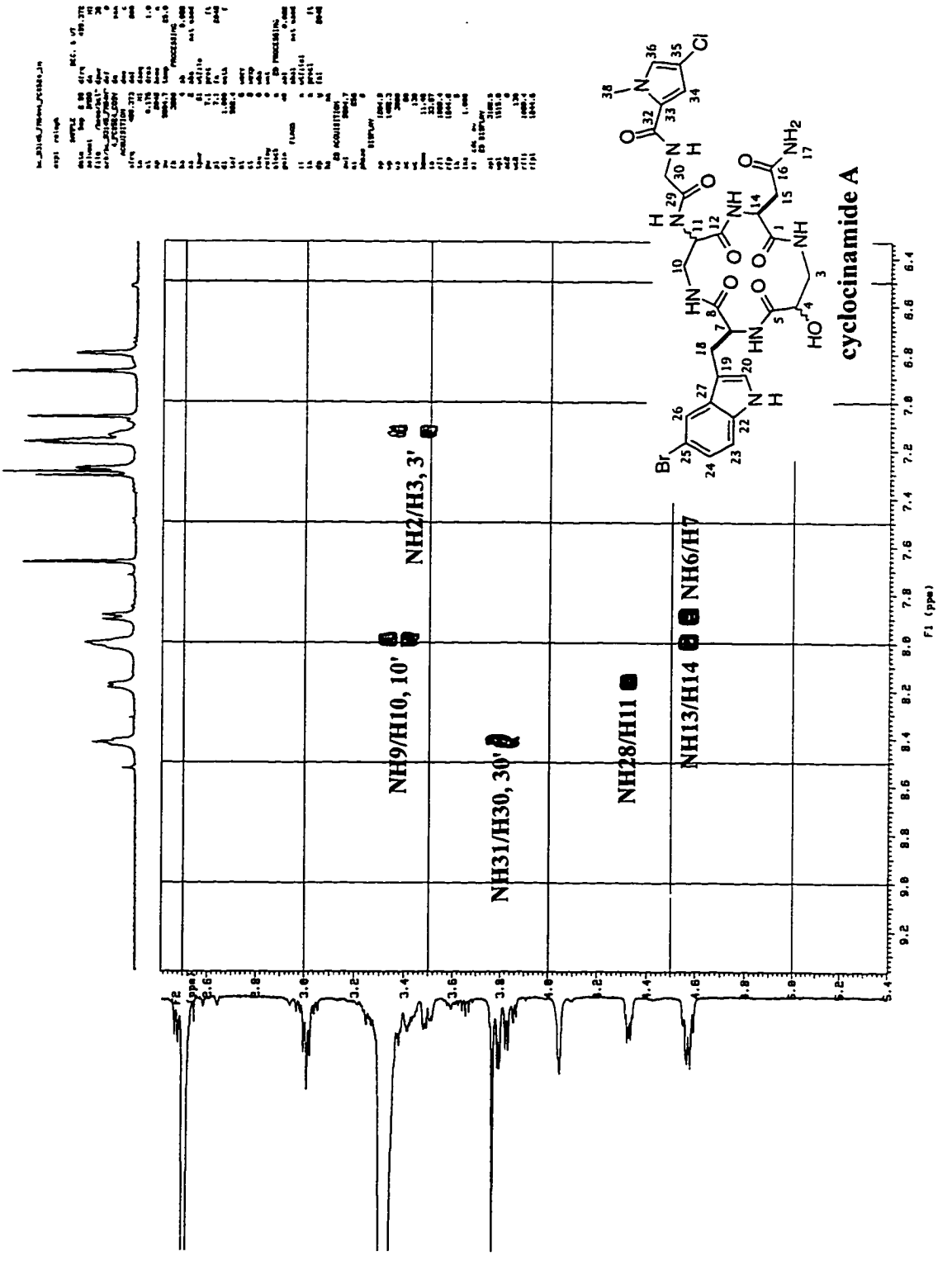


Figure 2.18. COSY Spectra (CD_3OD, 500 MHz)

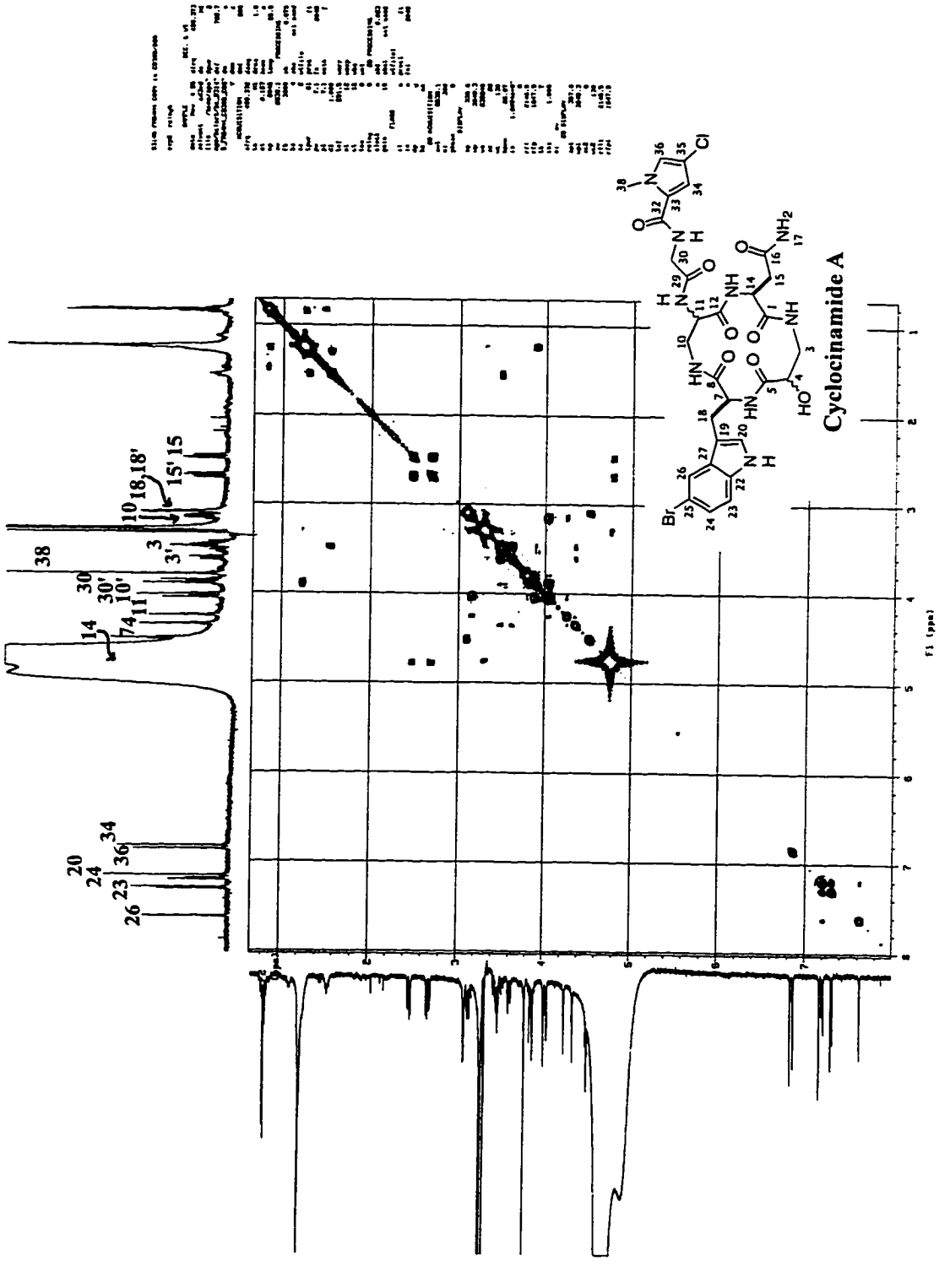


Figure 2.19. HMQC Spectra (DMSO-d6, 500 MHz)

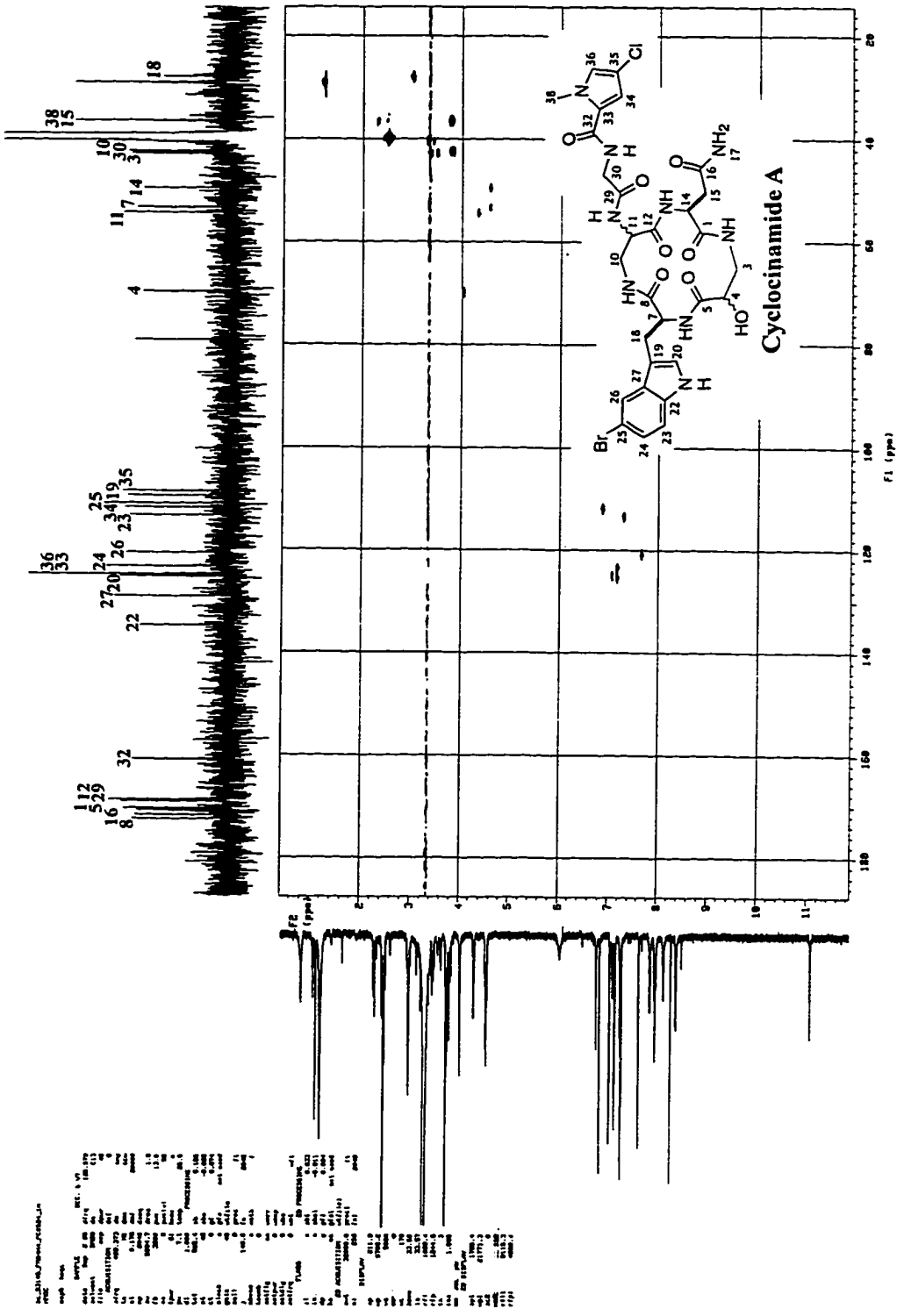


Figure 2.20. HMQC Expansion Spectra (DMSO-d6, 500 MHz)

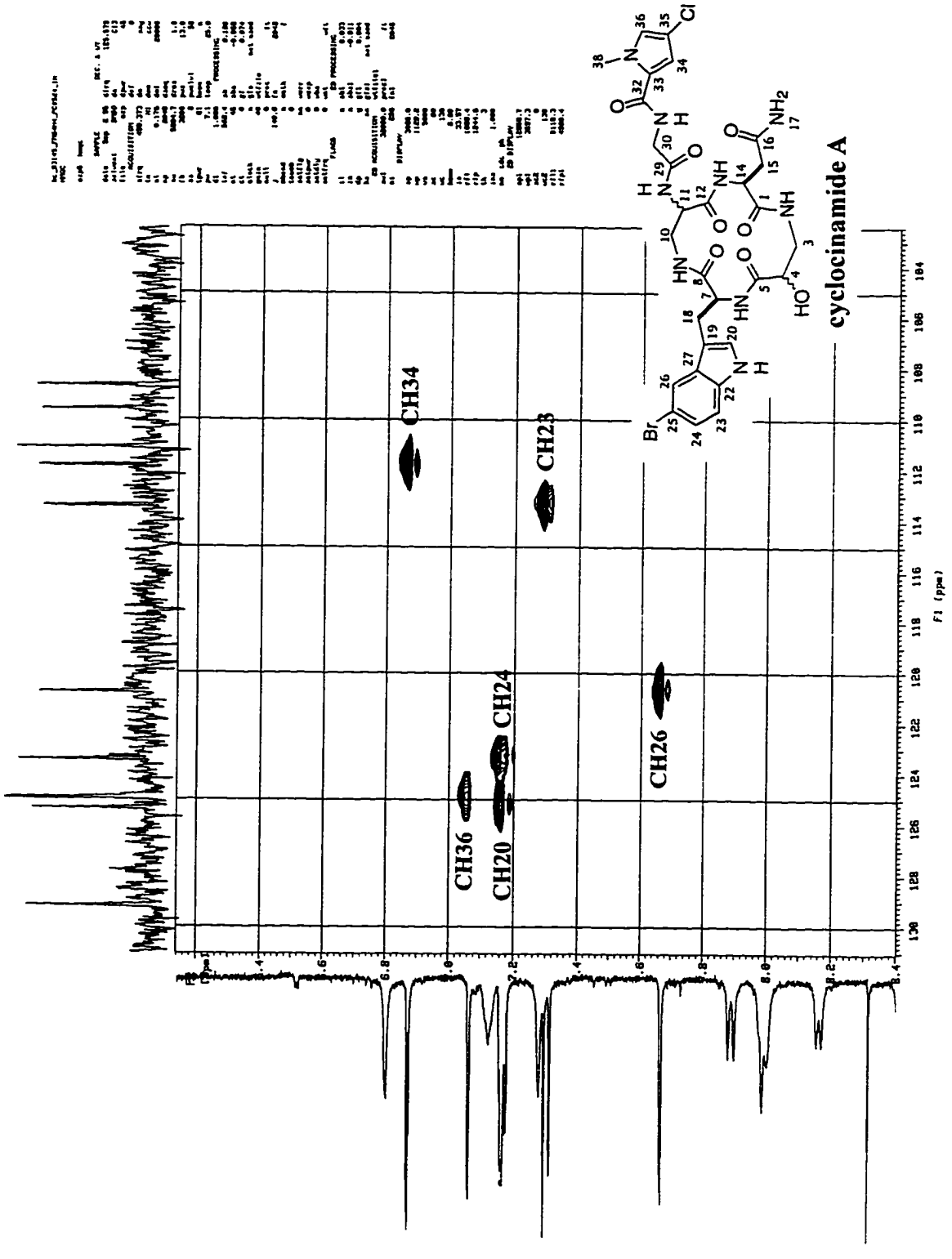


Figure 2.21. HMQC Expansion Spectra (DMSO-d6, 500 MHz)

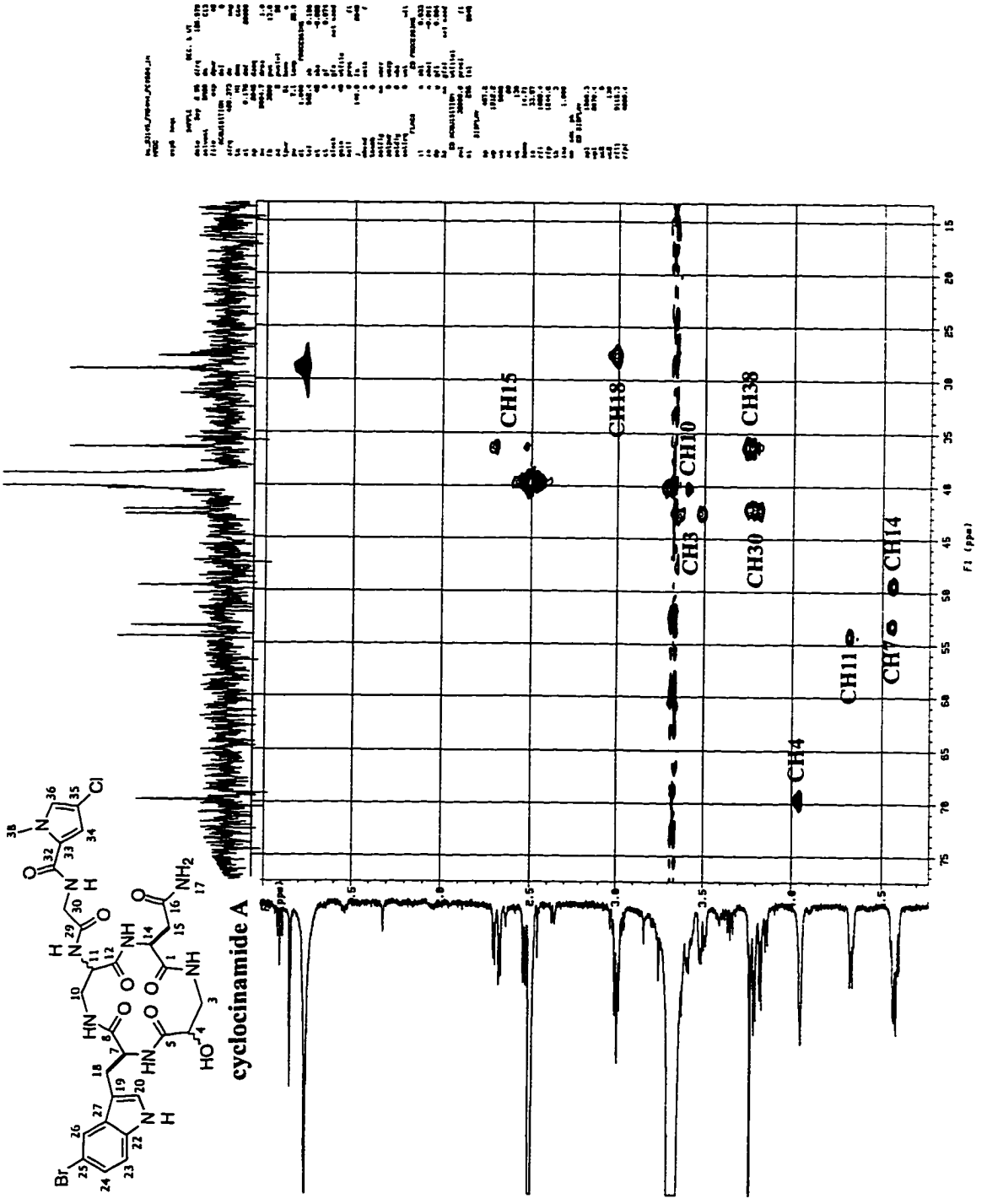


Figure 2.22. HMBC Spectra (J = 9 Hz, DMSO-d6, 500 MHz)

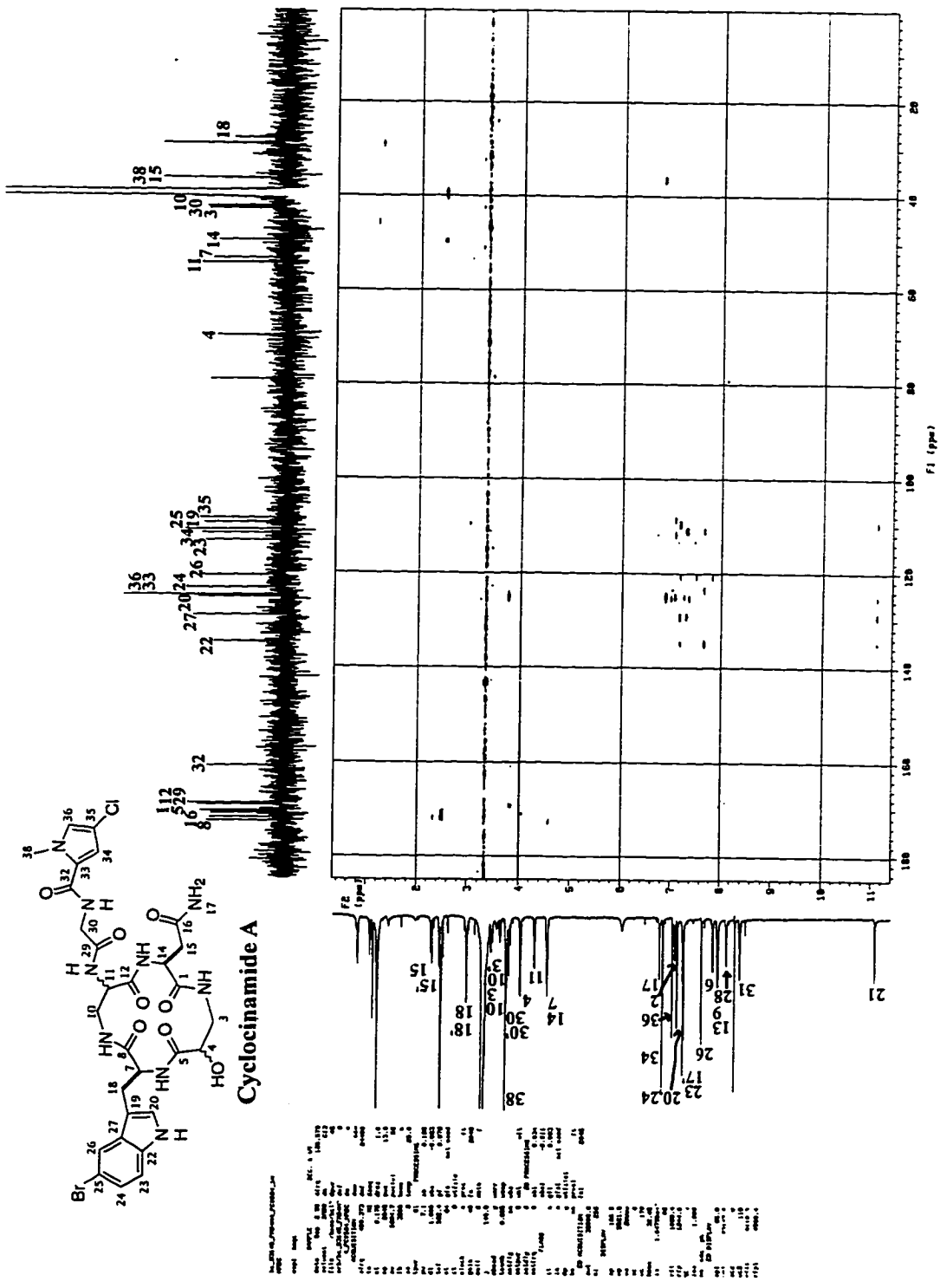


Figure 2.23. HMBC Expansion Spectra (J = 9 Hz, DMSO-d6, 500 MHz)

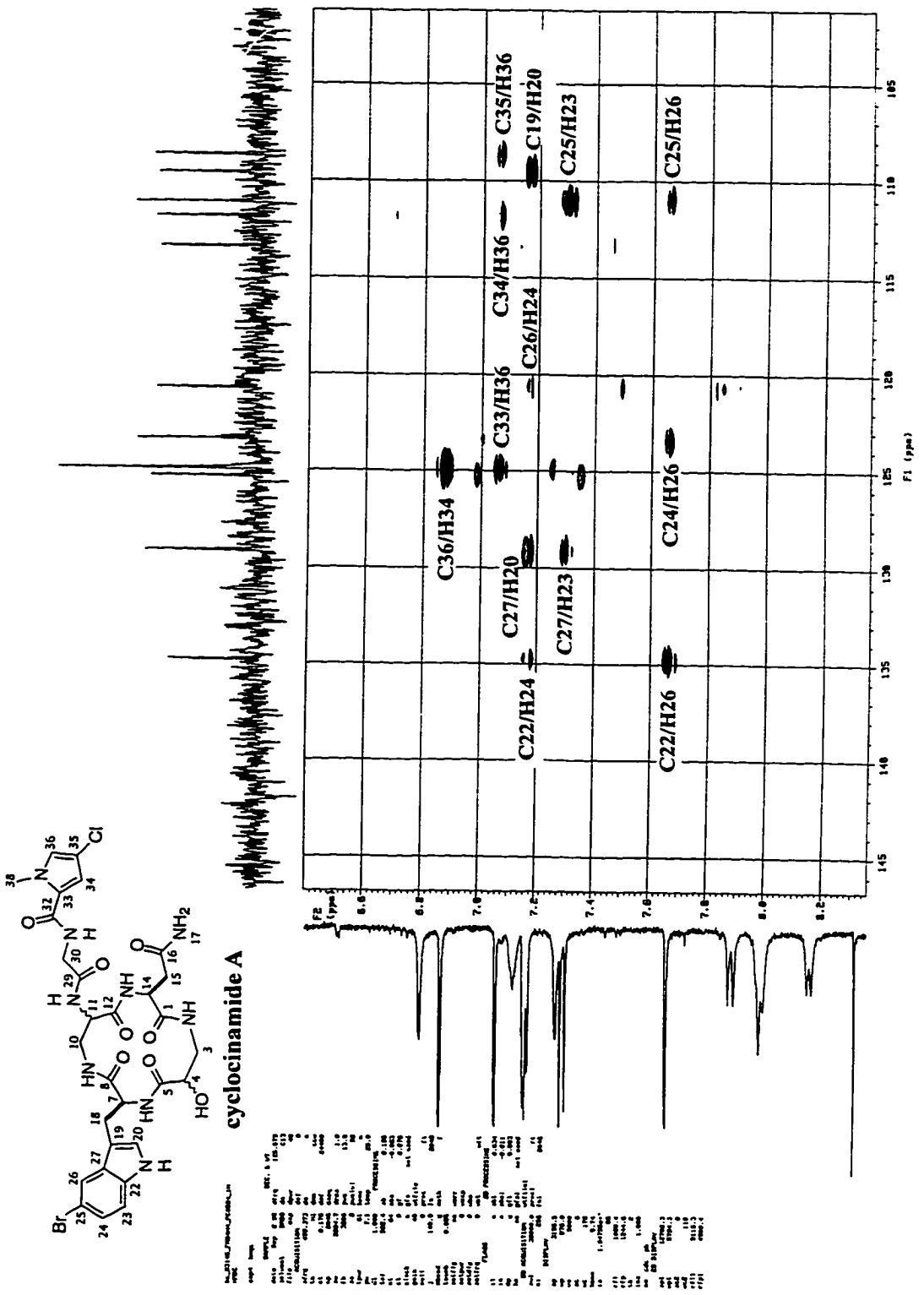


Figure 2.24. HMBC Expansion Spectra (J = 9 Hz, DMSO-d6, 500 MHz)

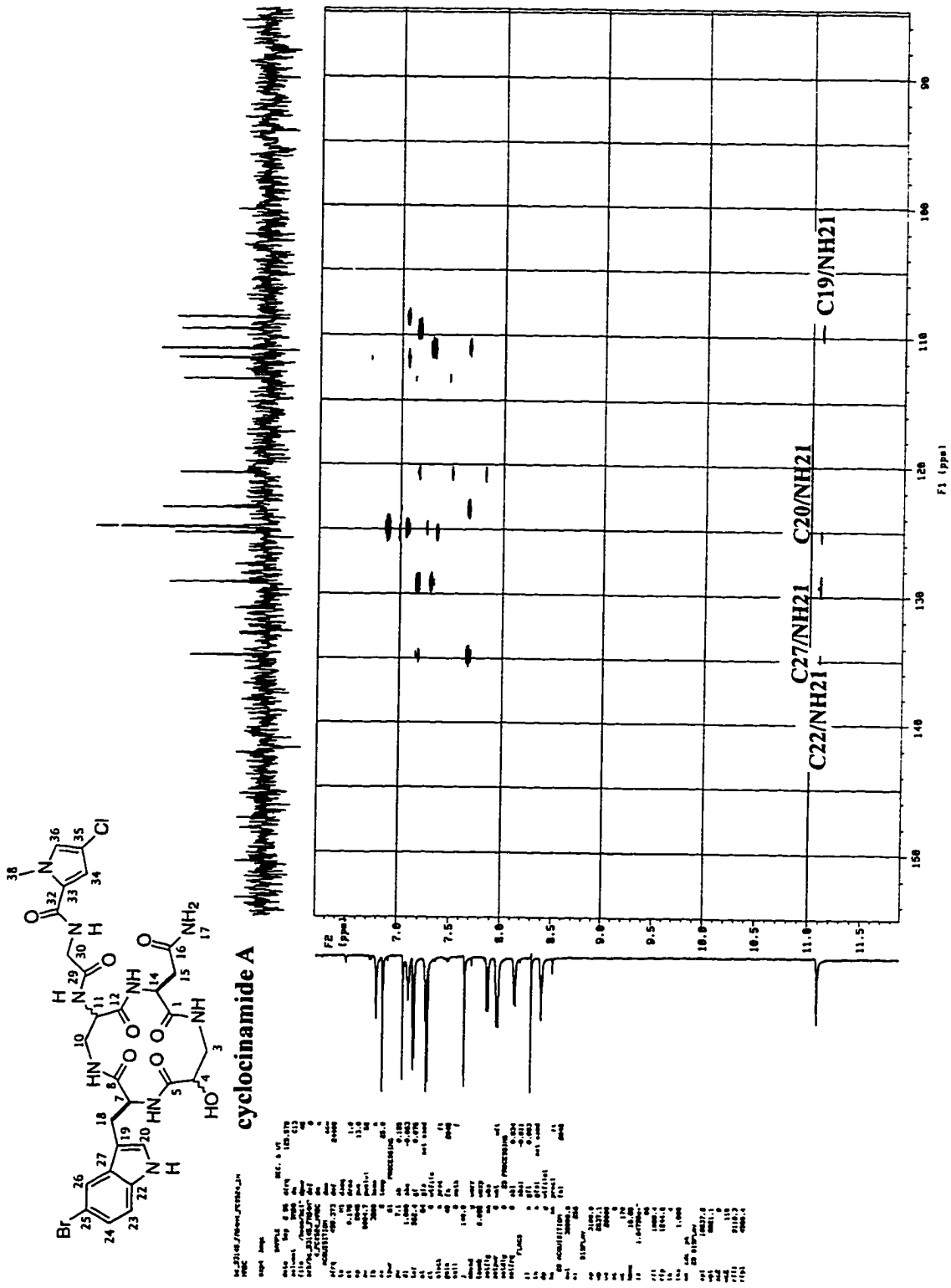


Figure 2.25. HMBC Expansion Spectra (J = 9 Hz, DMSO-d6, 500 MHz)

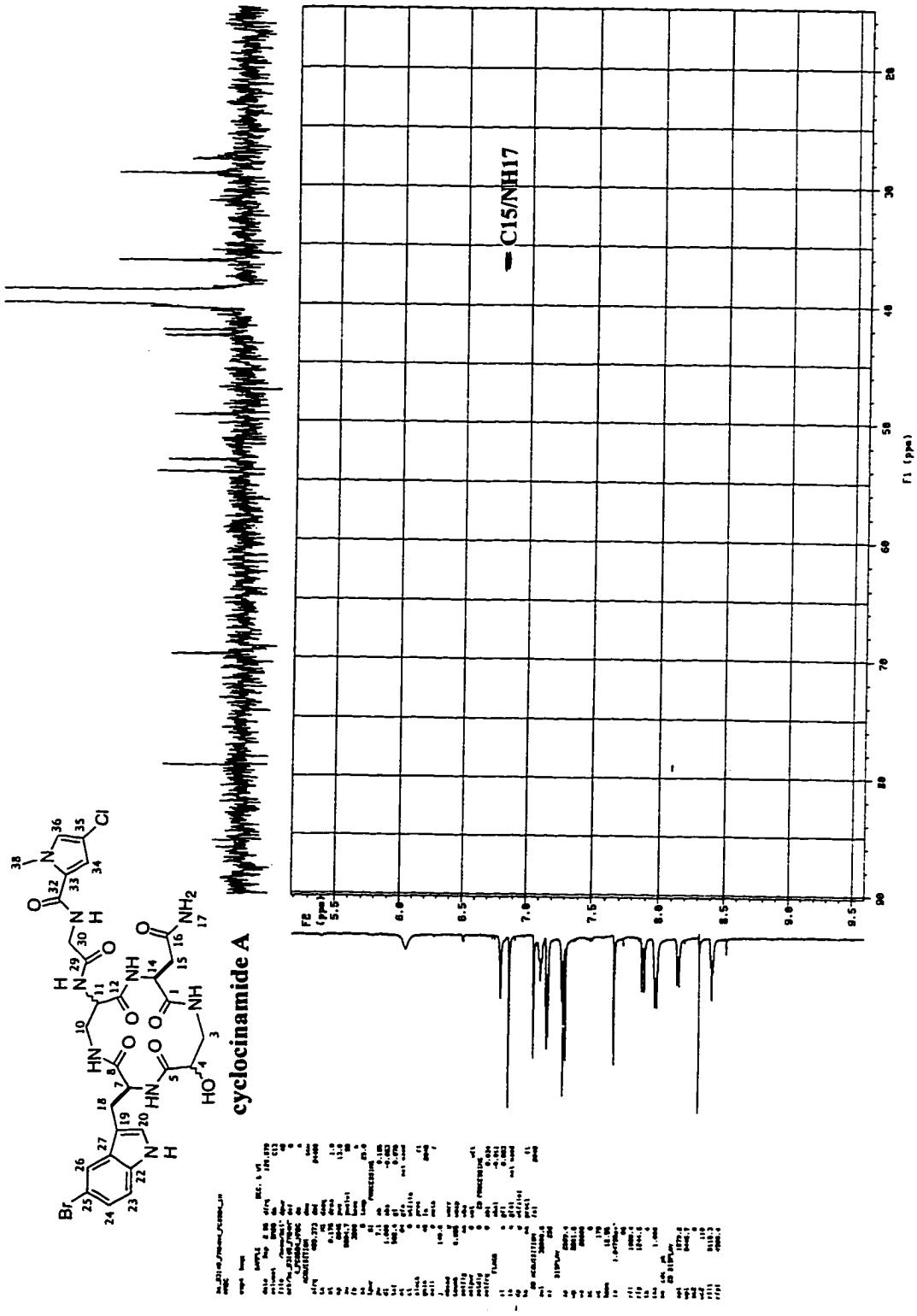


Figure 2.26. HMBC Expansion Spectra (J = 9 Hz, DMSO-d6, 500 MHz)

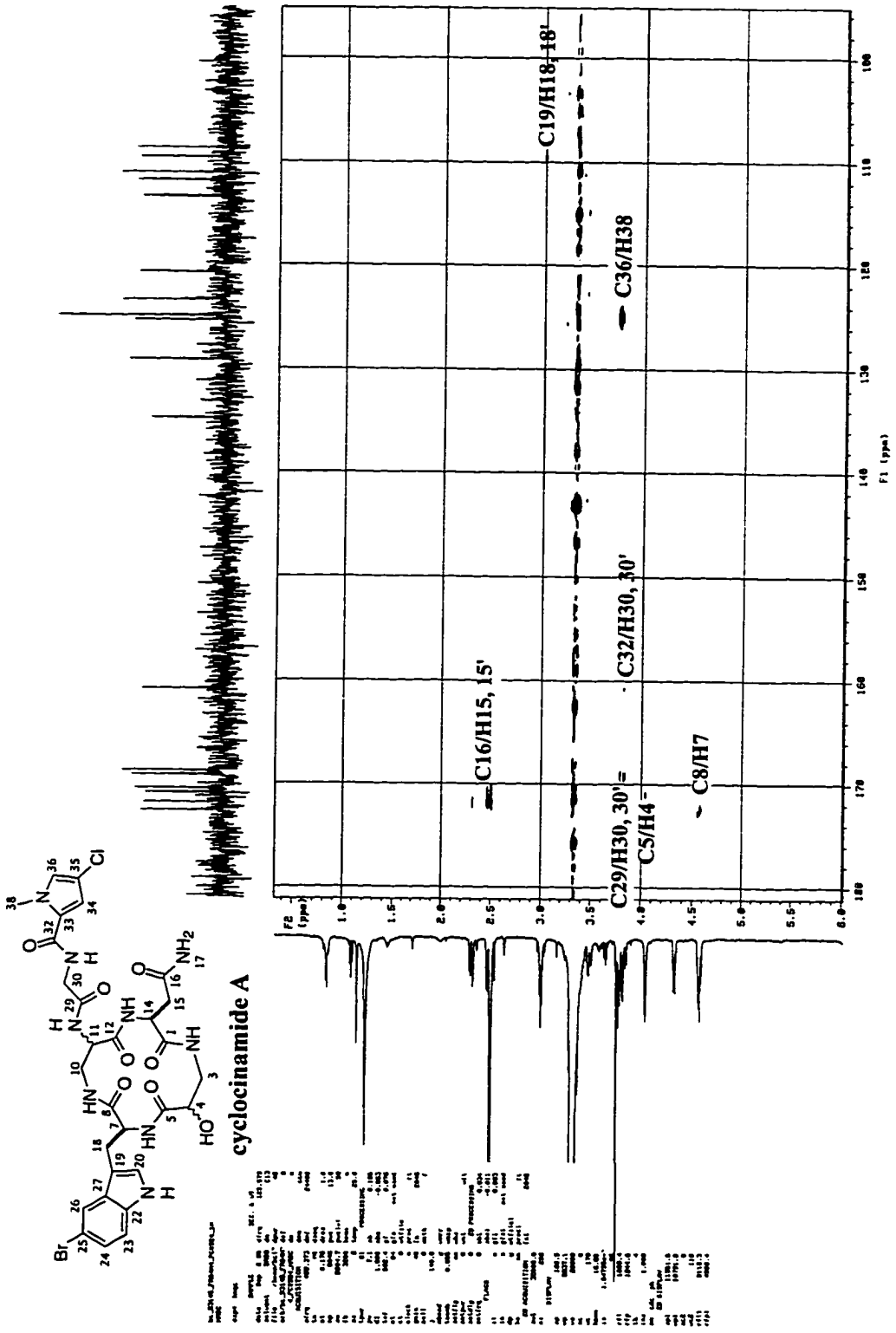


Figure 2.27. HMBC Expansion Spectra (J = 9 Hz, DMSO-d6, 500 MHz)

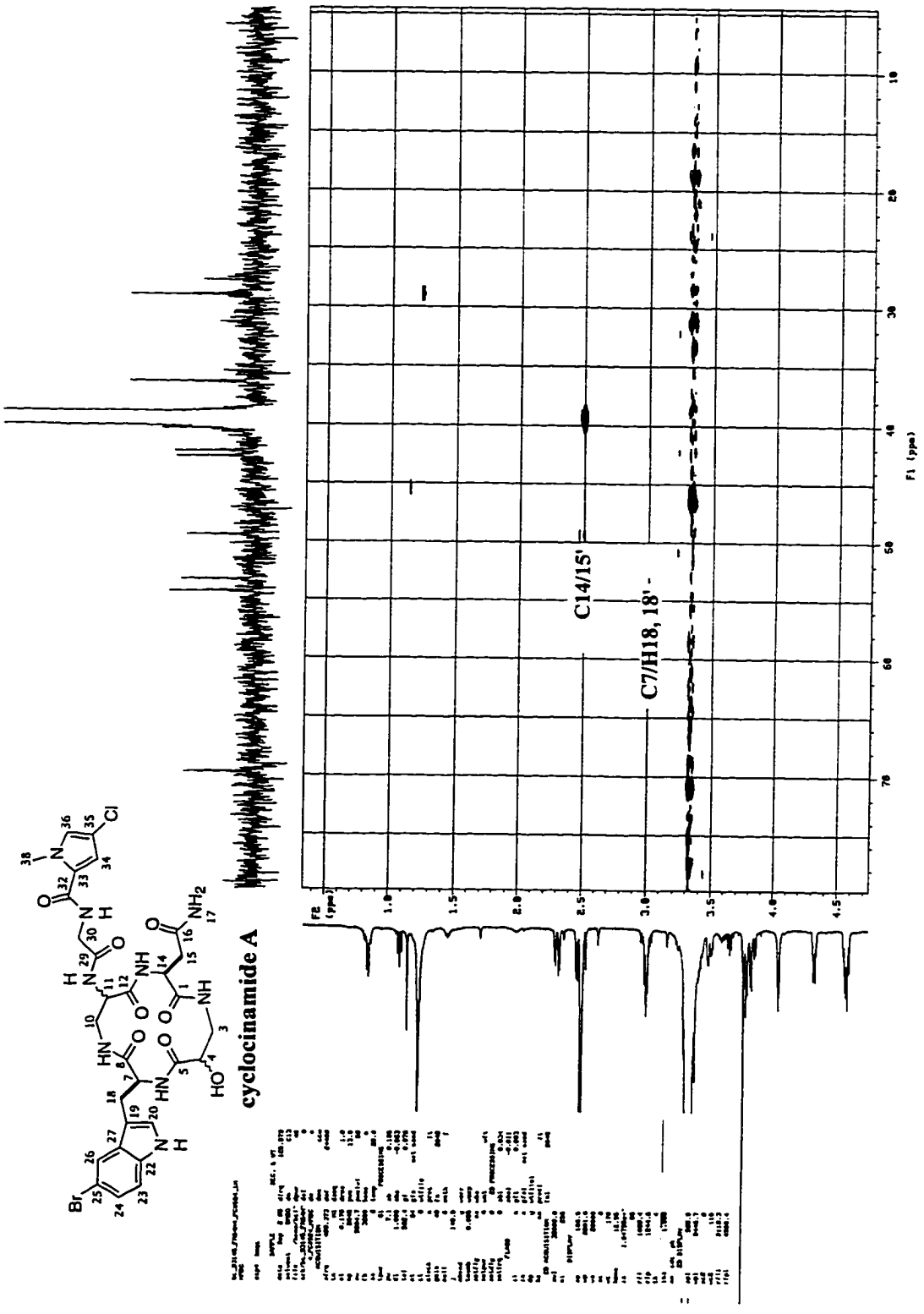


Figure 2.28. HMBC Spectra (J = 4 Hz, DMSO-d6, 500 MHz)

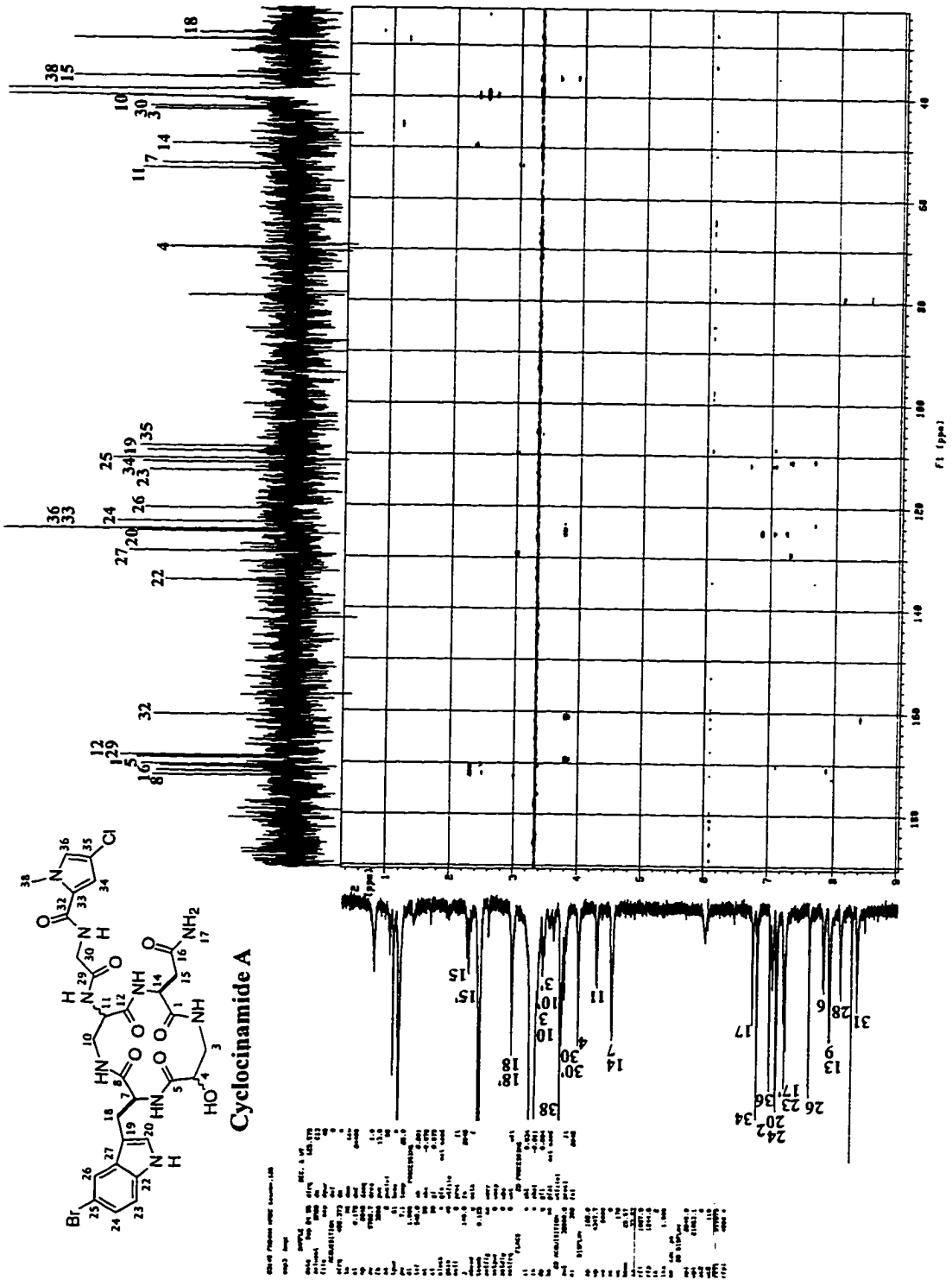


Figure 2.29. HMBC Expansion Spectra (J = 4 Hz, DMSO-d6, 500 MHz)

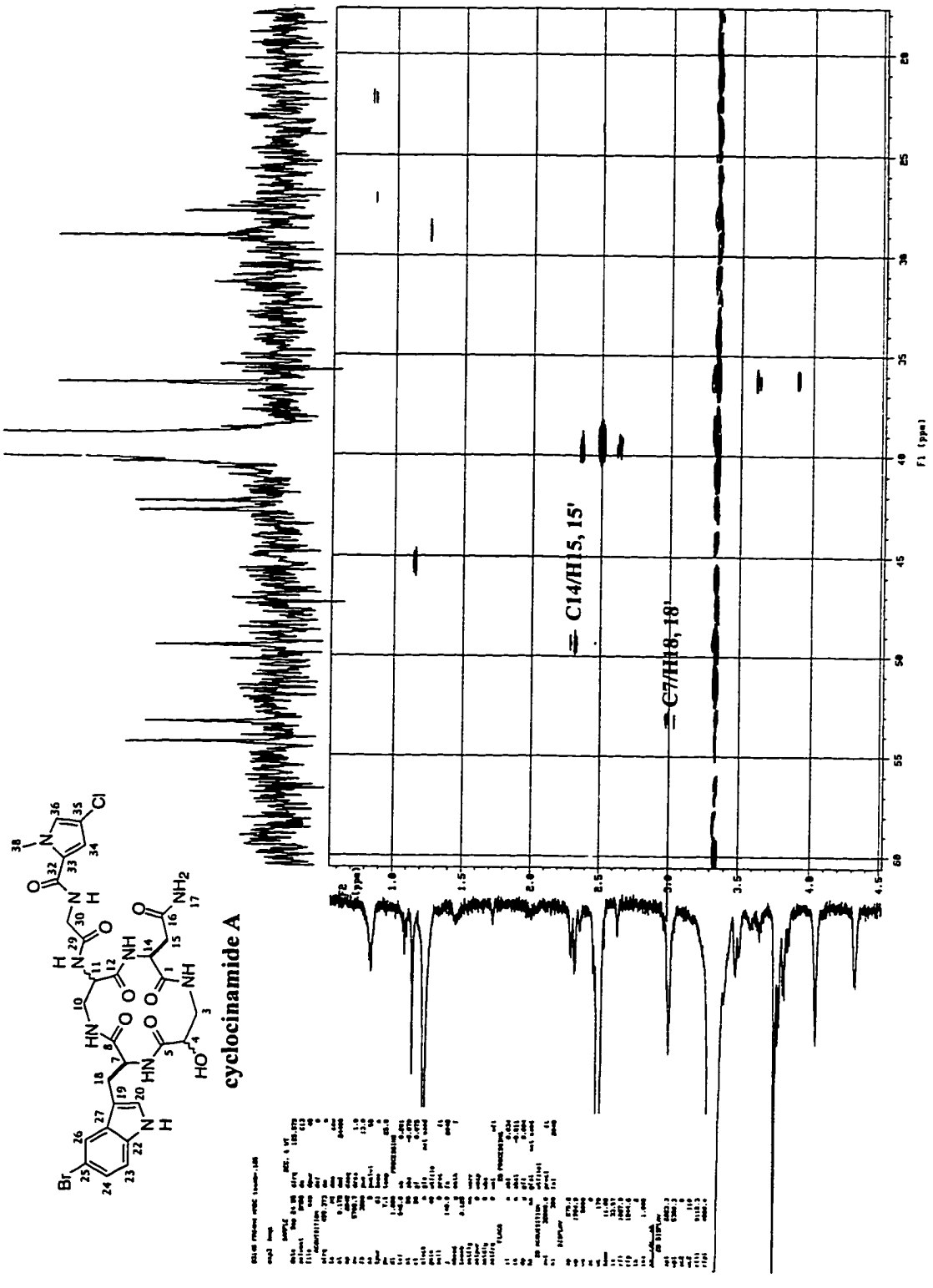


Figure 2.30. HMBC Expansion Spectra (J = 4 Hz, DMSO-d6, 500 MHz)

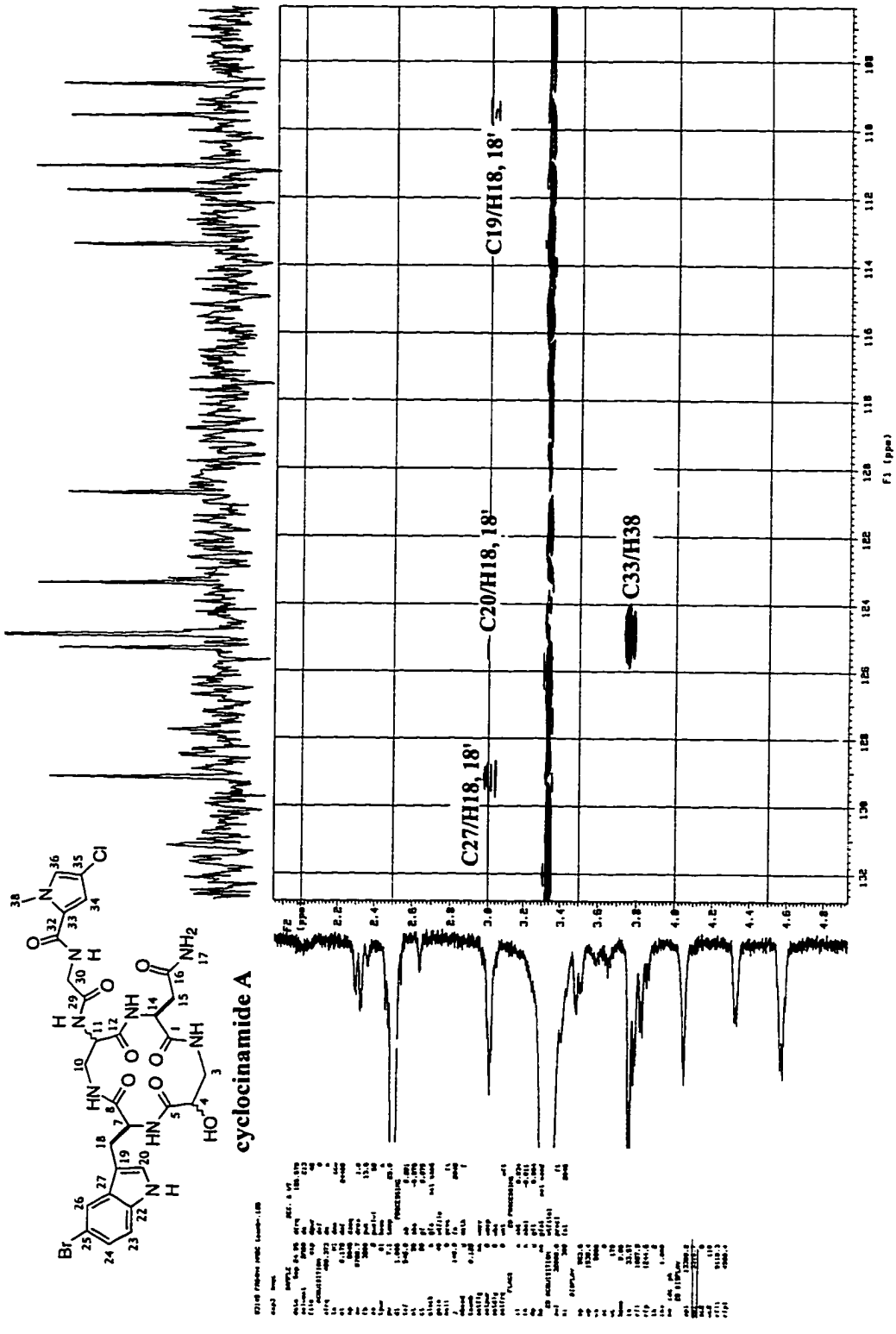


Figure 2.31. HMBC Expansion Spectra (J = 4 Hz, DMSO-d6, 500 MHz)

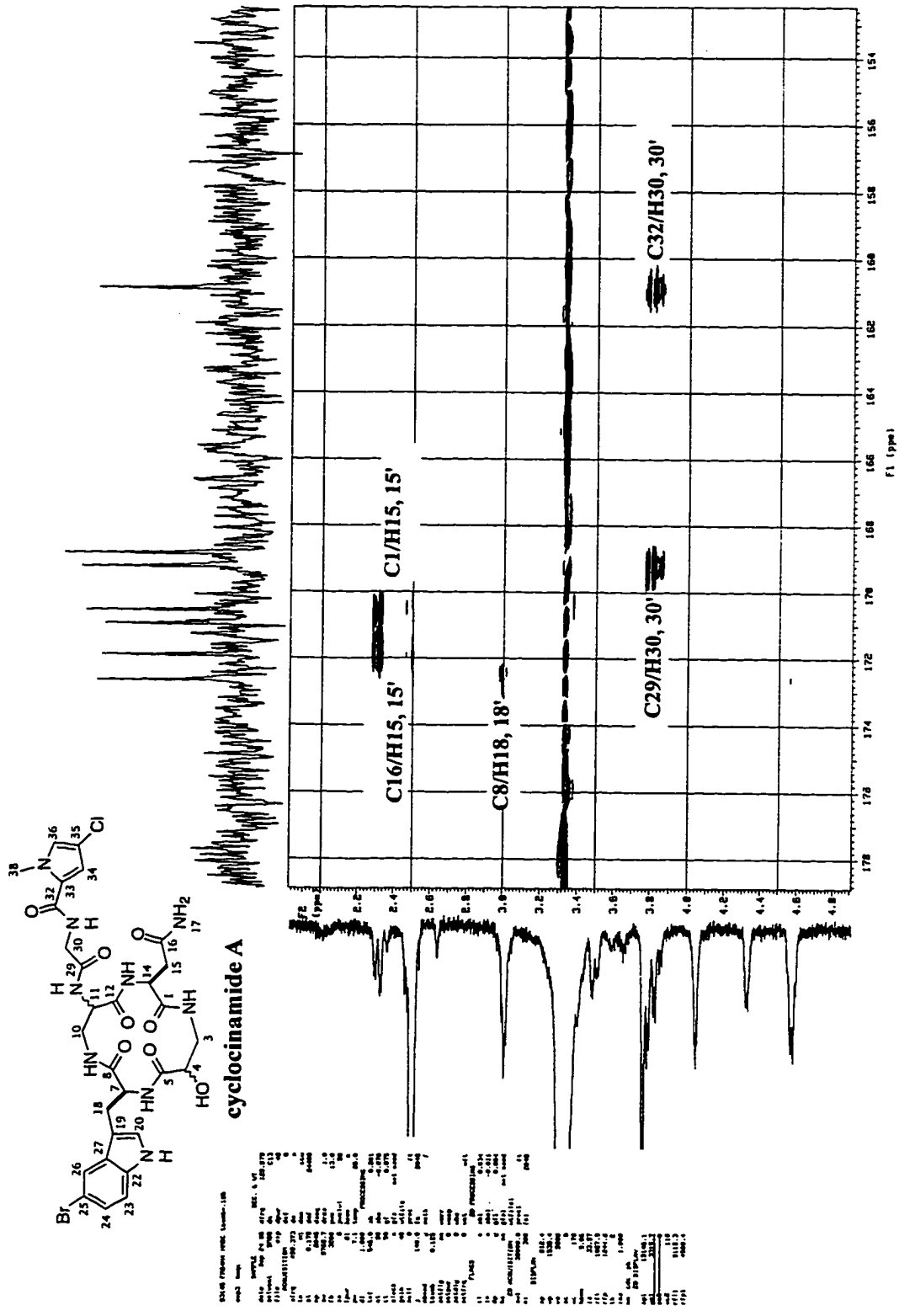


Figure 2.32. HMBC Expansion Spectra (J = 4 Hz, DMSO-d6, 500 MHz)

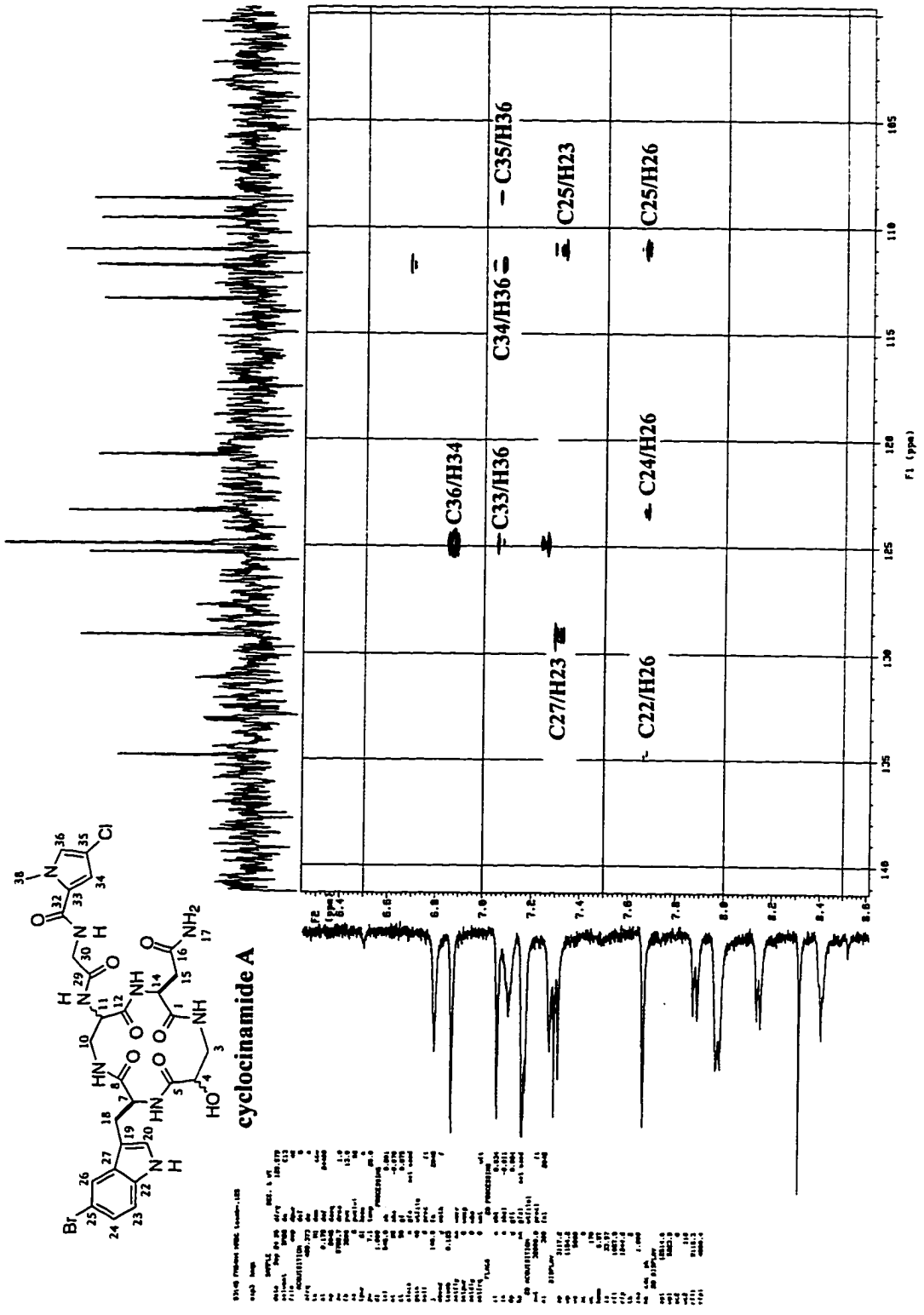


Figure 2.33. HMBC Expansion Spectra (J = 4 Hz, DMSO-d6, 500 MHz)

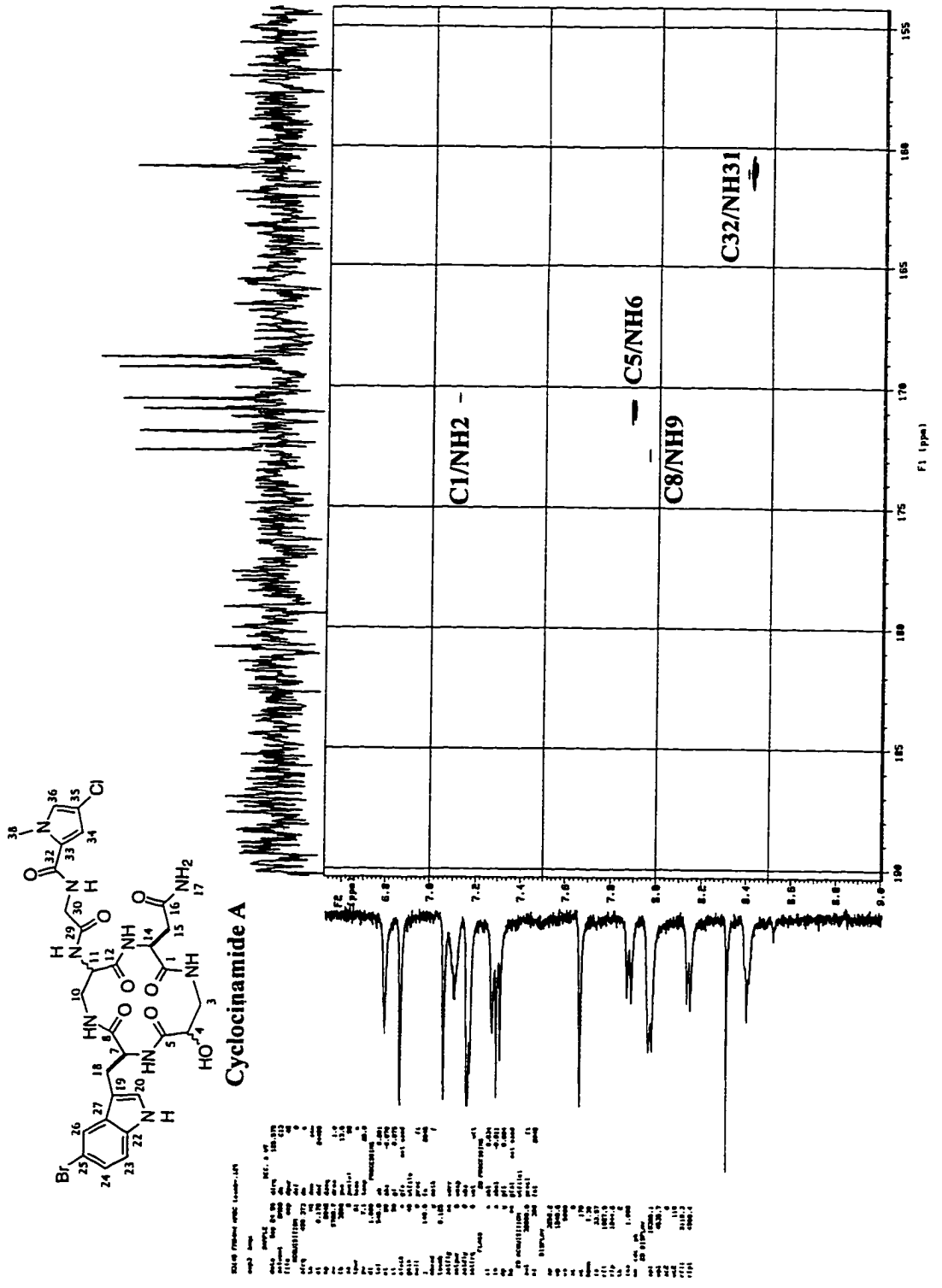


Figure 2.34. HMBC Spectra (DMSO-d6/Benzene-d6, 500 MHz)

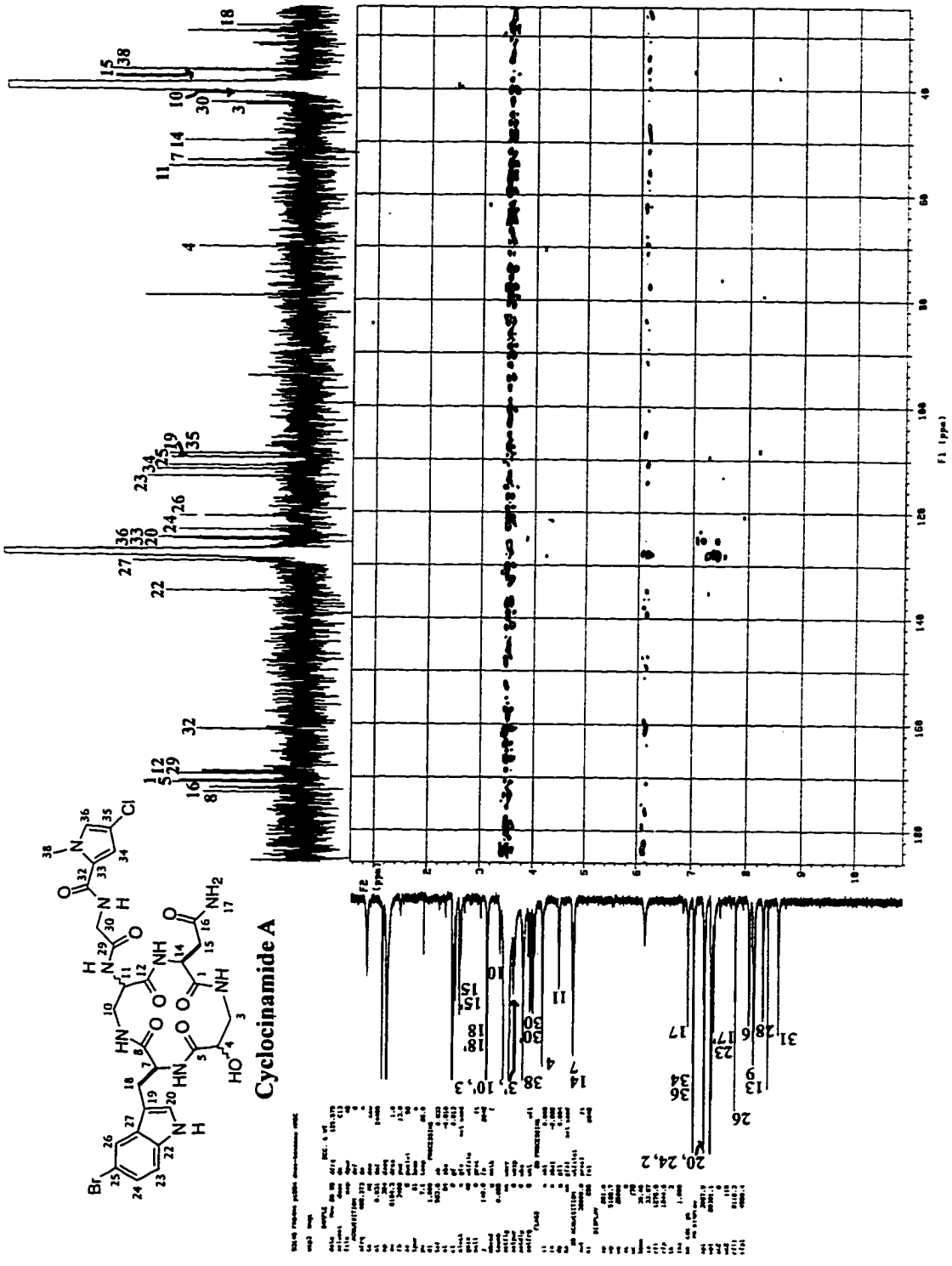


Figure 2.35. HMBC Expansion Spectra (DMSO-d6/Benzene-d6, 500 MHz)

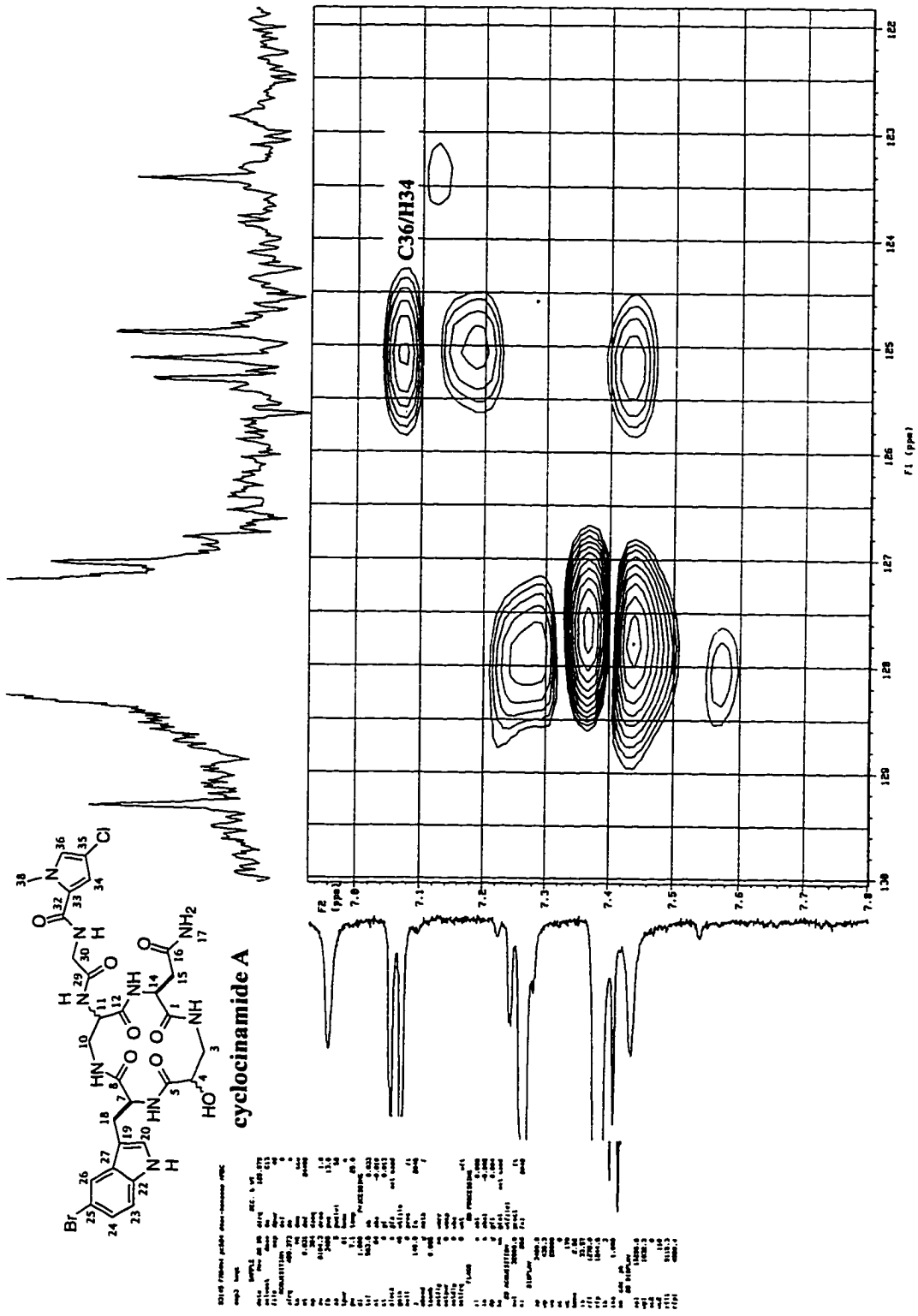


Figure 2.36. HMBC Expansion Spectra (DMSO-d6/Benzene-d6, 500 MHz)

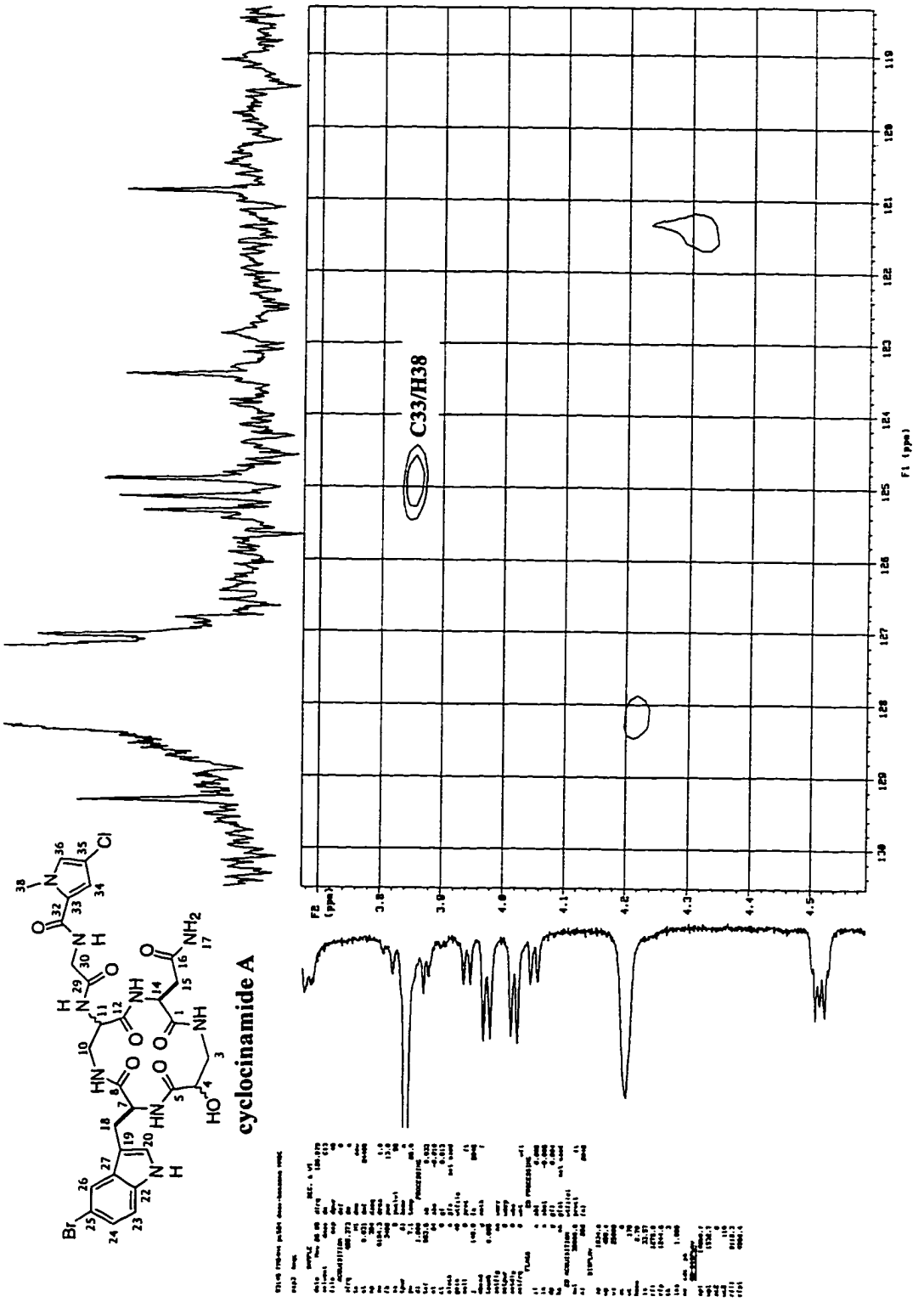


Figure 2.37. NOESY Spectra (DMSO-d6, 500 MHz)

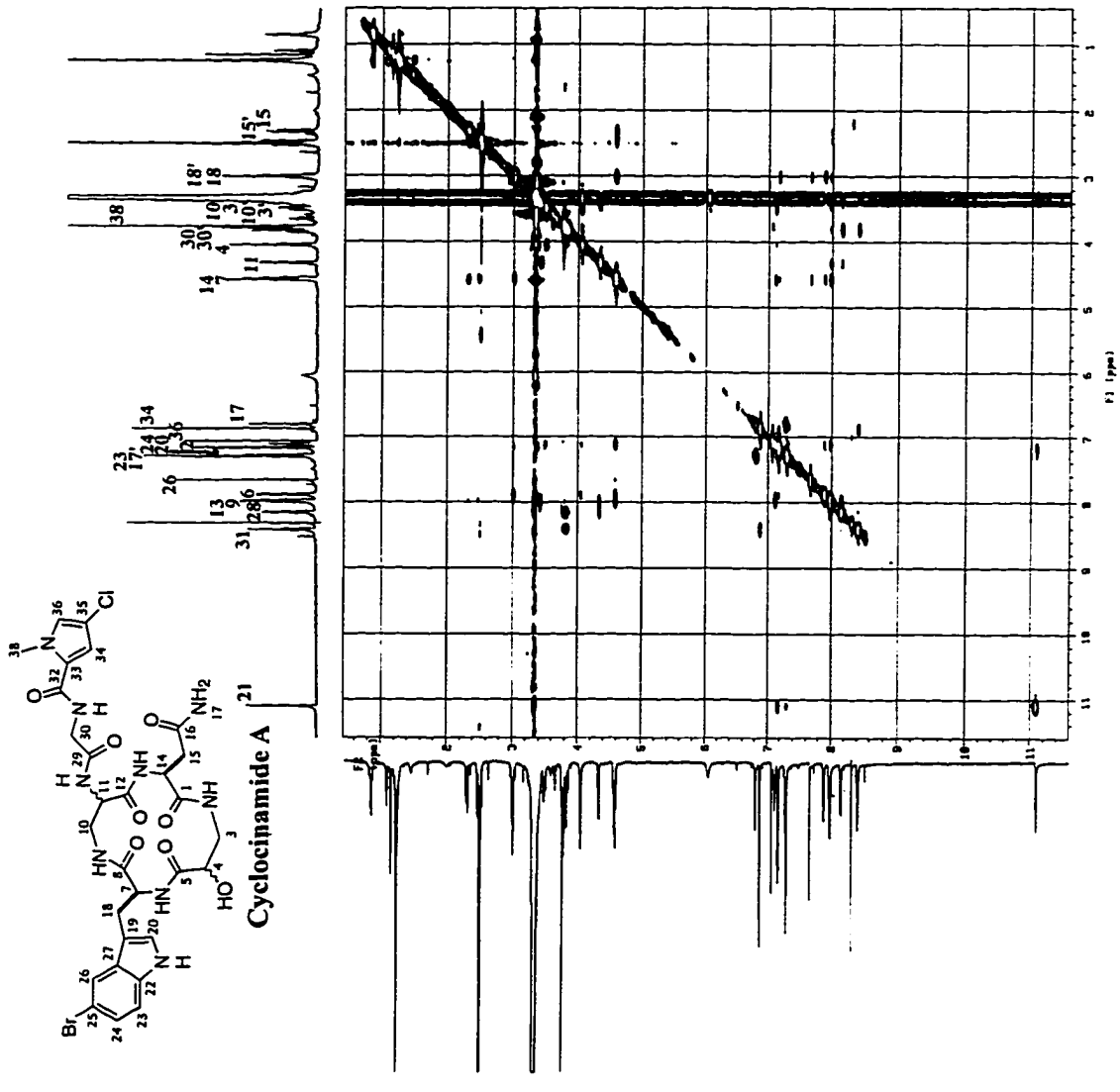


Figure 2.38. NOESY Expansion Spectra (DMSO-d6, 500 MHz)

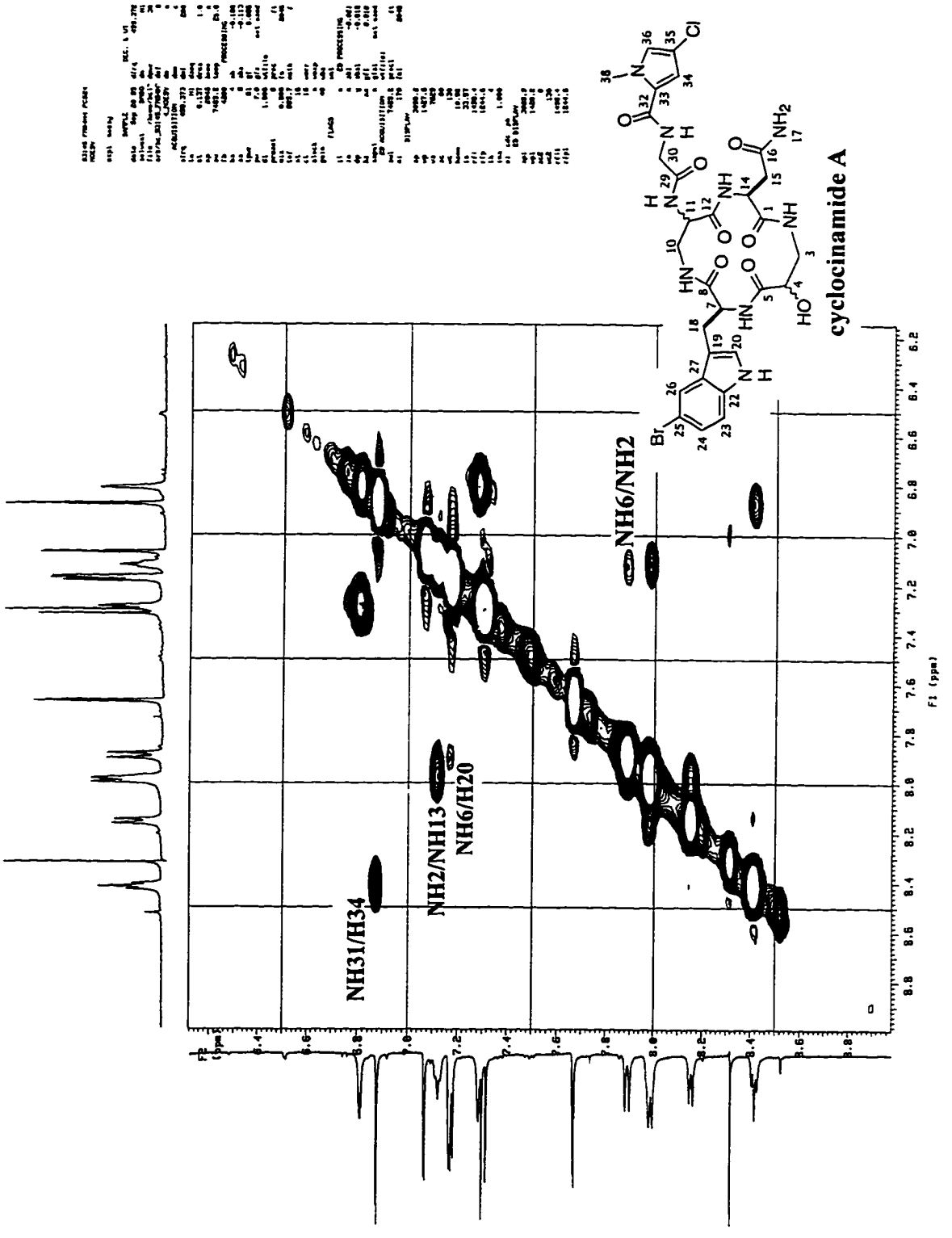


Figure 2.39. NOESY Expansion Spectra (DMSO-d6, 500 MHz)

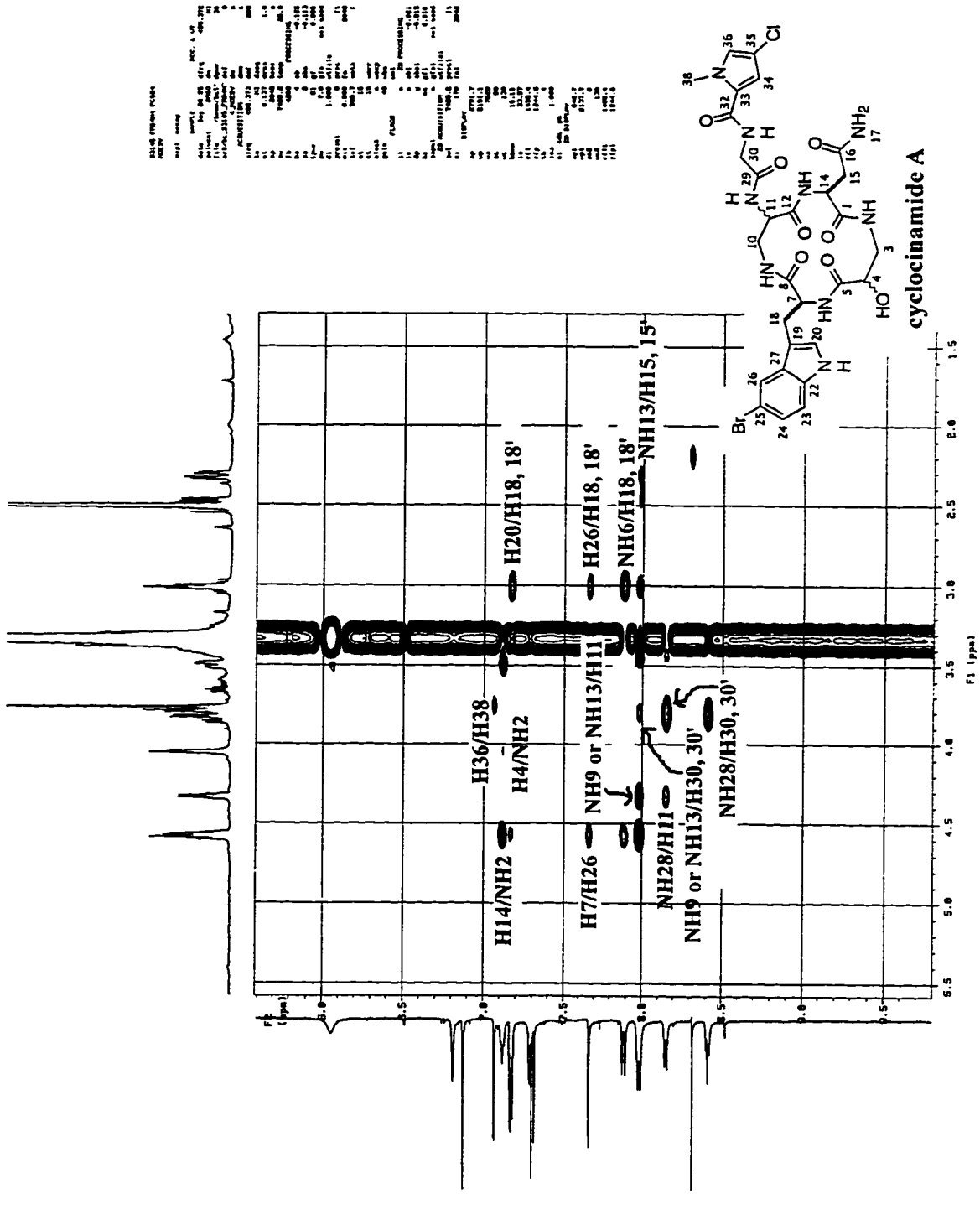


Figure 2.40. NOESY Expansion Spectra (DMSO-d6, 500 MHz)

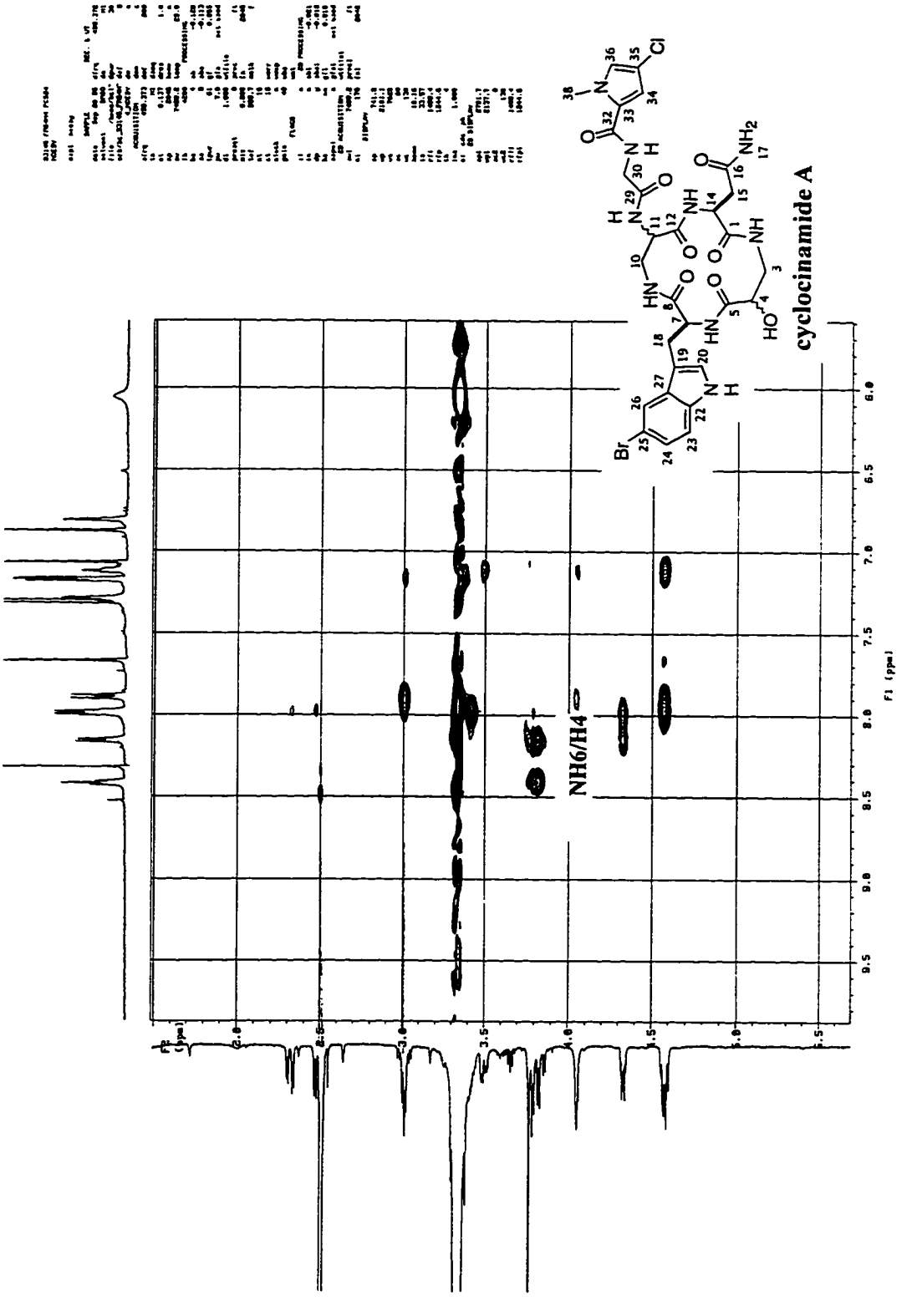


Figure 2.41. 4*R*, 7*S*, 11*R*, 14*S*-C8/C12 Conformer

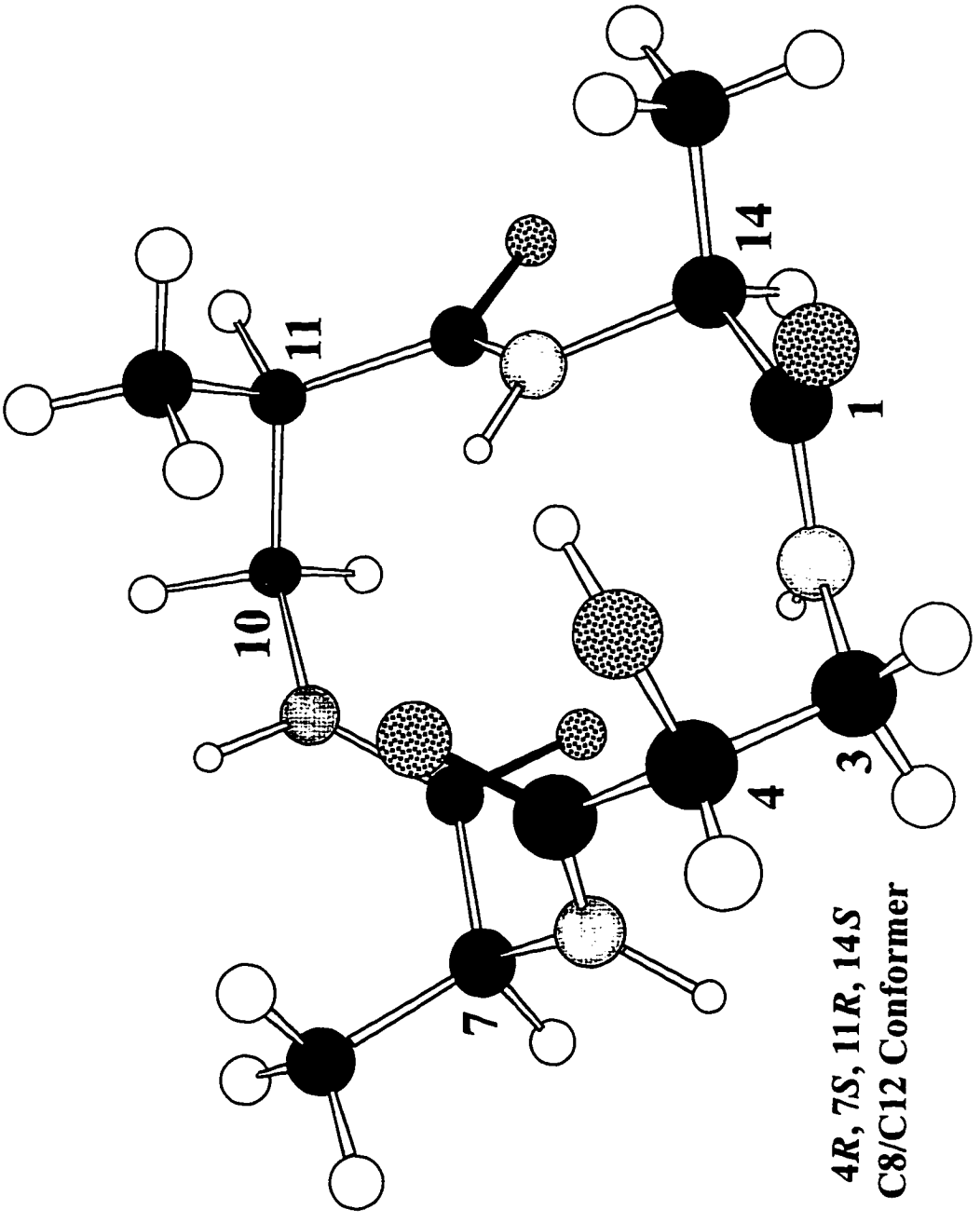


Figure 2.42. 4*R*, 7*S*, 11*R*, 14*S*-C8 Conformer

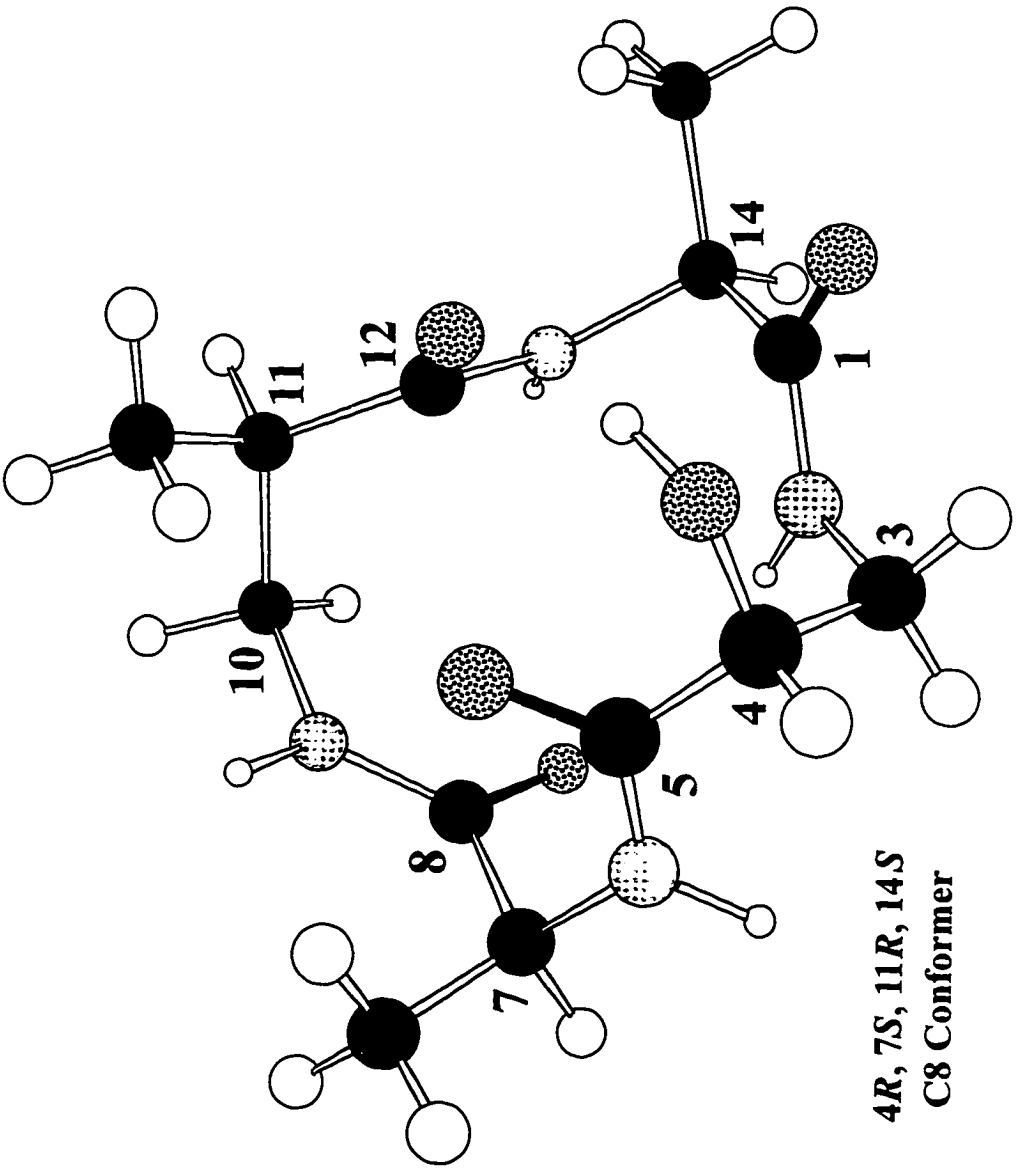


Figure 2.43. 4*R*, 7*S*, 11*R*, 14*S*-C12 Conformer

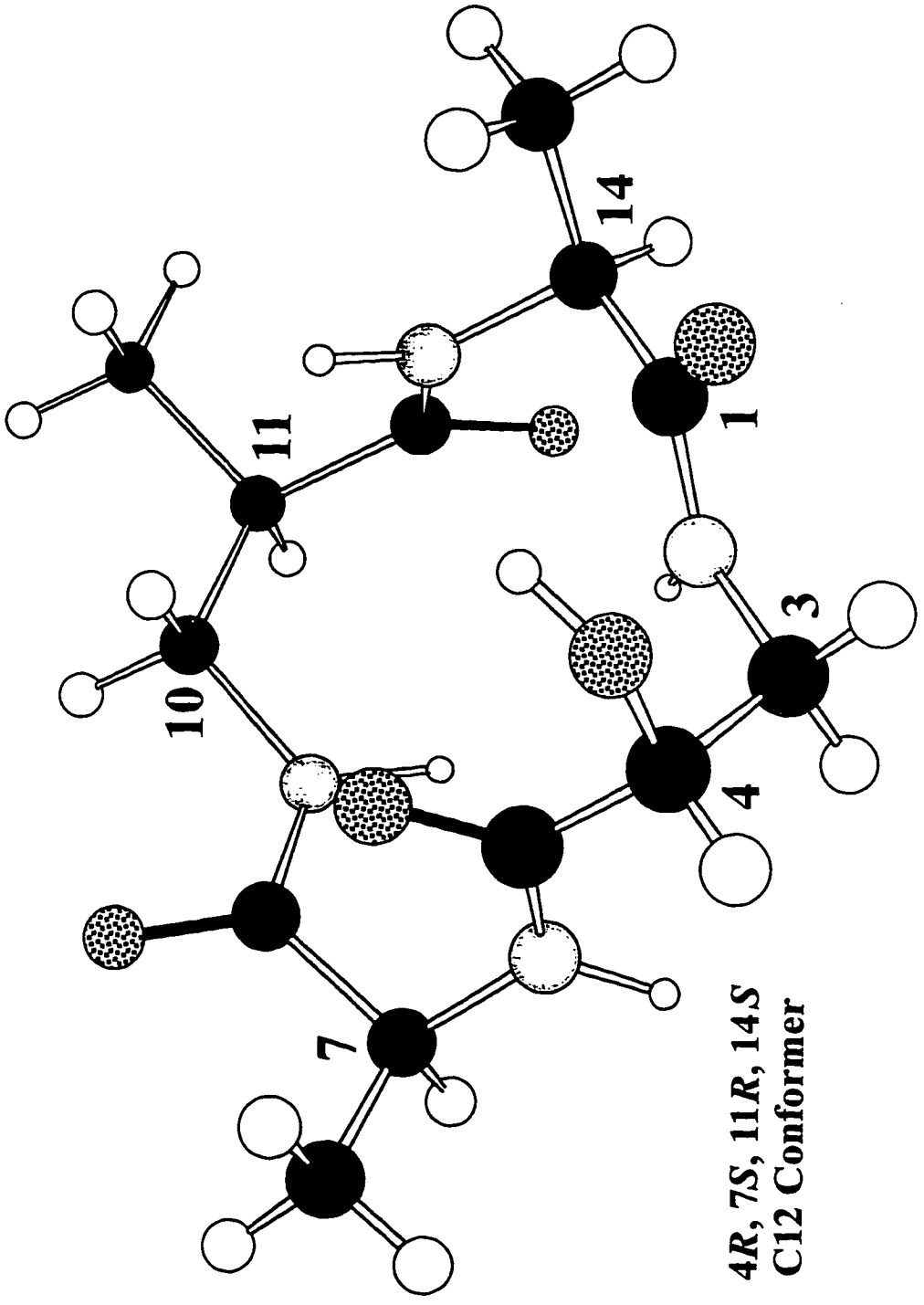


Figure 2.44. 4*R*, 7*S*, 11*R*, 14*S*-C1 Conformer

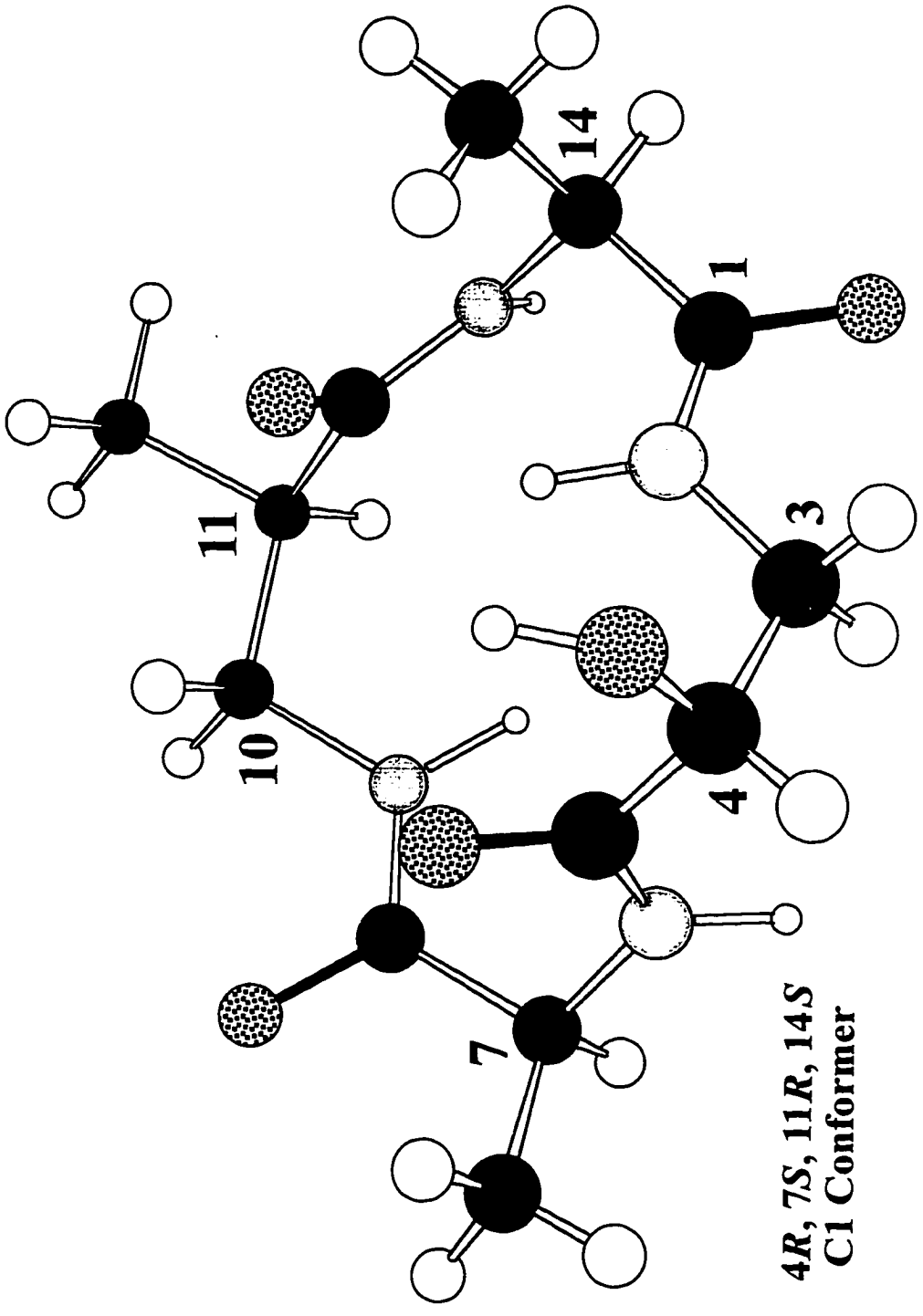


Figure 2.45. 4*R*, 7*S*, 11*R*, 14*S* Conformer

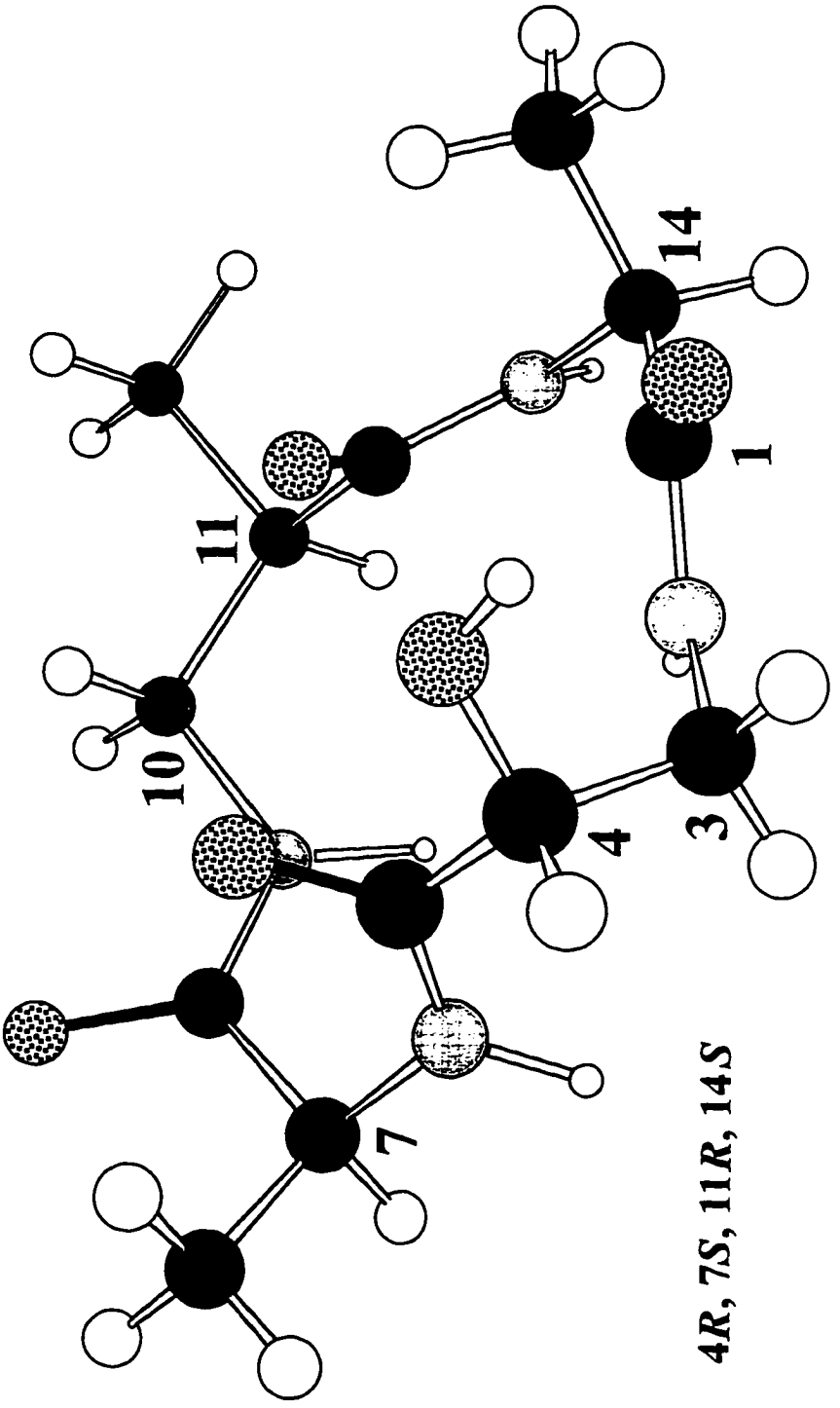


Figure 2.46. 4*R*, 7*S*, 11*R*, 14*S*-C3 Conformer

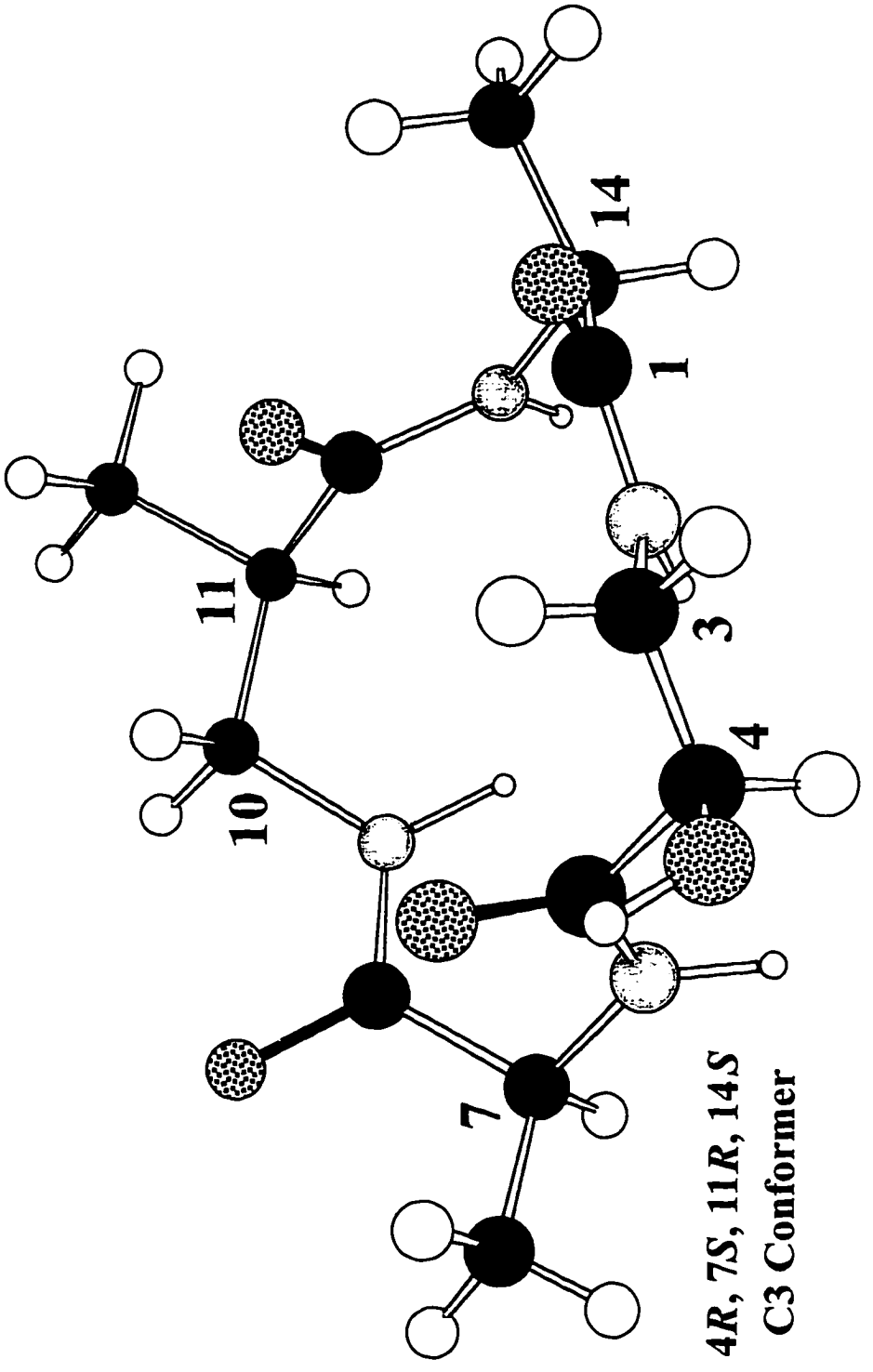


Figure 2.47. 4*R*, 7*S*, 11*S*, 14*S*-C8 Conformer

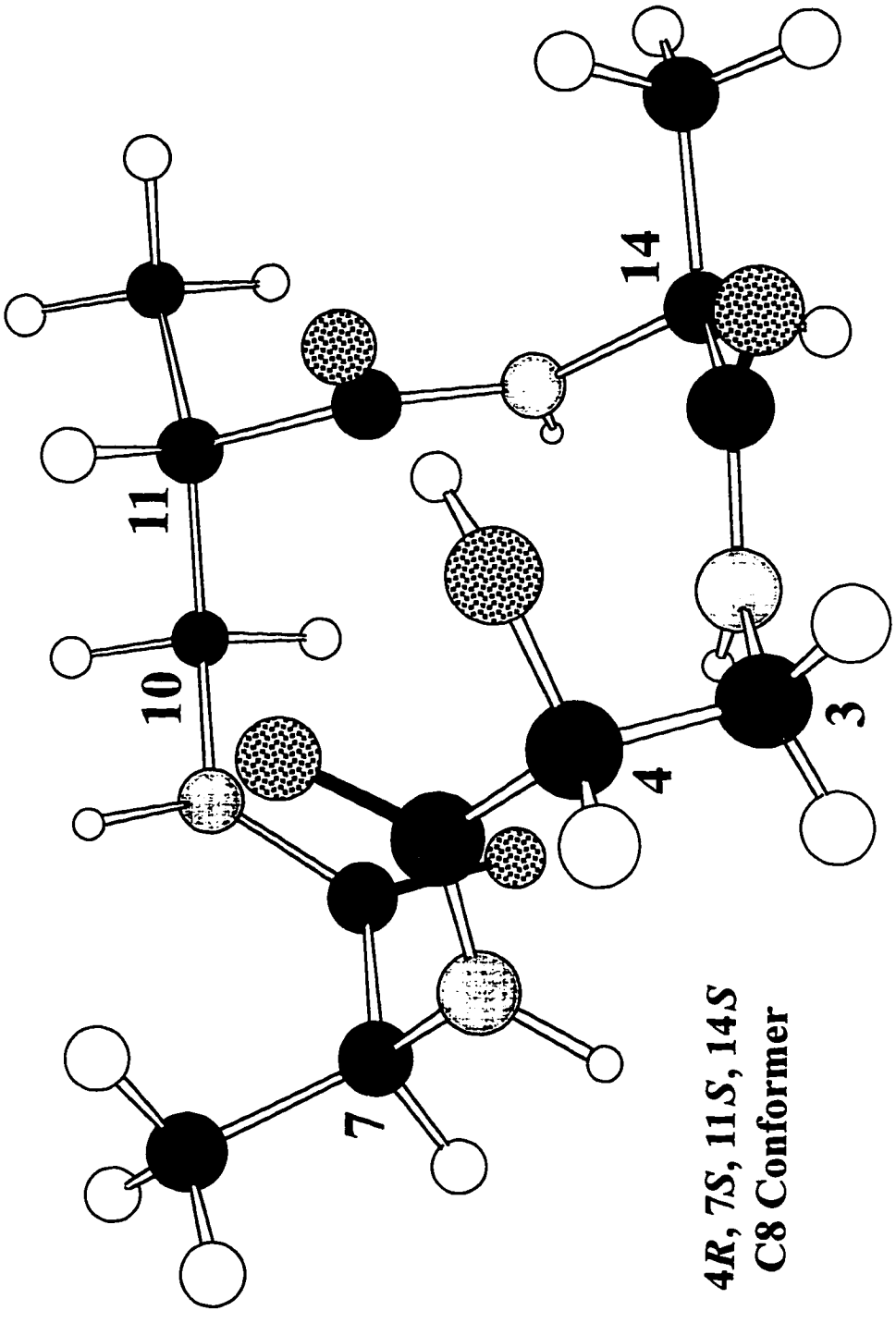


Figure 2.48. 4*R*, 7*S*, 11*S*, 14*S*-C8/C12 Conformer

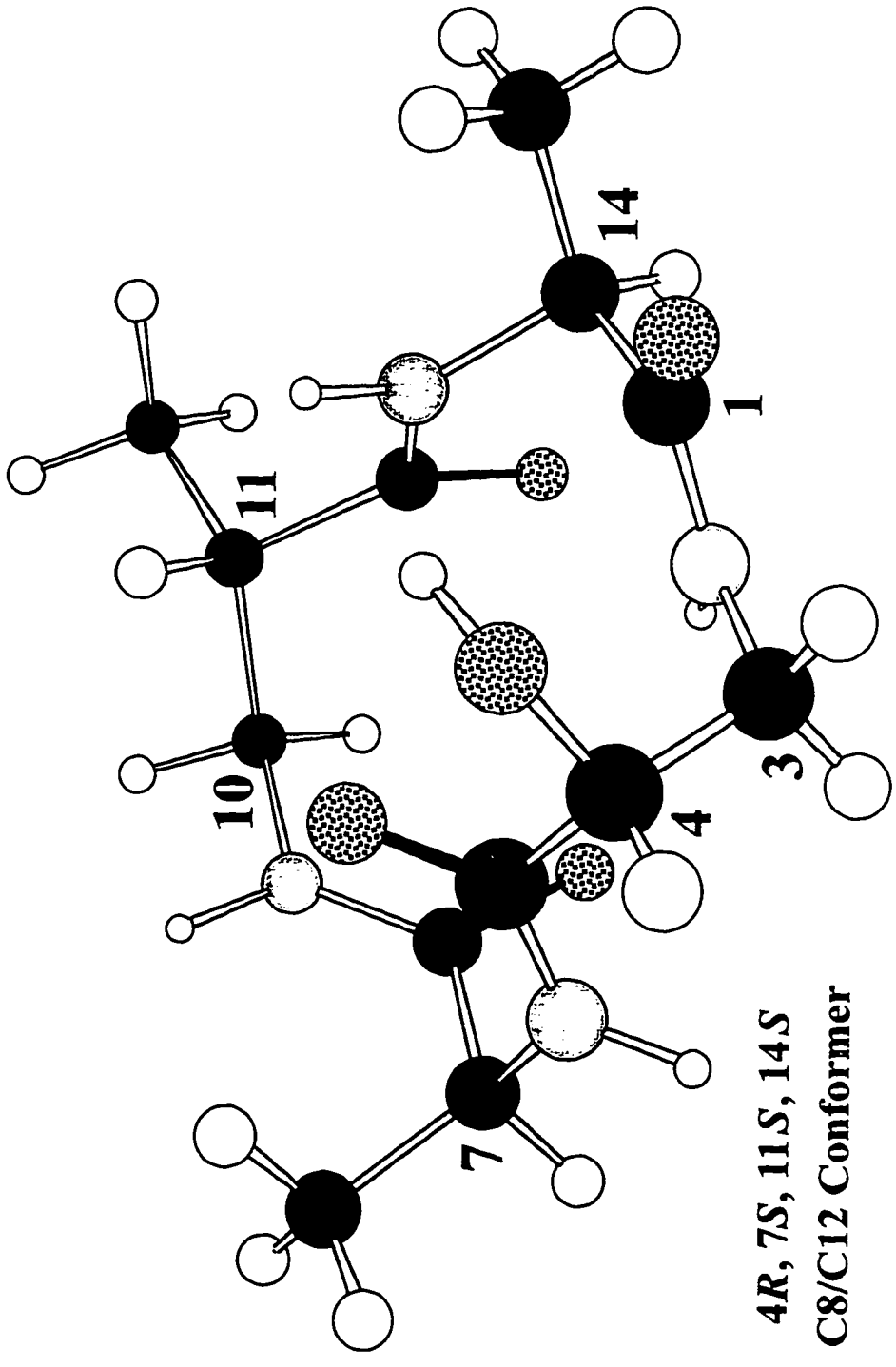


Figure 2.49. 4*R*, 7*S*, 11*S*, 14*S*-C12 Conformer

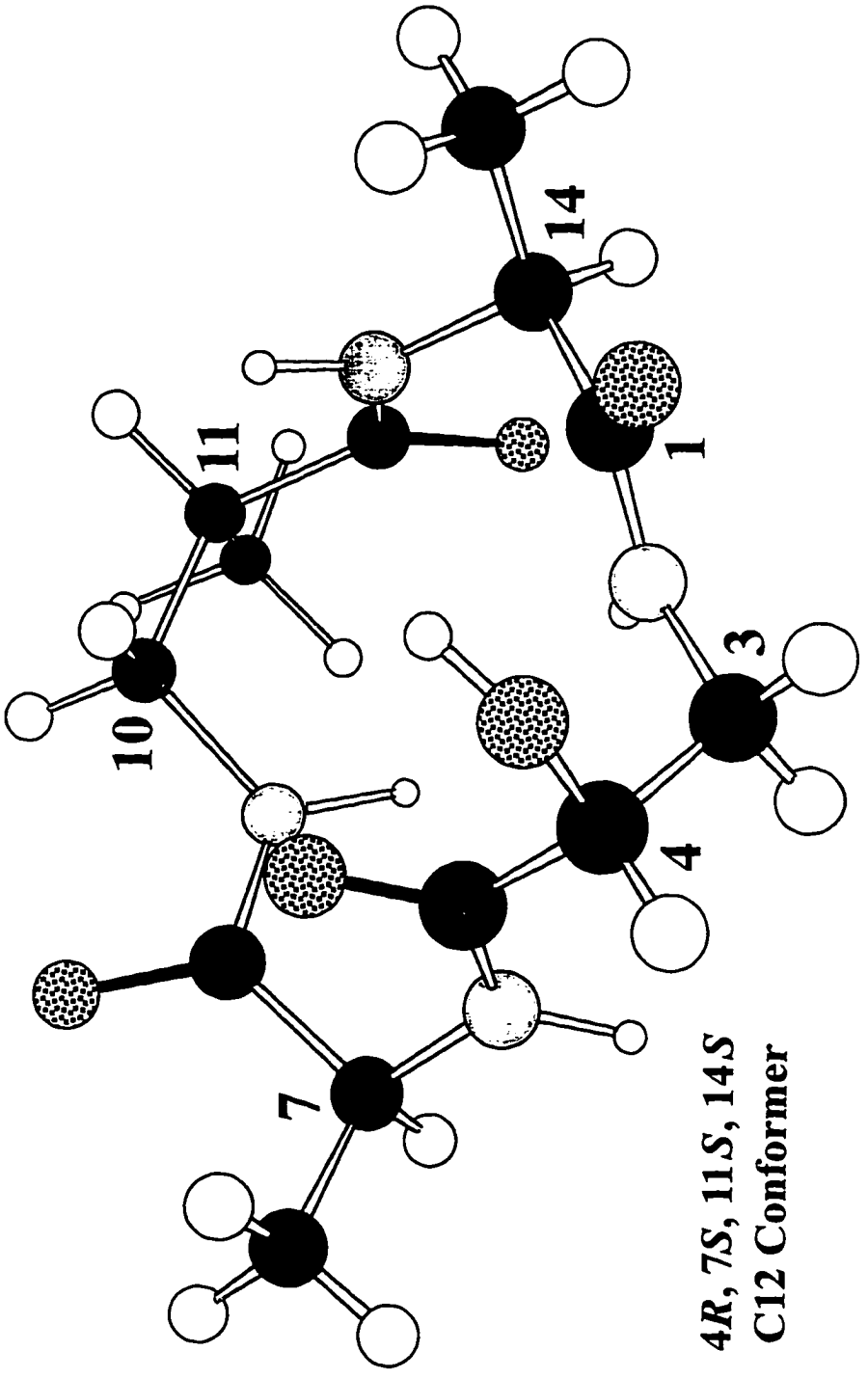


Figure 2.50. 4*R*, 7*S*, 11*S*, 14*S* Conformer

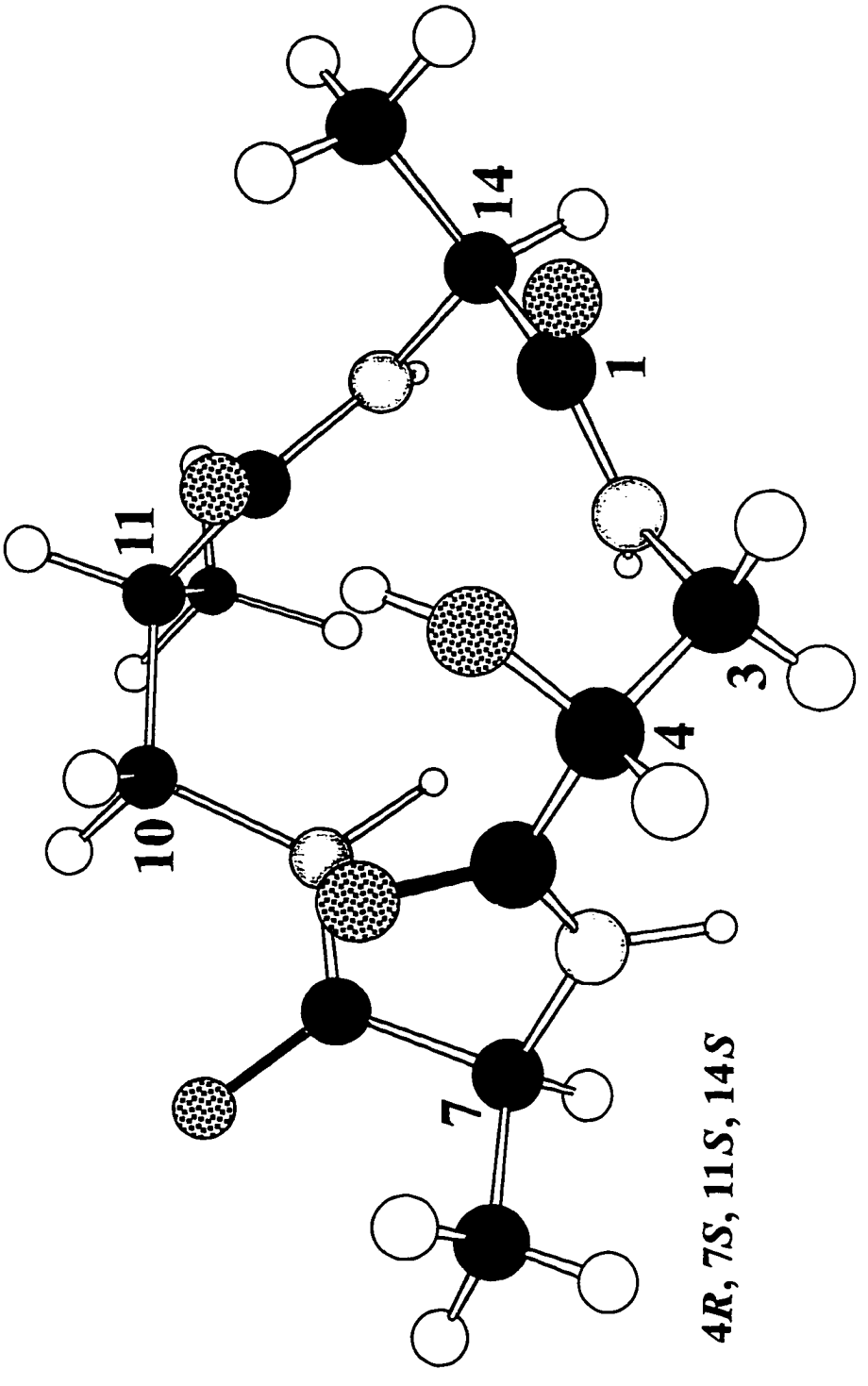


Figure 2.51. 4*S*, 7*S*, 11*R*, 14*S*-C3/C8 Conformer

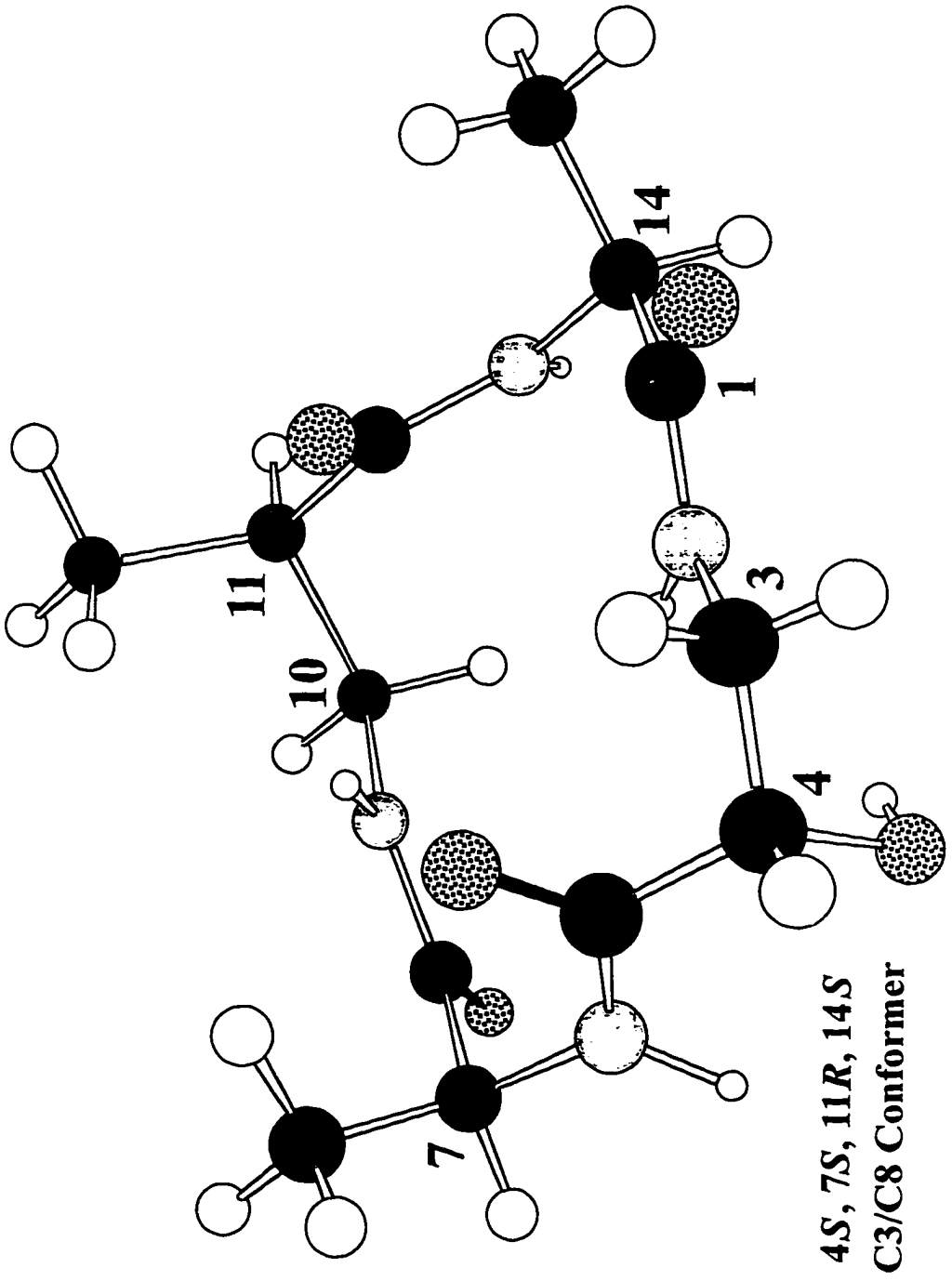


Figure 2.52. 4*S*, 7*S*, 11*R*, 14*S*-C3 Conformer

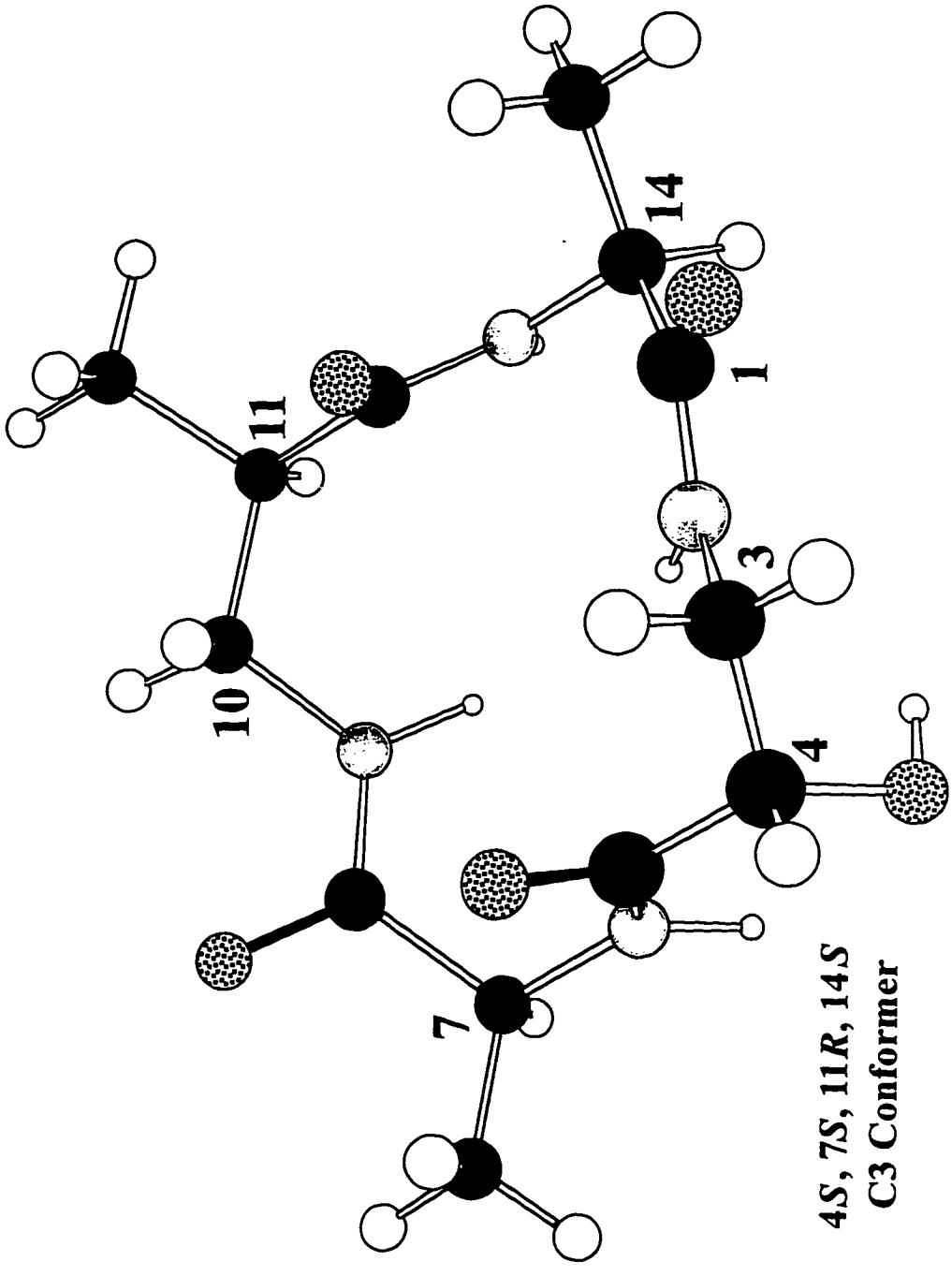


Figure 2.53. 4*S*, 7*S*, 11*R*, 14*S* Conformer

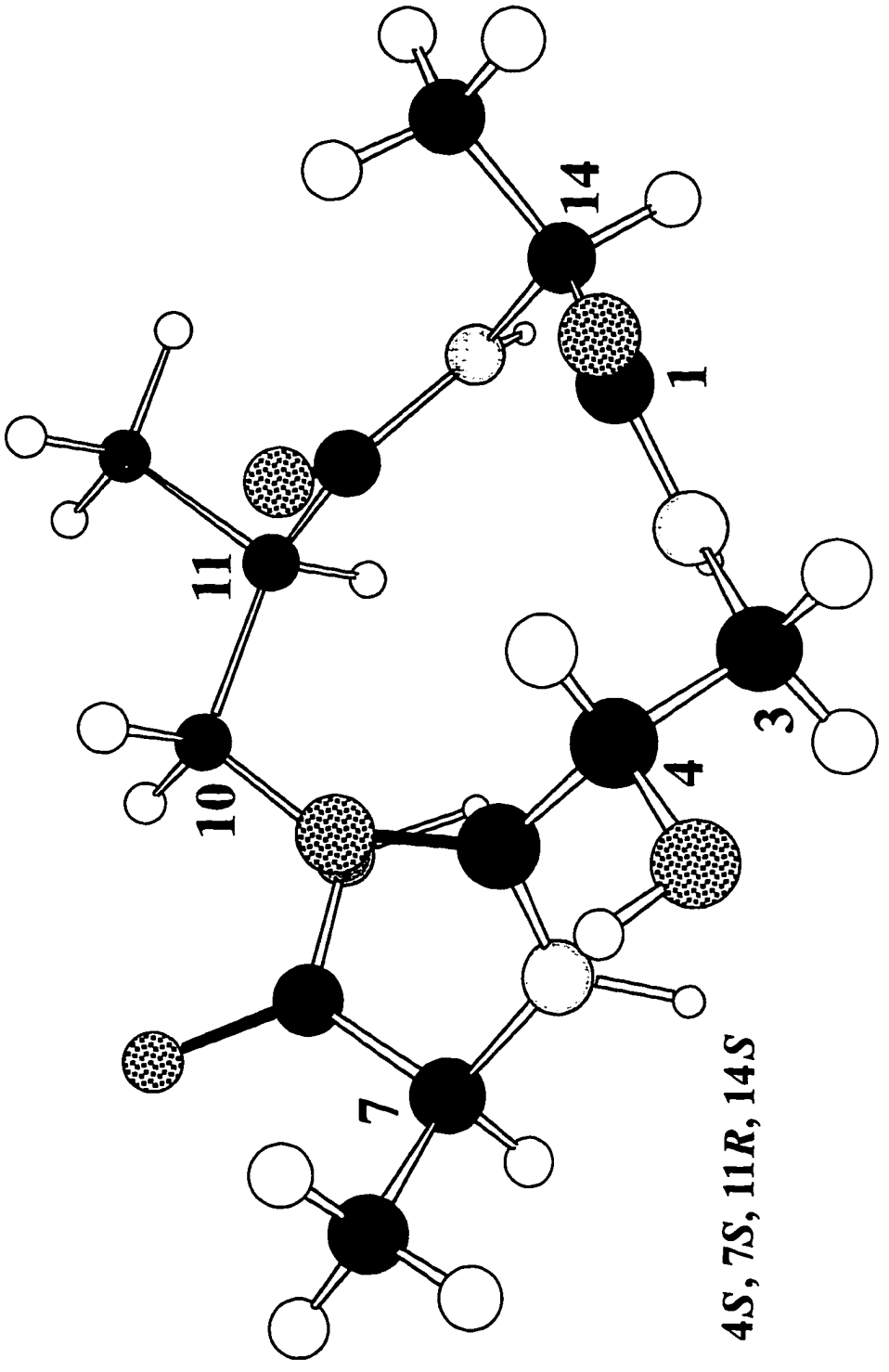


Figure 2.54. 4*S*, 7*S*, 11*R*, 14*S*-C1 Conformer

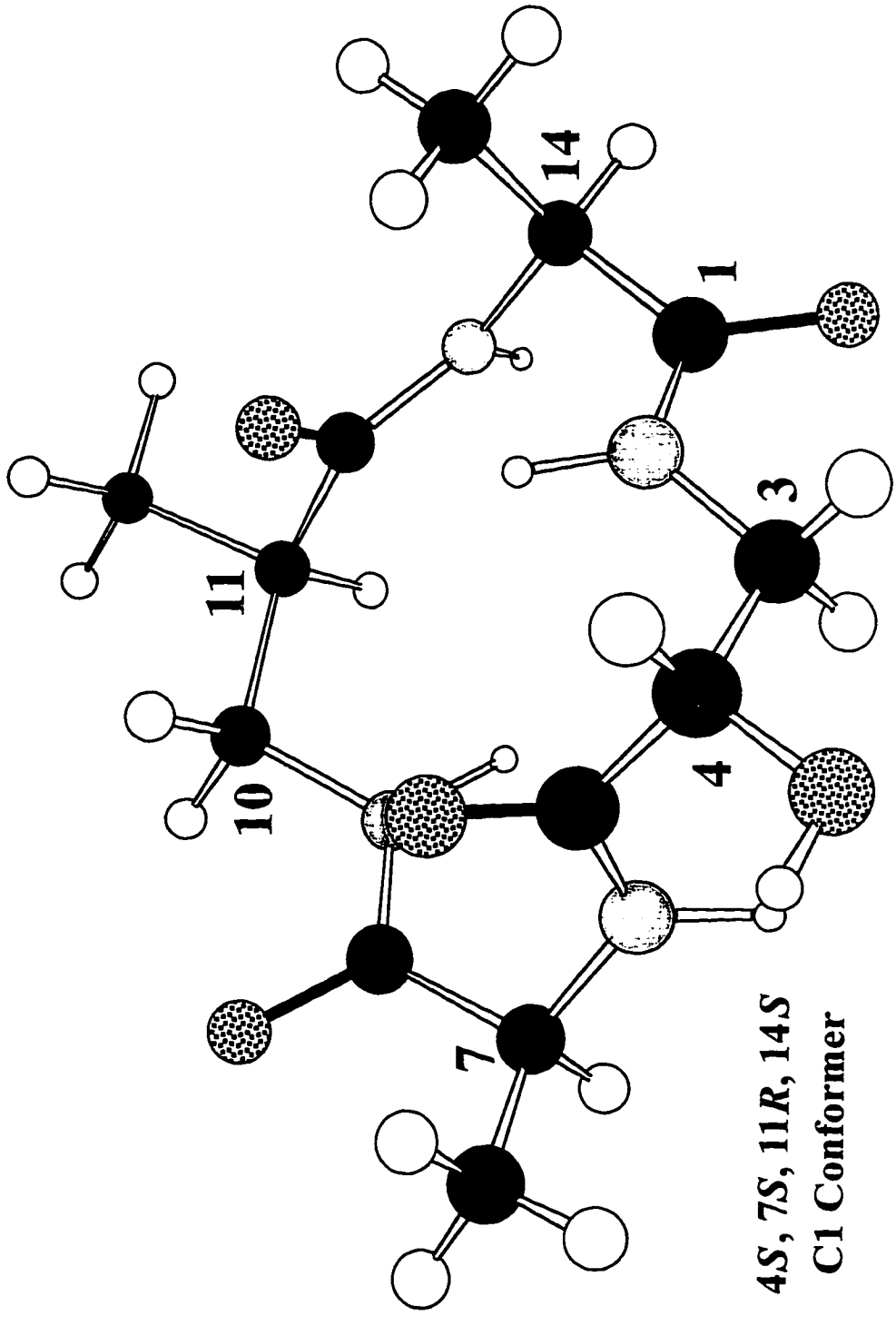


Figure 2.55. 4*S*, 7*S*, 11*S*, 14*S*-C1/C3/C12 Conformer

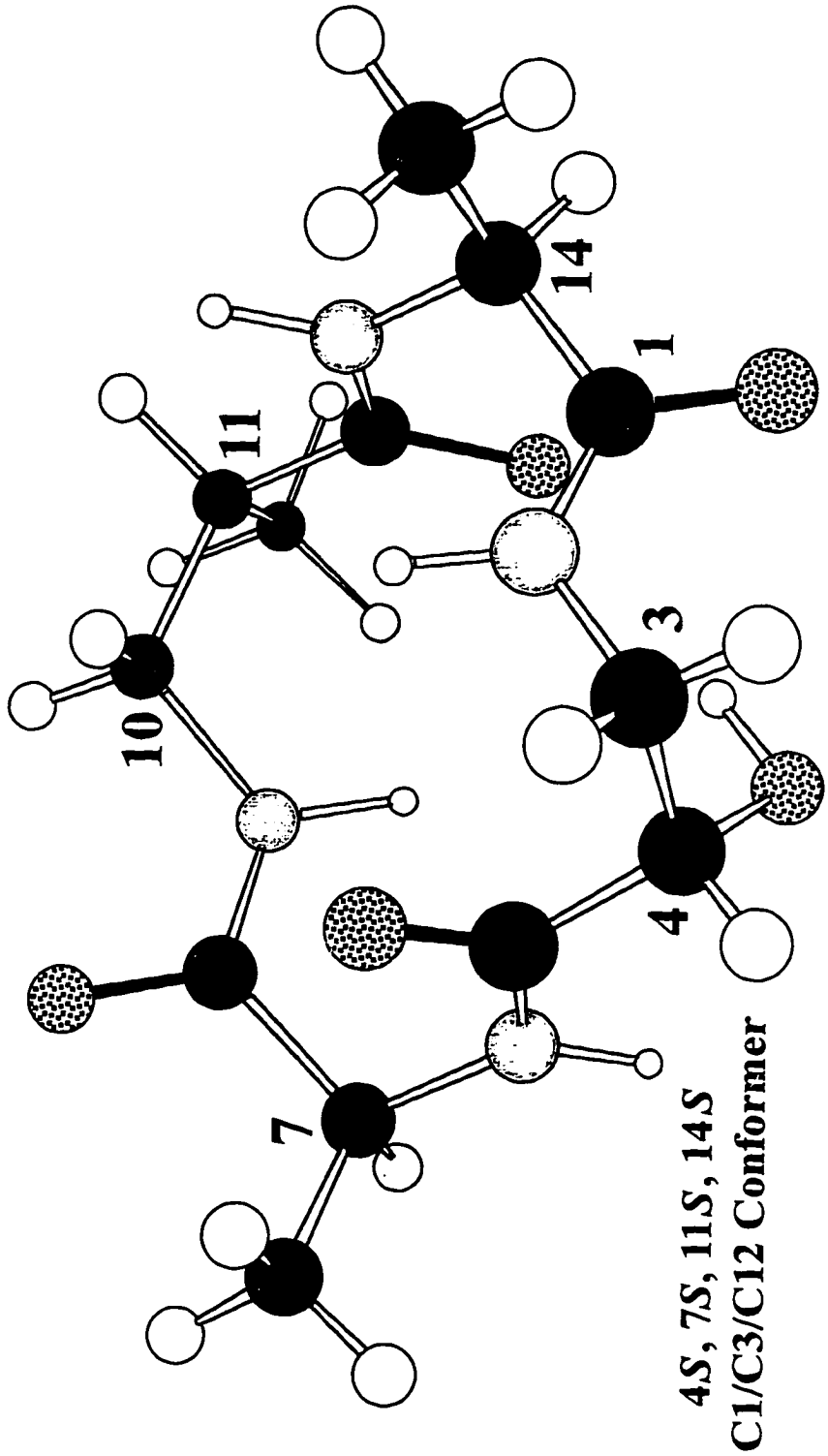


Figure 2.56. 4*S*, 7*S*, 11*S*, 14*S*-C3/C12 Conformer

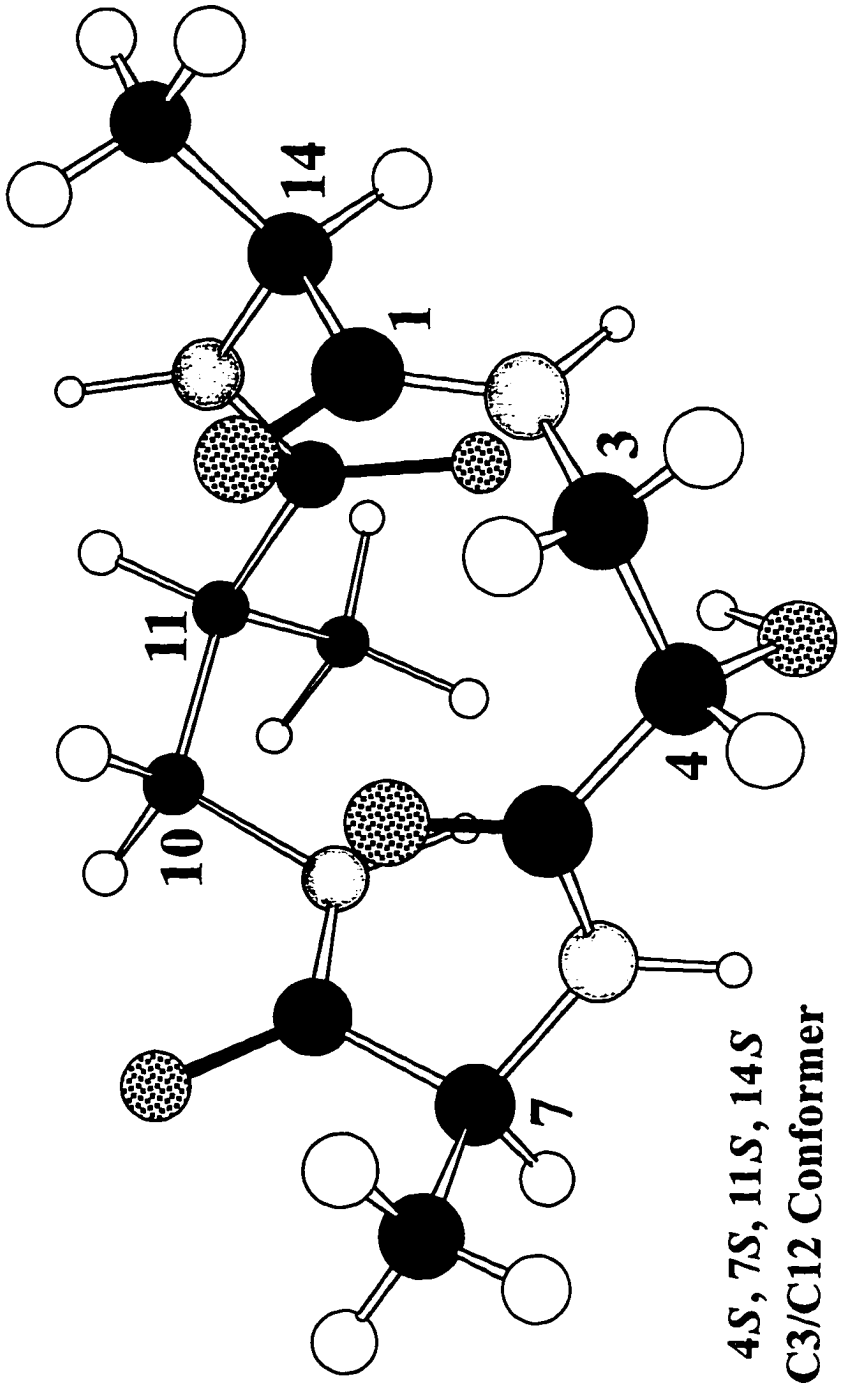


Figure 2.57. 4*S*, 7*S*, 11*S*, 14*S*-C3/C5/C12 Conformer

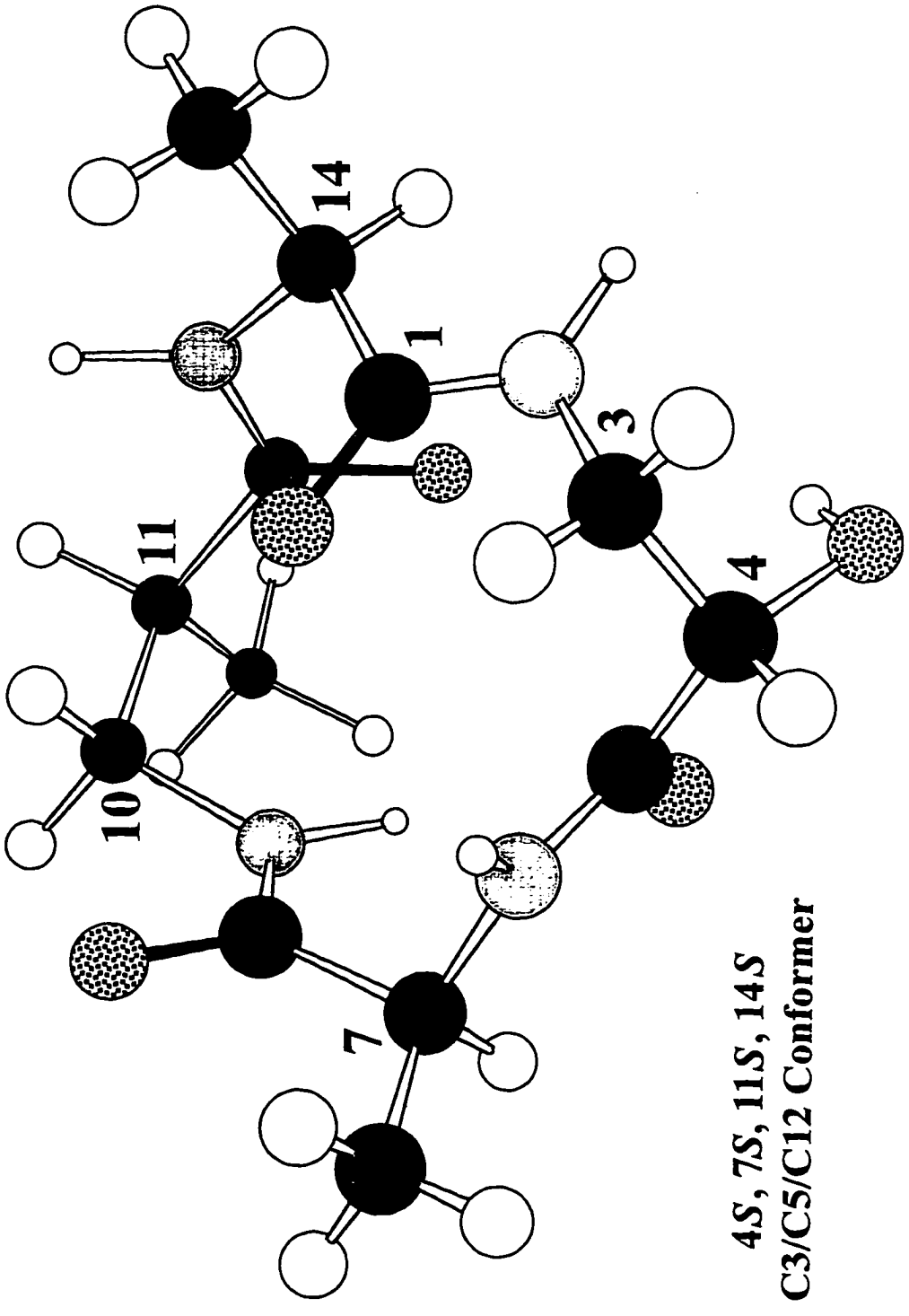
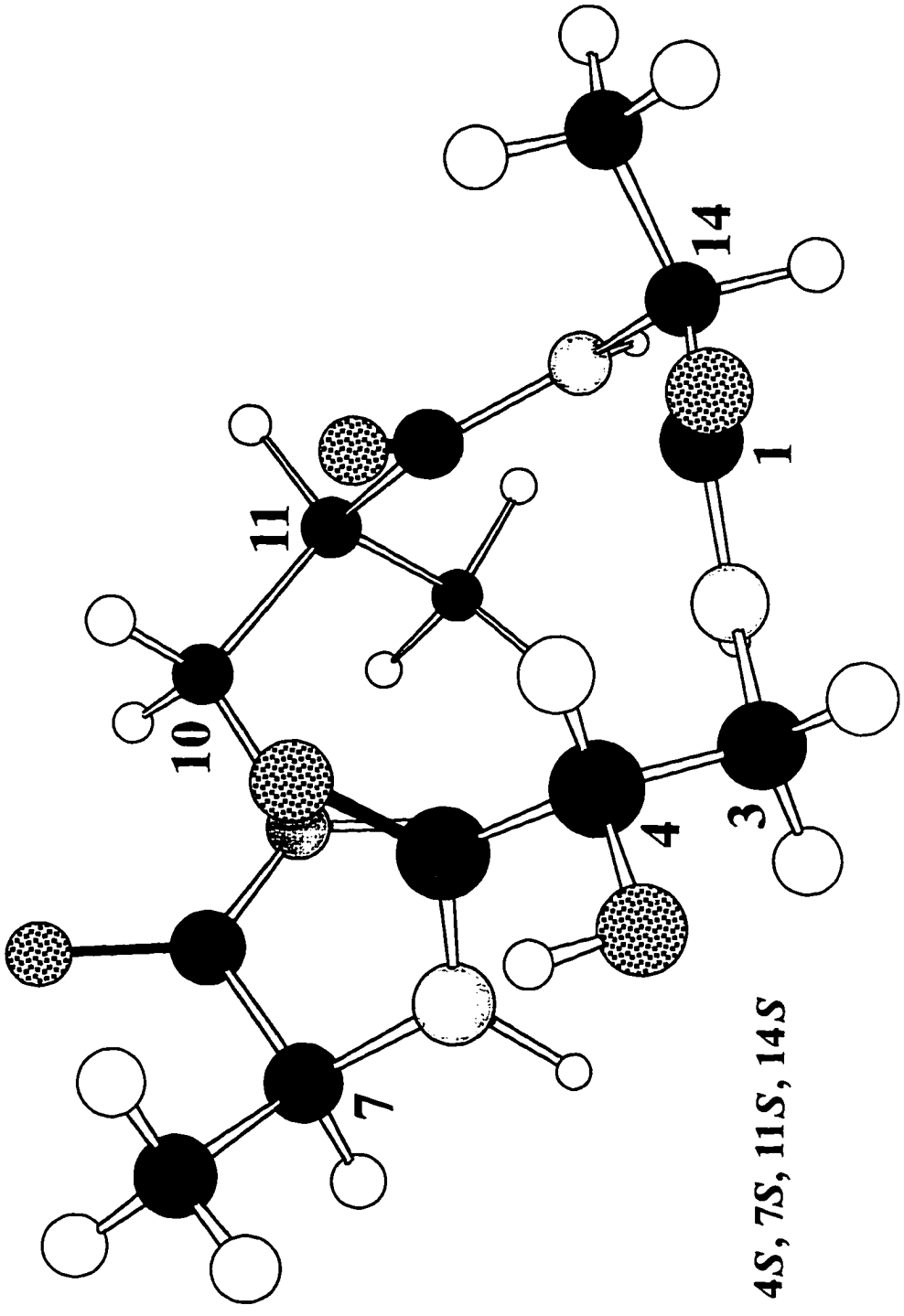


Figure 2.58. 4*S*, 7*S*, 11*S*, 14*S* Conformer



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21. (a) Valeriote, F.; Corbett, T.; LoRusso, P.; Moore, R. E.; Scheuer, P. J.; Patterson, G.; Paul, V.; Grindey, G.; Bonjouklian, R.; Pearce, H.; Suffness, M. *I. J. Pharmacognosy* **1995**, *33, Supplement* 59-66. (b) Valeriote, F.; Corbett, T.; Edelstein, M.; Baker, L. *Cancer Invest.* **1996**, *14*, 124-141. (c) Corbett, T. H; Valeriote, F. A.; Polin, L.; Panchapor, C.; Pugh, S.; White, K.; Lowichik, N.; Knight, J.; Bissery, M.-C.; Wozniak, A.; LoRusso, P.; Biernat, L.; Polin, D.; Knight, L.; Biggar, S.; Looney, D.; Demchik, L.; Jones, J.; Jones, L.; Scott, B.; Palmer, K.; Essenmacher, S.; Lisow, L.; Mattes, K. C.; Cavanaugh, P. R.; Rake, J. B.; Baker, L. In *Cytotoxic Anticancer Drugs: Models and Concepts for Drug Discovery and Development*, Valeriote, F. A.; Corbett, T. H.; Baker, L. H., Eds.; Kluwer Academic Publishers, 1992: Norwell, MA, pp 35 - 87.
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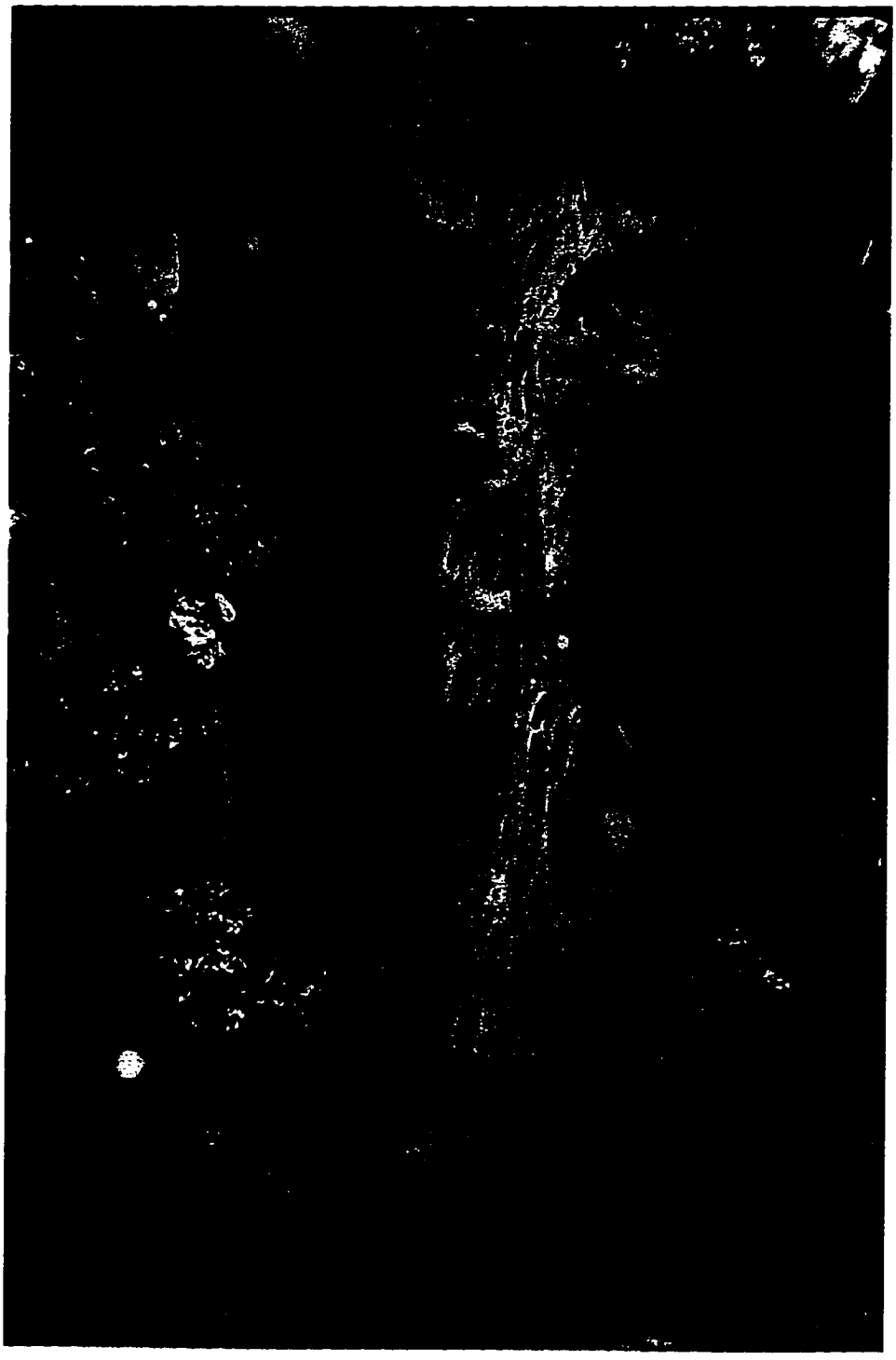
Chapter 3-A Novel Chlorinated Ketide Amino Acid, Herbamide A, from the Marine Sponge *Dysidea herbacea*



Marine Sponge-Papua New Guinea Photo Courtesy of Jay Burreson, Ph.D.

Abstract

A collection of the marine sponge *Dysidea herbacea* (coll. # 93153), containing cyanobacterial symbionts, from Papua New Guinea yielded a new ketide amino acid, herbamide A (**1**), along with the previously reported polychlorinated tetrapeptide, dysidenin (**2**) as major components. The structure of herbamide A (**1**) was determined after analysis of various one and two-dimensional NMR spectra. The ^{1}H and ^{13}C-NMR data of dysidenin (**2**) matched that reported in the literature. The possible biogenesis of herbamide A and a similar compound, barbamide (**4**), is discussed.

Background

During my first year of graduate research (1992-93) I began to investigate the chemistry of various specimens of the Indo-Pacific marine sponge *Dysidea herbacea* (order Dictyoceratida). The examination of this sponge had a threefold purpose. First and foremost was to try and identify new metabolites that were novel and bioactive. However, I did not expect any compounds from this particular sponge to exhibit any interesting biological activity. As discussed in Chapter 1 the known metabolites from *D. herbacea*, bromophenols[1] and chlorinated peptides[2], were shown to have antimicrobial activity only. The focus of the UCSC marine natural products program was, at the time, to find therapeutic leads for cancer in mechanism-based assays.[3]

The second reason I was investigating the chemistry of this sponge was to isolate any chlorinated peptides that could be found because the UCSC repository of known compounds was lacking this structure class. The only constituents isolated in the past by the UCSC marine natural products research group were bromophenols[4] and furanosesquiterpenes[5] collected in Fiji and Papua New Guinea in the Indo-Pacific. In the beginning, my research focused on *Dysidea* sponges collected from new areas of Papua New Guinea where we had hoped to find these metabolites during our expeditions.

Finally, I was comparing the chemistry of *D. herbacea* and the sponge *Phyllospongia folliscens* (order Dictyoceratida) in an effort to define these sponges chemotaxonomically due to their morphological similarities. The chemotaxonomy study of *D. herbacea* developed much later and was the subject of Chapter 2. Consequently, during early investigations of *Dysidea* sponges from Papua New Guinea I isolated the first chlorinated peptides of the UCSC marine natural products program and identified a new class of chlorinated peptides, the ketide amino acid herbamides. This resulted in a

publication in *Tetrahedron Letters* **1995**, *36*, 1185-1188 on the isolation and structure elucidation of herbamide A, the first ketide amino acid in the series. I have already discussed the chemistry of *Dysidea herbacea* in great detail in Chapters 1 and 2. Therefore, I will continue with a discussion on the isolation and structure elucidation of herbamide A (**1**) and dysidenin (**2**) followed by a discussion of the biogenesis of herbamide A (**1**) and barbamide[6] (**4**).

Collection and Isolation

During our expedition to Papua New Guinea in the Spring of 1993 the UCSC research group performed thin layer chromatography (TLC) analyses in the field of the various sponge specimens collected. The goal was to examine those sponges that appeared rich in cyanobacteria as indicated by the presence of chlorophylls in the TLC analysis and by the characteristic blue-green color on the surface of the sponge. One specimen, collection number 93153, appeared heavily-laden with cyanobacteria as is visible in the topside photographs of the sponge (Figure 3.1). It also fit the taxonomic description of *Dysidea herbacea*.[7]

The sponge was preserved in the field according to our standard procedure and brought back to the UCSC labs for further workup (Appendix A).[8] The sponge was soaked in MeOH three times with the solvent being removed from each pour-off by rotary evaporation. The crude MeOH extracts were combined and subjected to our liquid-liquid extraction scheme (Scheme 3.1). The MeOH extract was partitioned between H_2O and CH_2Cl_2. After rotary evaporation of the organic phase, the crude dichloromethane fraction was further partitioned between hexanes and 10% aqueous MeOH to remove fats. Water was added to the 10% aqueous MeOH fraction to give a 50% aqueous MeOH solvent partition fraction which was extracted with dichloromethane to give the FD fraction. The FD fraction was further explored because its ^{1}H-NMR spectra exhibited lowfield resonances at ~ δ 7.80 and 7.30 indicating that there may be a thiazole ring, a characteristic functionality of most of the chlorinated peptides normally isolated from this sponge. Subsequent purification of this fraction by HPLC (ODS, MeOH:THF:MeCN:H_2O, 54:9:13:24, Appendix B) afforded two compounds; herbamide A (**1**) a new minor component and the known major component dysidenin (**2**).

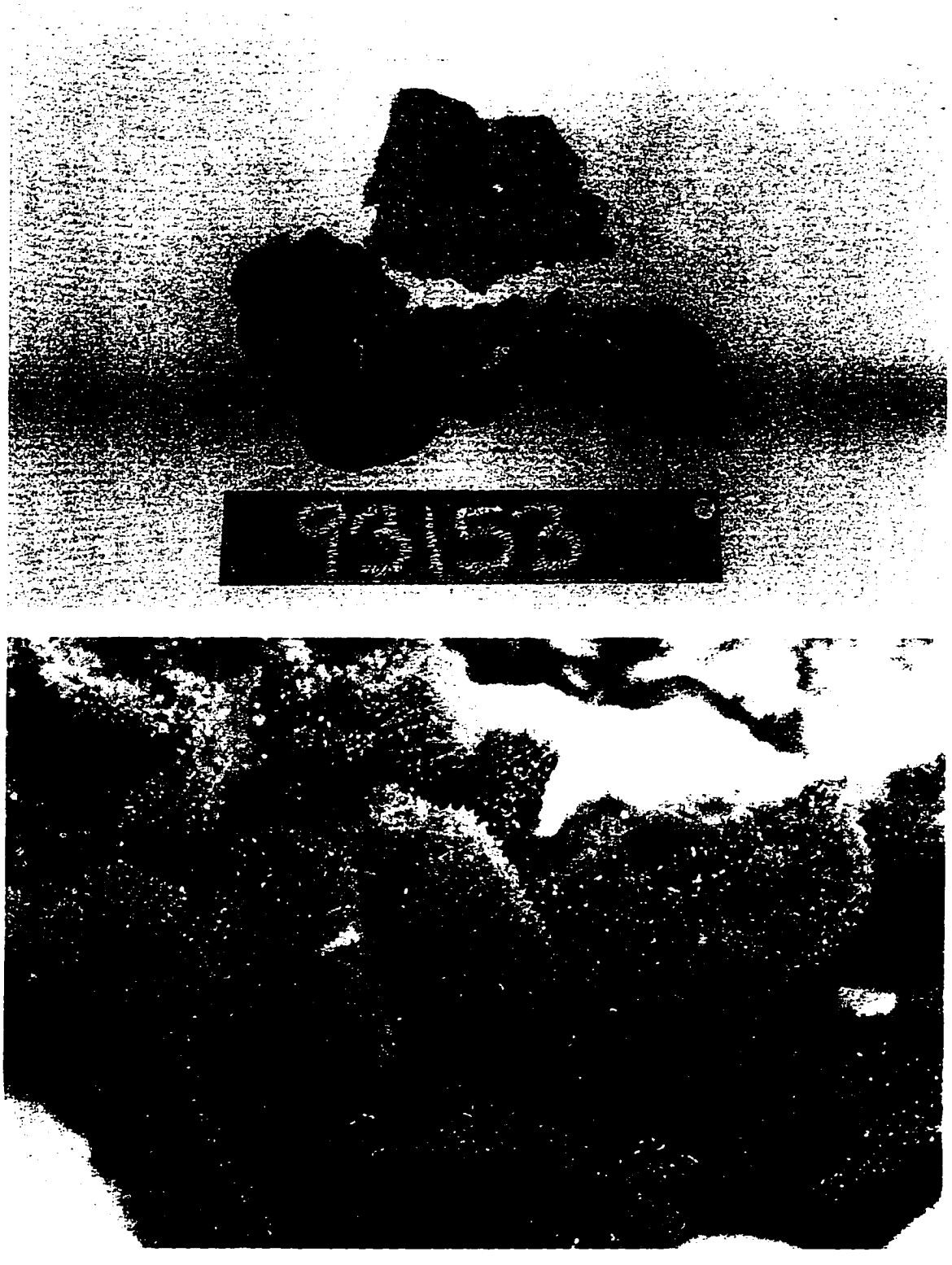
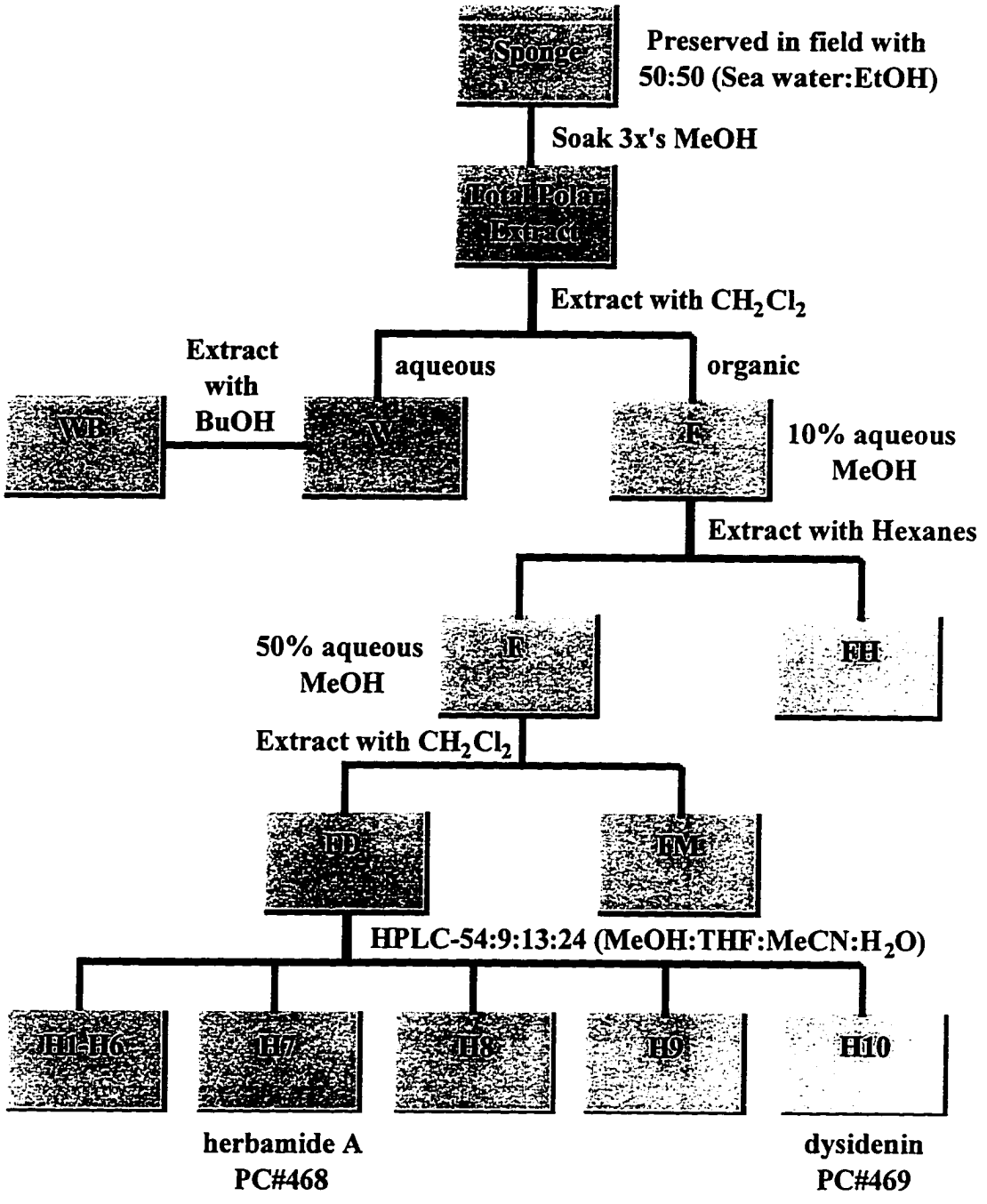


Figure 3.1. Topside and Closeup Photographs of 93153.



Scheme 3.1. Extraction Scheme for 93153.

Structure Elucidation

The structural elucidation work on herbamide A (**1**) began once I recognized that this compound had additional resonances in the δ 5.00 - 6.50 region of the ^{1}H-NMR spectra not normally associated with the chlorinated peptides. The only examples known of chlorinated peptides with vinylic proton resonances were herbaceamide, dysinin, dysideapyrrolidone and some diketopiperazines (see p. 30-Ch. 1), but my compound did not match the proton data of any of these compounds. This indicated that I had isolated something new. Further evidence that I had isolated a chlorinated peptide was shown in the LRFABMS (positive mode) where the molecular ion peak exhibited an isotope distribution pattern characteristic of three chlorine atoms (Figure 3.15 in Supp. Mat.).

A molecular formula of $C_{16}H_{21}Cl_3N_2OS$ was established by a HRFABMS $[MH]^+$ peak at *m/z* 394.0442 (Δ 0.3 mmu of calcd.) indicating an unsaturation number of six. The presence of an NH was evident by comparison of the mass spectral data to the ^{1}H, ^{13}C and APT NMR spectra which gave an APT formula of $C_{16}H_{20}$ along with the assignment of the δ 165.6 resonance to an amide moiety (Table 3.1; Figures 3.4, 3.5, 3.6 in Supp. Mat.) Seven ^{13}C resonances were identified as being characteristic of a thiazole ring (δ 170.0, 142.3 and 118.8) and two disubstituted double bonds (δ 141.2, 138.8, 131.1 and 122.8) which accounted for the remaining five elements of unsaturation. The UV (λ_{max} 258) and IR (ν 1672, 1602 cm^{-1}) spectra were consistent with extended conjugation represented by a dieneamide functionality (Figures 3.14, 3.16 in Supp. Mat.)

Analysis of ^{1}H-^{1}H COSY NMR data confirmed the presence of three substructures; a thiazole ring (**A**) and two acyclic chains consisting of respectively a *N*H-CH(CH$_3$)$_2$ (**C**) and C1 through C7 (**A**) as shown in Figure 3.2 (Table 3.1; Figures 3.7, 3.8 in Supp. Mat.). Substructure (**A**) was further supported by the ^{1}H-NMR doublet

Table 3.1. ^{1}H (300 MHz), ^{13}C (75.5 MHz), COSY (300MHz) and HMBC (500 MHz, DMSO-d6) NMR Data ($CDCl_3$) of herbamide A.

Position	^{13}C δ	^{1}H δ, mult., (J= Hz)	COSY (H → H)	HMBC (C → H)
1	16.2	1.29, d, (6.0)	H2	–
2	54.6	2.58, m	H1, H3'	H1, H3
3	36.6	2.96, m;	H3', H4;	H5
		2.19, m	H2, H3, H4	
4	138.8	6.02, ddd, (14.7, 8.4, 2.4)	H3, H3', H5	H3, H3', H6
5	131.1	6.25, dd, (14.7, 11.0)	H4, H6	H7
6	141.2	7.24, dd, (14.7, 11.0)	H5, H7	H4, H5,
7	122.8	5.90, d, (14.7)	H6	H5
8	165.6	–		H6, H7, NH
9	56.0	5.33, dd, (8.7, 6.3)	H10, NH	H11, H12
10	34.1	2.33, m	H9, H11, H12	H9, H11, H12
11	18.2	0.97, d, (6.9)	H10	H9, H12
12	19.2	0.93, d, (6.9)	H10	H9, H11
13	105.7	–		–
14	170.0	–		H9, H15, H16
15	142.3	7.74, d, (3.0)	H16	H16
16	118.8	7.26, d, (3.0)	H15	H15
NH	–	6.46, bd, (8.4)	H9	–

resonances at δ 7.74 and 7.26 with a coupling of J = 3 Hz as indicative of a thiazole ring. Acyclic substructure (**A**) consisting of a C1-C7 diene was confirmed to be in an *E*, *E* stereochemical relationship based on the $^{3}J_{4,5}$ and $^{3}J_{6,7}$ couplings each of J = 14.7 Hz as characteristic of trans-coupled protons in a double bond.[9]

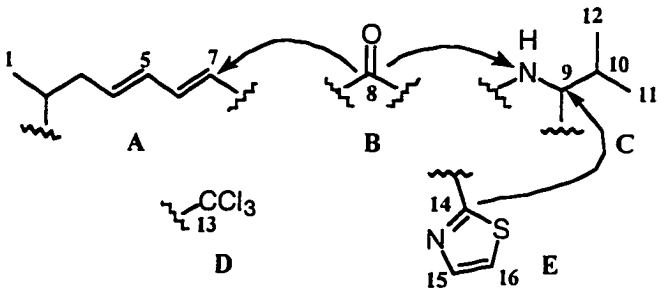
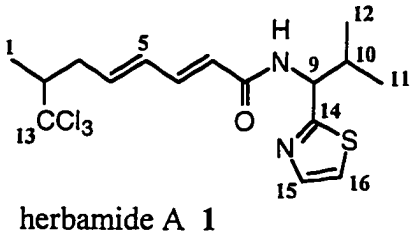


Figure 3.2. Substructures (**A-E**) Established after Analysis of ^{1}H, ^{13}C, APT and ^{1}H-^{1}H COSY NMR Data ($CDCl_3$, 75.5/300 MHz). Arrows Indicate Diagnostic HMBC (DMSO-d6, 500 MHz) Correlations.

Substructures (**A, B, C** and **E**) were connected together based on analysis of HMBC NMR spectral data (DMSO-d6, 500 MHz) (Table 3.1, Figure 3.2; Figures 3.9-3.13 in Supp. Mat.). A correlation between C8 (δ 165.6) and vinyl proton H7 (δ 5.90) allowed connection of the amide carbonyl (**B**) to the C1-C7 diene (**A**) which was further supported by the characteristic chemical shifts at C7 (δ 122.8) and C6 (δ 141.2) for an α, β unsaturated system, respectively. Likewise, a correlation from amide *N*H (δ 6.46) to carbonyl C8 (δ 165.6) connected substructure (**C**) to (**A-B**). An important HMBC correlation from thiazole ring carbon C14 (δ 170.0) to methine proton H9 (δ 5.33) allowed connection of substructure (**E**) to (**C**) rather than to (**A**) at position C2.

Unfortunately, no HMBC correlations were observed from the trichloro carbon C13 (δ 105.7) to the rest of the structure. This was probably due to the slow relaxation (long T_1) of this particular carbon. Evidence for attaching the Cl_3C residue (**D**) to C2 came from a ^{13}C NMR chemical shift analysis (Table 3.2), as agreement between the shift of CZ in the substructure shown and C2 (δ 54.6) in herbamide A (**1**) was achieved for the case where R = Cl_3 giving the calculated shift of δ 53.2. This final attachment completed the planar structure of herbamide A (**1**).



herbamide A **1**

$[\alpha]_D = +13°$ (c 0.013, $CHCl_3$)

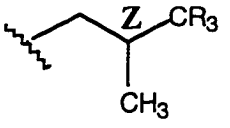
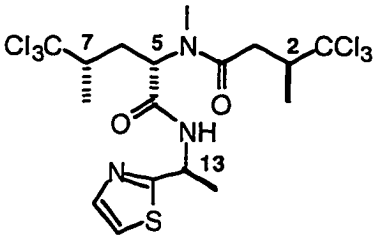


Table 3.2. ^{13}C NMR Comparison Data of Trichloroisopropyl Group.

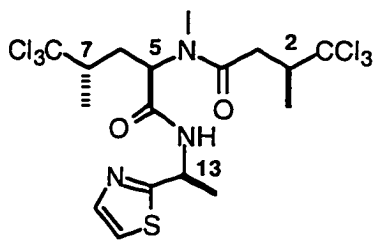
R_3	Z(δ)	CH_3(δ)	Ref.
H_3	28.2	22.3	10a
H_2Cl	34.5	17.2	10a
HCl_2	42.2	15.1	10b
Cl_3	53.2	16.1	10c

The planar structure of the major component, dysidenin (**2**), was rapidly deduced by comparing my ^1H, ^{13}C-NMR and mass spectral data to that reported in the literature (Table 3.3).[11,12] Less straightforward was the assignment of the absolute stereochemistry at C2, C7 and C13. I measured an $[\alpha]_D$ = -68° which was close to the $[\alpha]_D$ = -98° reported for (-)-dysidenin (**2**)[11a], 2*S*, 5*S*, 7*S*, 13*S*, but distinctly different compared to the $[\alpha]_D$ = +47° for (+)-isodysidenin (**3**)[11b], 2*S*, 5*R*, 7*S*, 13*S*. The differences in the ^1H NMR chemical shifts at H2, $H_2$3, H5, $H_2$6 and H7 reported in the literature for (-)-dysidenin (**2**) and (+)-isodysidenin (**3**) diastereomers reflects the stereochemical difference at C5 between these two compounds (Table 3.3). The shifts measured for these hydrogens in my material were nearly identical to those reported for (-)-dysidenin (**2**) suggesting that the stereochemistry at C2, C5, and C7 is identical for this pair of compounds (Table 3.3). Unfortunately, the reference data for the protons attached to C13 in the dysidenin framework are incomplete, because no chemical shift assignments have been made for CH13 of the *R* isomer.[13] Also, comparison of the $[\alpha]_D$ between (-)-dysidenin (-98°)[11a] and (+)-13-demethyldysidenin (+96°)[12] shows that this parameter is probably insensitive to epimeric changes at C13. Combining all of the above considerations with

the added assumption that there must be a chemotaxonomic analogy between this and the past work on *D. herbacea* prompts the provisional conclusion that my compound is identical to (-)-dysidenin (**2**).



dysidenin [11a] **2**



isodysidenin [11b] **3**

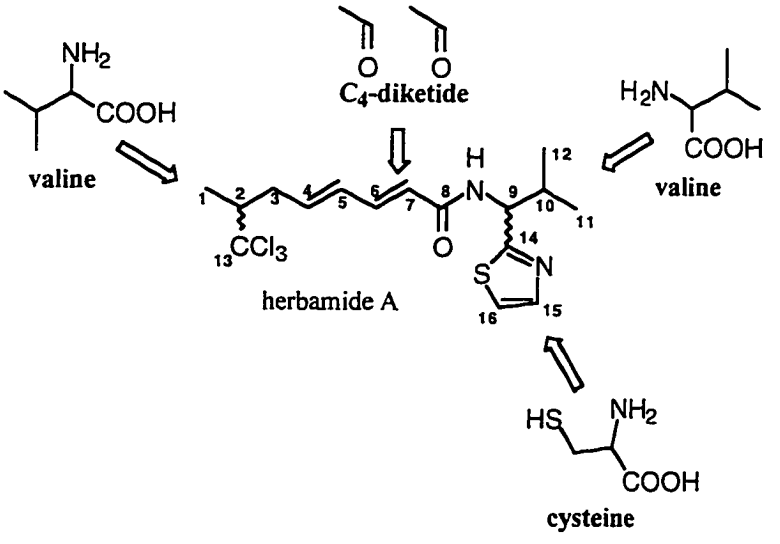
Table 3.3. Comparison of ^{1}H and ^{13}C-NMR Data Between dysidenin (**2**) and Literature.

Position	^{13}C δ	^{1}H δ, mult., (*J* = Hz)	^{13}C δ Lit.*	^{1}H δ Lit*	^{1}H δ Lit‡
1	17.4	1.39, d, (6.5)	17.3	1.36	1.39
2	51.5	3.38, ddq, (10.0, 6.5, 2.5)	51.4	3.30	3.30
3	37.5	3.13, dd, (16.3, 2.5)	37.4	3.15	3.08
		2.49, dd, (16.3, 10.0)		2.51	2.51
4	172.1		171.9	-	-
5	54.1	5.37, dd, (10.5, 2.5)	54.0	5.27	5.34
6	31.0	2.62, dd, (14.8, 10.5)	31.0	2.64	2.94
		1.92, ddd, (14.8, 10.5, 2.5)		1.94	1.49
7	51.9	2.22, ddq, (10.5, 6.5, 2.5)	51.9	2.20	2.68
8	16.3	1.36, d, (6.5)	16.2	1.33	1.36
9	105.6	-	105.5	-	-
10	30.9	3.04, s	30.8	3.04	2.98
11	105.2	-	105.1	-	-
12	168.9	-	168.2	-	-
13	47.3	5.36, q, (6.8)	47.3	5.20	5.43
14	21.9	1.59, d, (6.8)	21.8	1.56	1.65
15	172.0	-	171.2	-	-
16	142.5	7.71, d, (3.3)	142.3	7.60	7.70
17	119.2	7.28, d, (3.3)	118.9	7.26	7.31
NH	-	6.84, bd, (7.0)	-	6.86	6.97

* dysidenin; ‡ isodysidenin.

Discussion

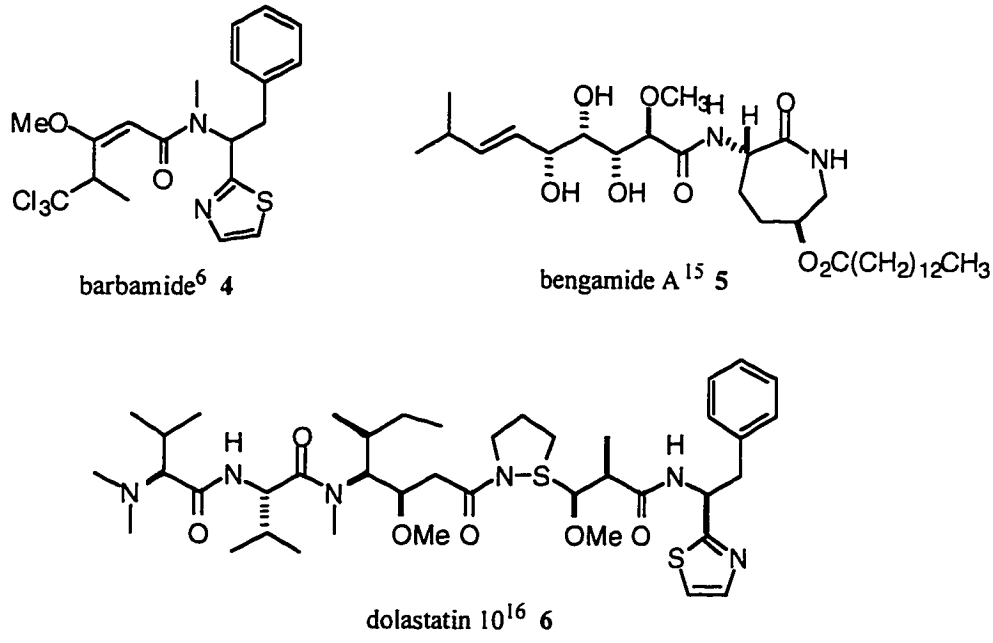
Herbamide A (**1**) has an interesting structure that introduces a new subclass to the known chlorinated peptides from *Dysidea* sponges containing cyanobacterial symbionts. I believe herbamide A has a similar biogenesis to dysidenin and isodysidenin and contains the same trichlorovaline subunit proposed by Garson[14] for 13-demethyldysidenin discussed in Chapter 1. Additionally, herbamide A appears to be the product of a mixed biogenesis due to the presence of a diketide portion in the structure and can be envisioned as a condensation product between this subunit and the amino acids valine and cysteine (Scheme 3.2).



Scheme 3.2. Possible Biogenesis of herbamide A.

Another compound, barbamide (**4**)[6], was isolated from the free-floating filamentous cyanobacteria *Lyngbya majuscula* and appears to be derived from a parallel biogenesis comprised of phenylalanine/valine dipeptide unit condensed with a single

ketide unit and trichlorovaline (Figure 3.3). Likewise, bengamide A (**5**) isolated by the UCSC labs can be imagined as derived from a leucine subunit condensed with a diketide portion instead of a trichlorovaline and diketide as in herbamide A.[15] Another compound from the sea hare, dolastatin 10 (**6**) an antineoplastic peptide, contains the same dipeptide phenylalanine/cysteine portion as in barbamide (**4**).[16] These biogenetic analogies are interesting and it is possible they are products of cyanobacterial metabolism. As mentioned in Chapter 1, barbamide exhibited molluscicidal activity, but herbamide A which was also tested by Gerwick in this assay, did not have any bioactivity.[17] Herbamide A was also inactive in the NCI disease oriented cytotoxicity screen.



barbamide[6] **4**

bengamide A[15] **5**

dolastatin 10[16] **6**

Figure 3.3. Marine Metabolites with Similar Biogenetic Pathways.

Supplementary Material

herbamide A

Figure 3.4. ^{1}H-NMR Spectra-herbamide A (300 MHz, $CDCl_3$).

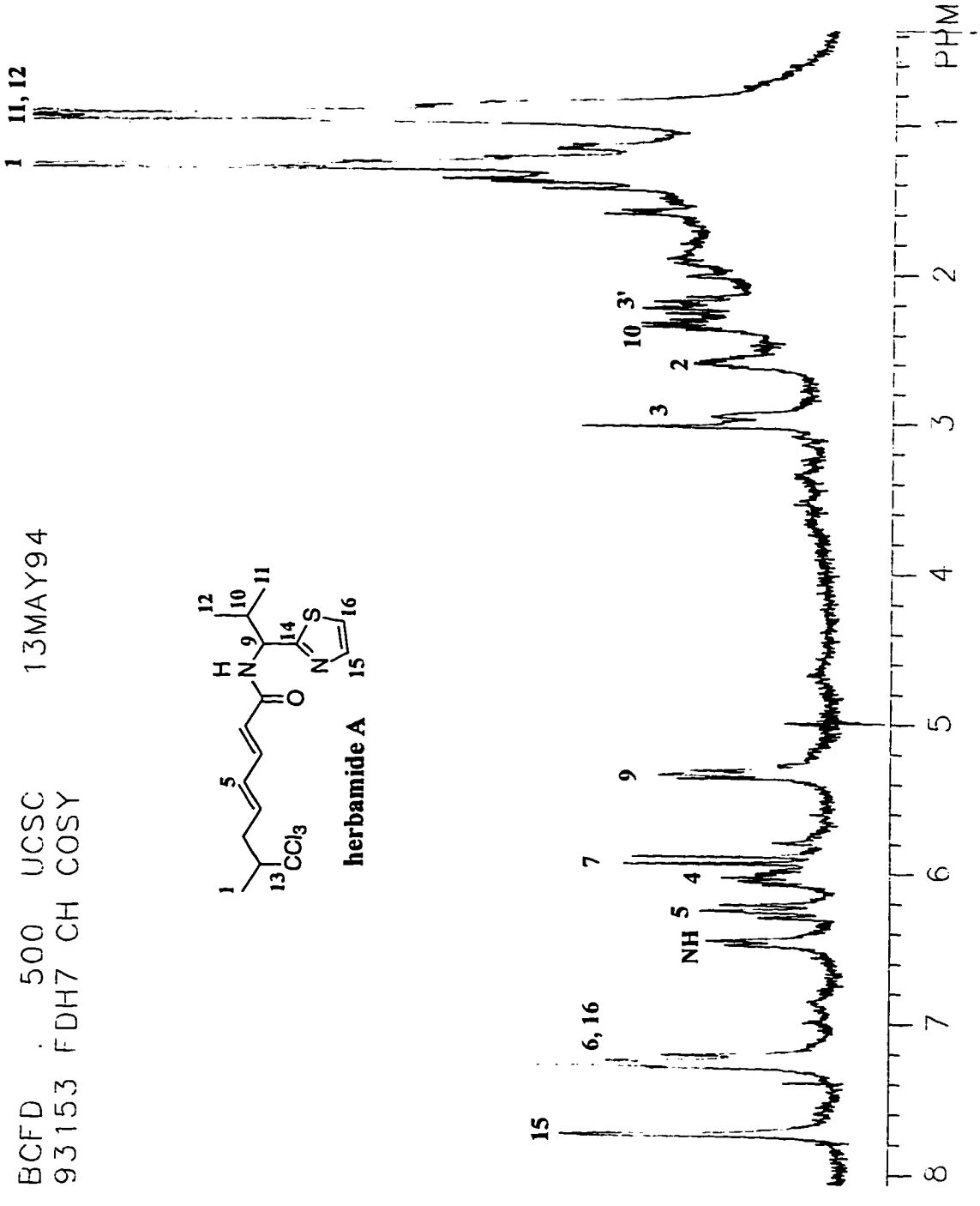


Figure 3.5. ^{13}C-NMR Spectra-herbamide A (75.5 MHz, $CDCl_3$).

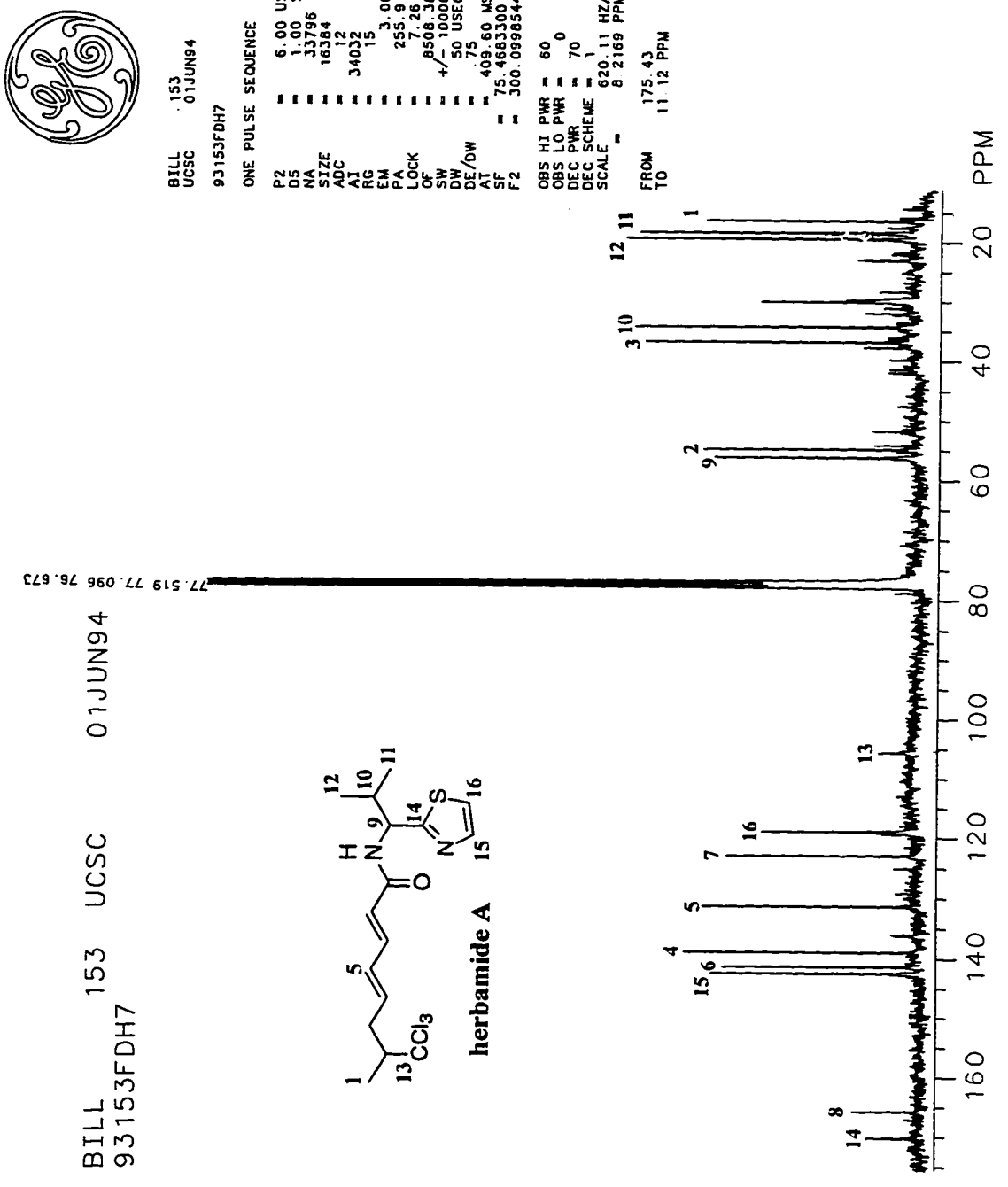


Figure 3.6. APT Spectra-herbamide A (75.5 MHz, $CDCl_3$).

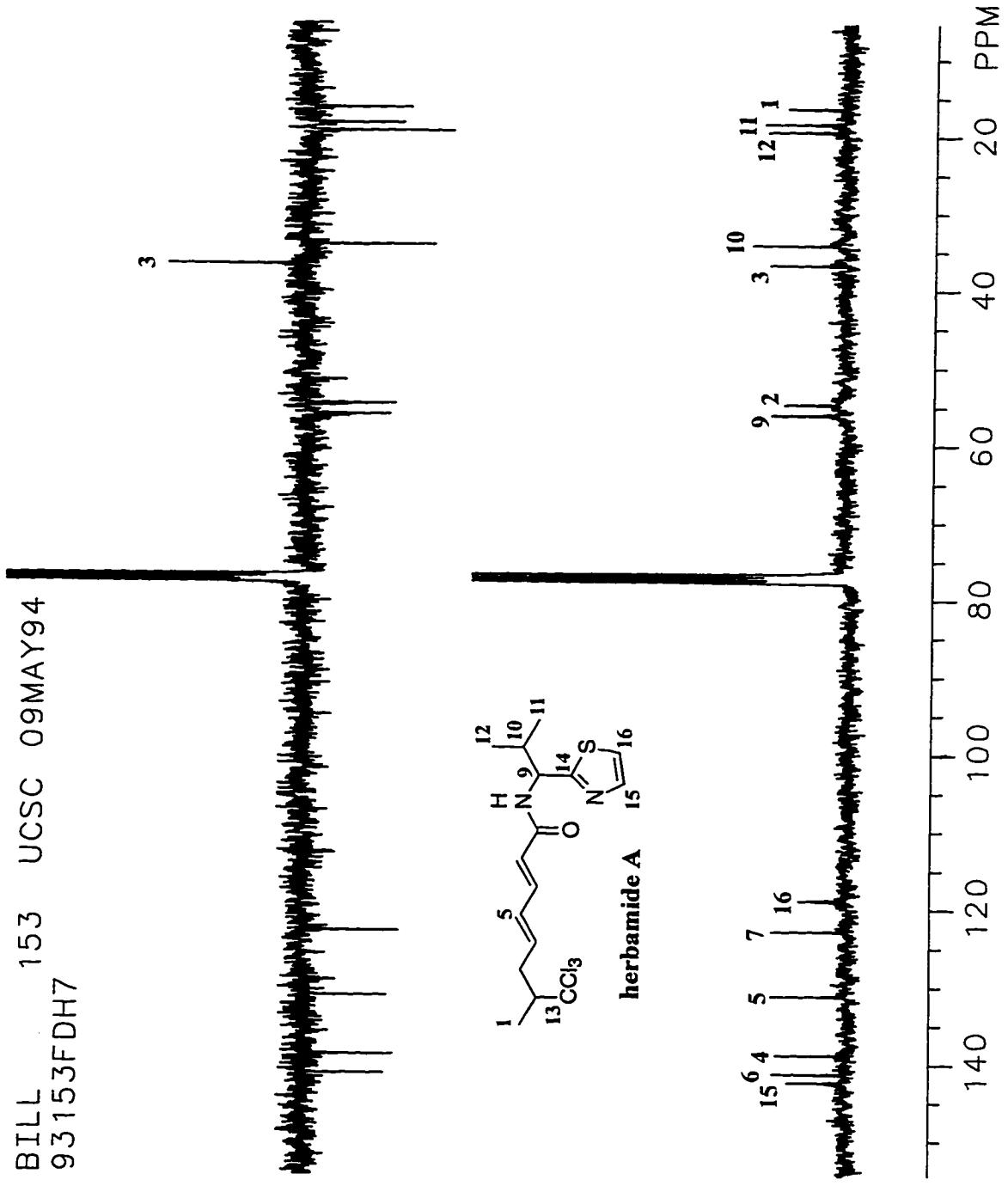


Figure 3.7. COSY Spectra-herbamide A (300 MHz, $CDCl_3$).

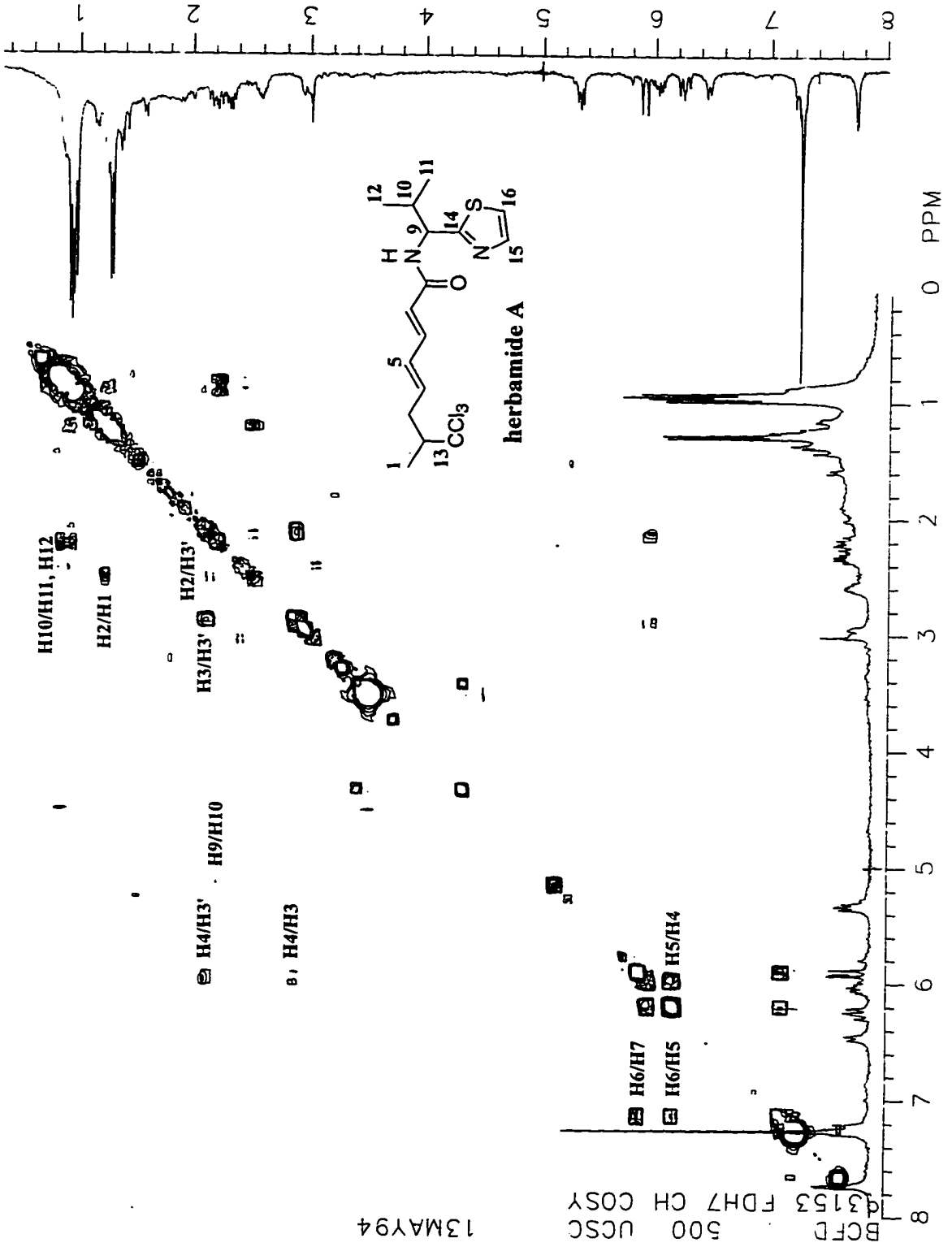


Figure 3.8. COSY/Expansion Spectra-herbamide A (300 MHz, $CDCl_3$).

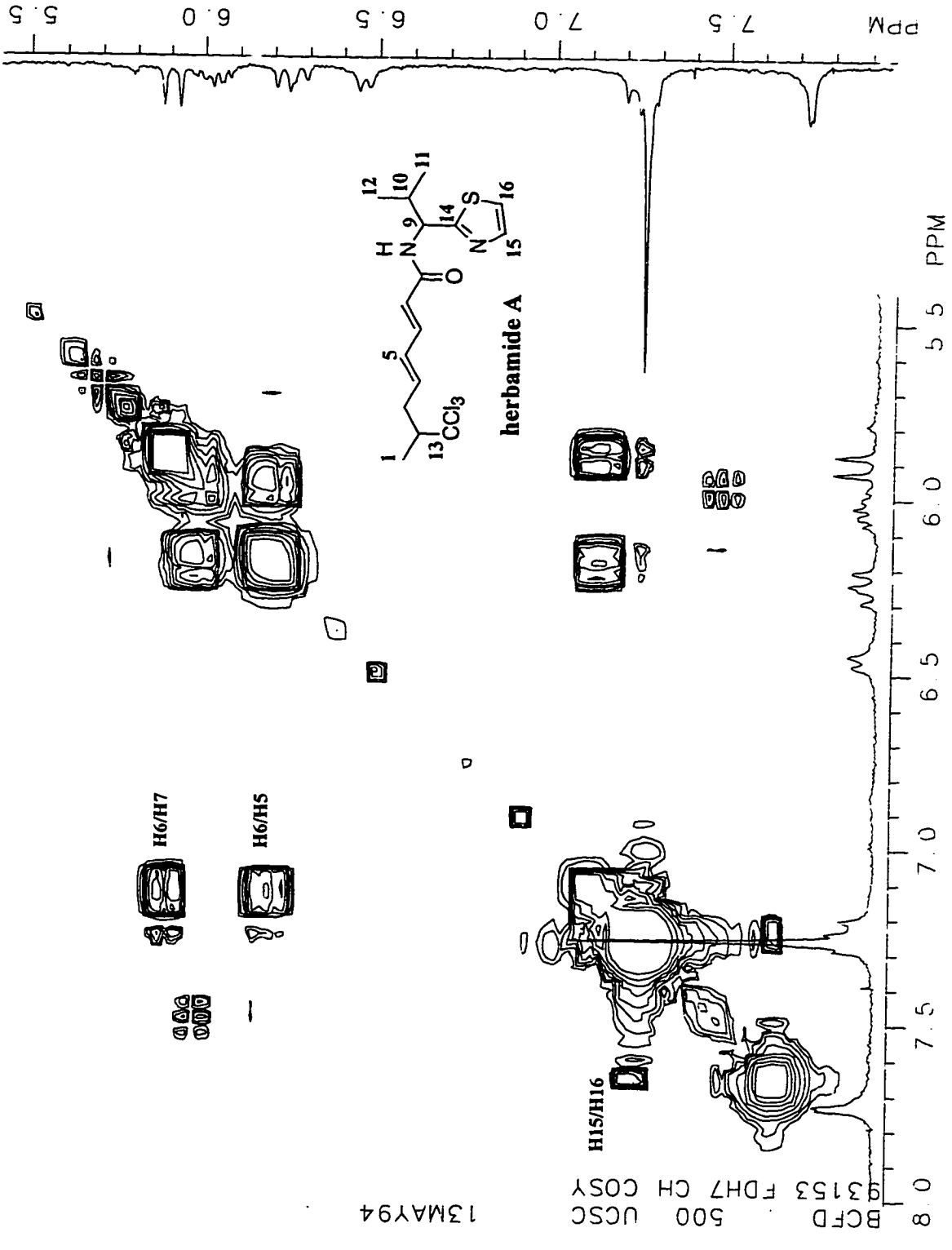


Figure 3.9. ^{1}H-NMR Spectra-herbamide A (500 MHz, DMSO-d6).

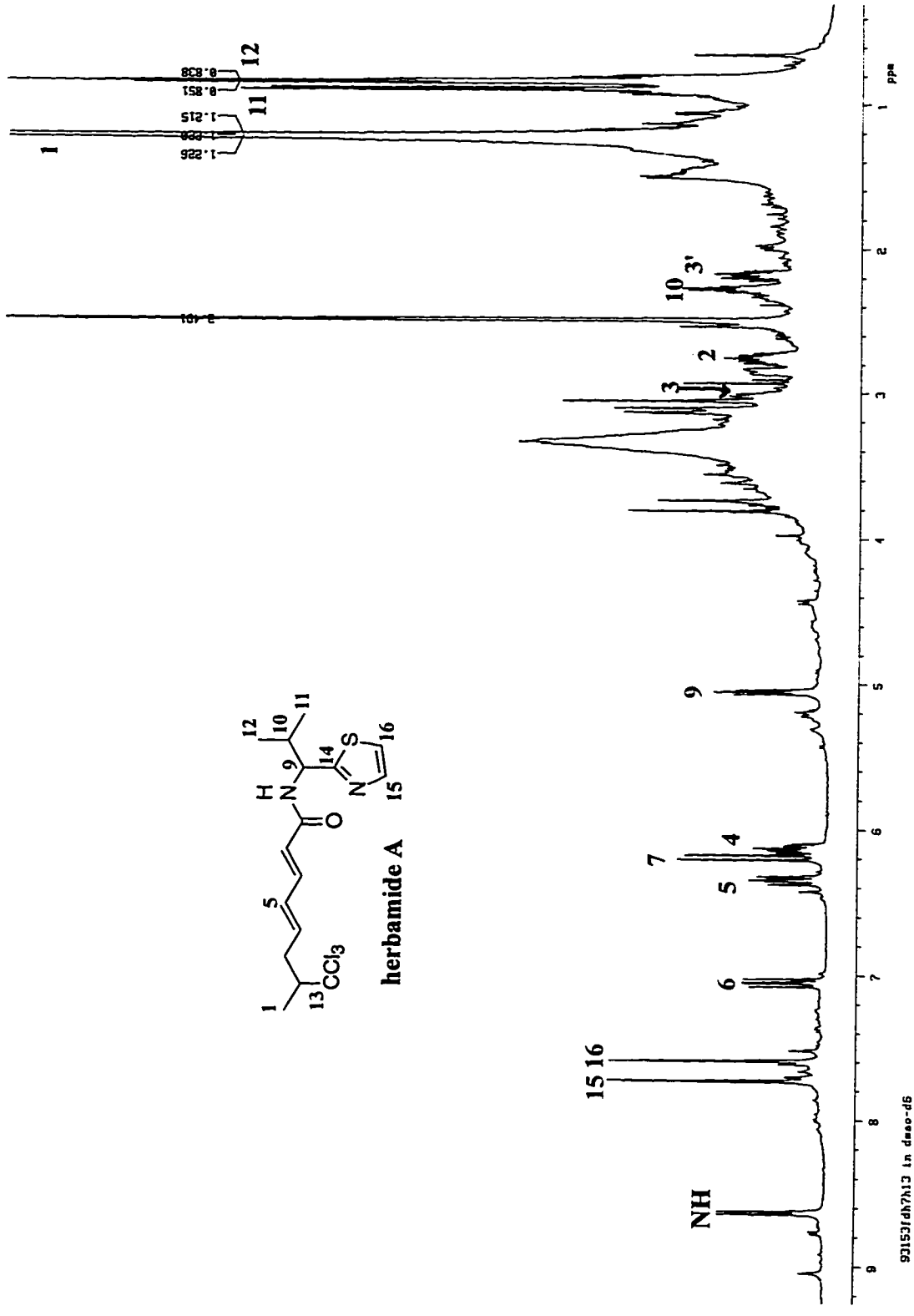


Figure 3.10. HMBC Spectra-herbamide A (500 MHz, DMSO-d6).

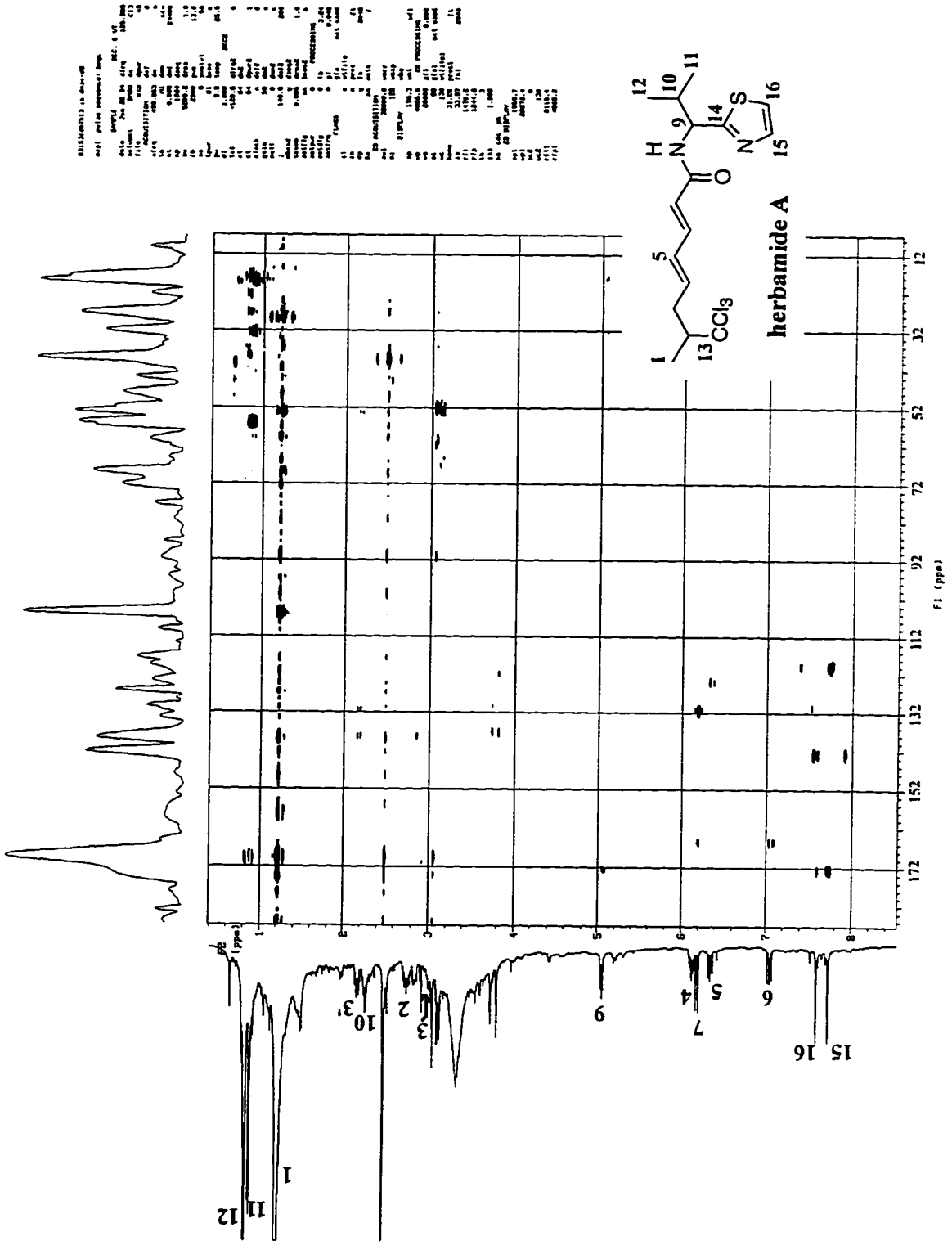


Figure 3.11. HMBC/Expansion Spectra-herbamide A (500 MHz, DMSO-d6).

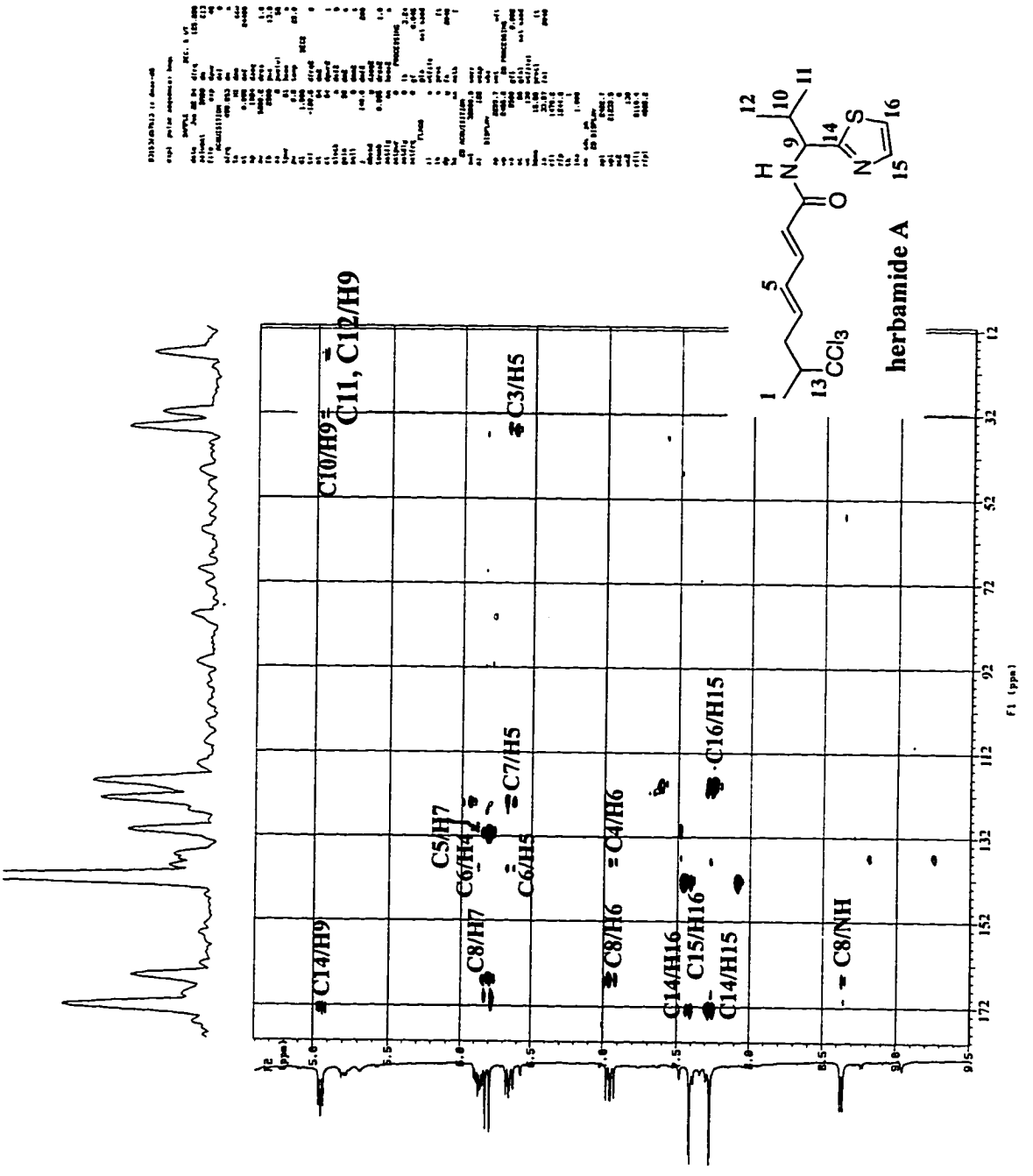


Figure 3.12. HMBC/Expansion Spectra-herbamide A (500 MHz, DMSO-d6).

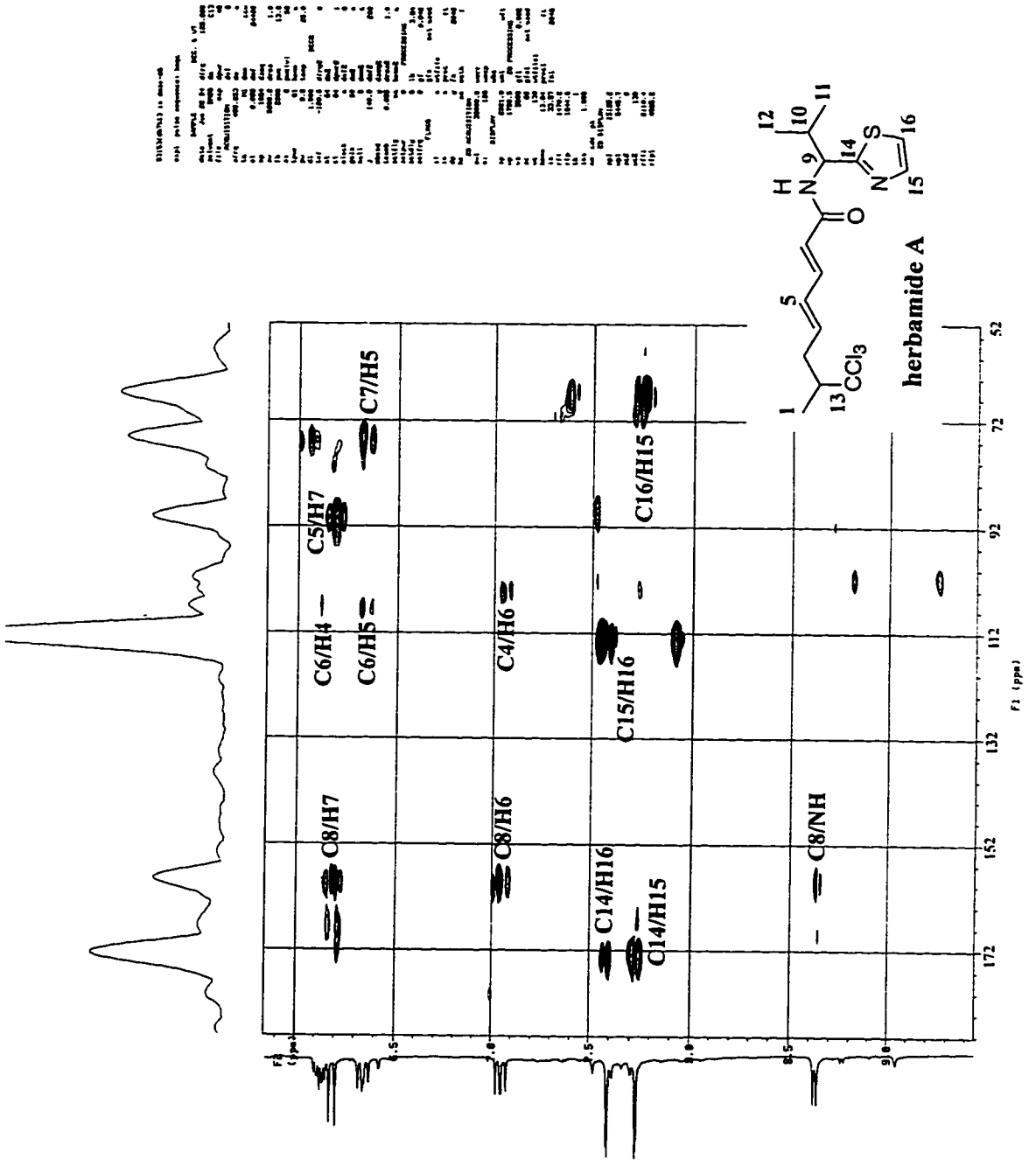


Figure 3.13. HMBC/Expansion Spectra-herbamide A (500 MHz, DMSO-d6).

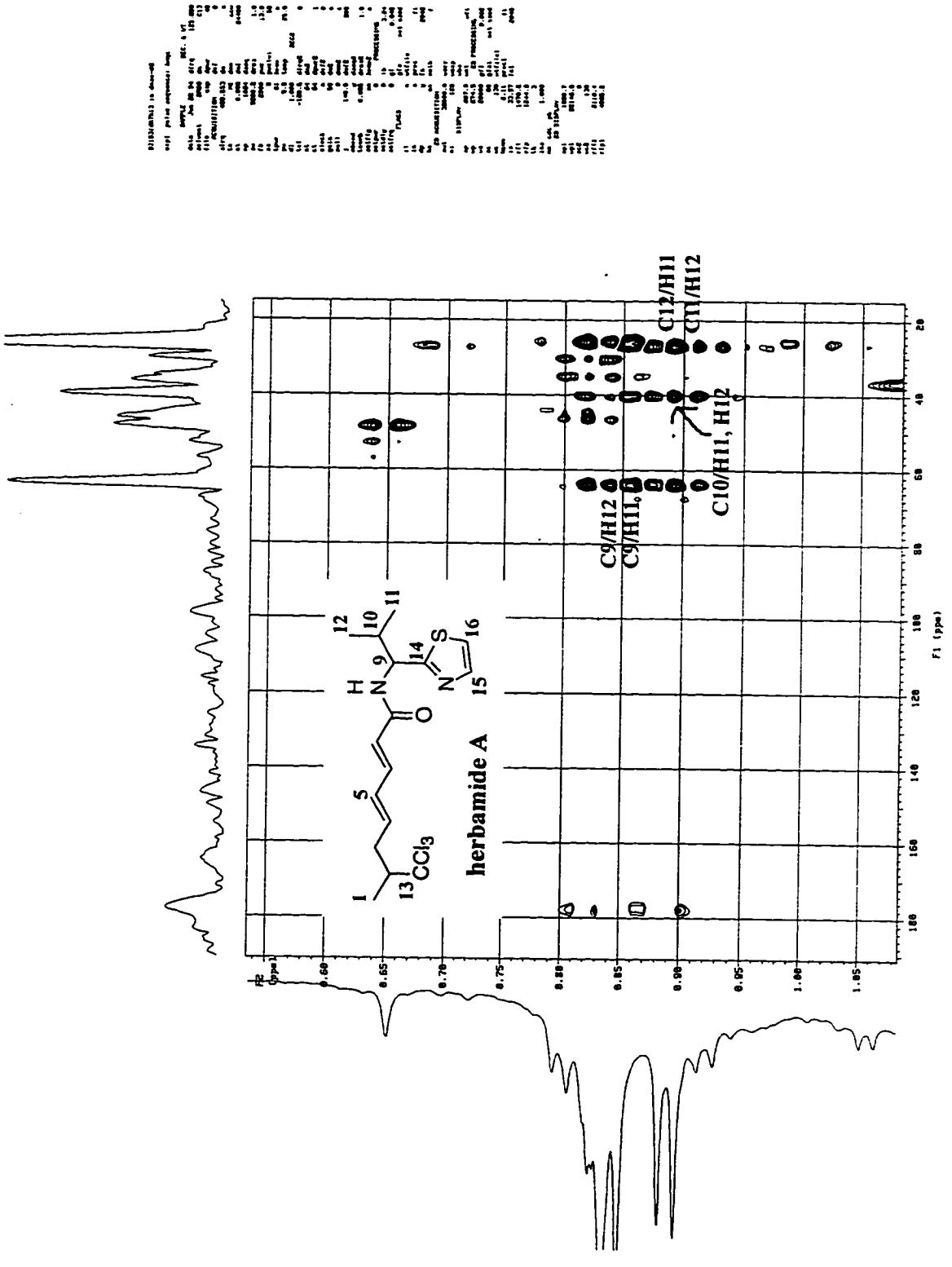


Figure 3.14. IR Spectra-herbamide A.

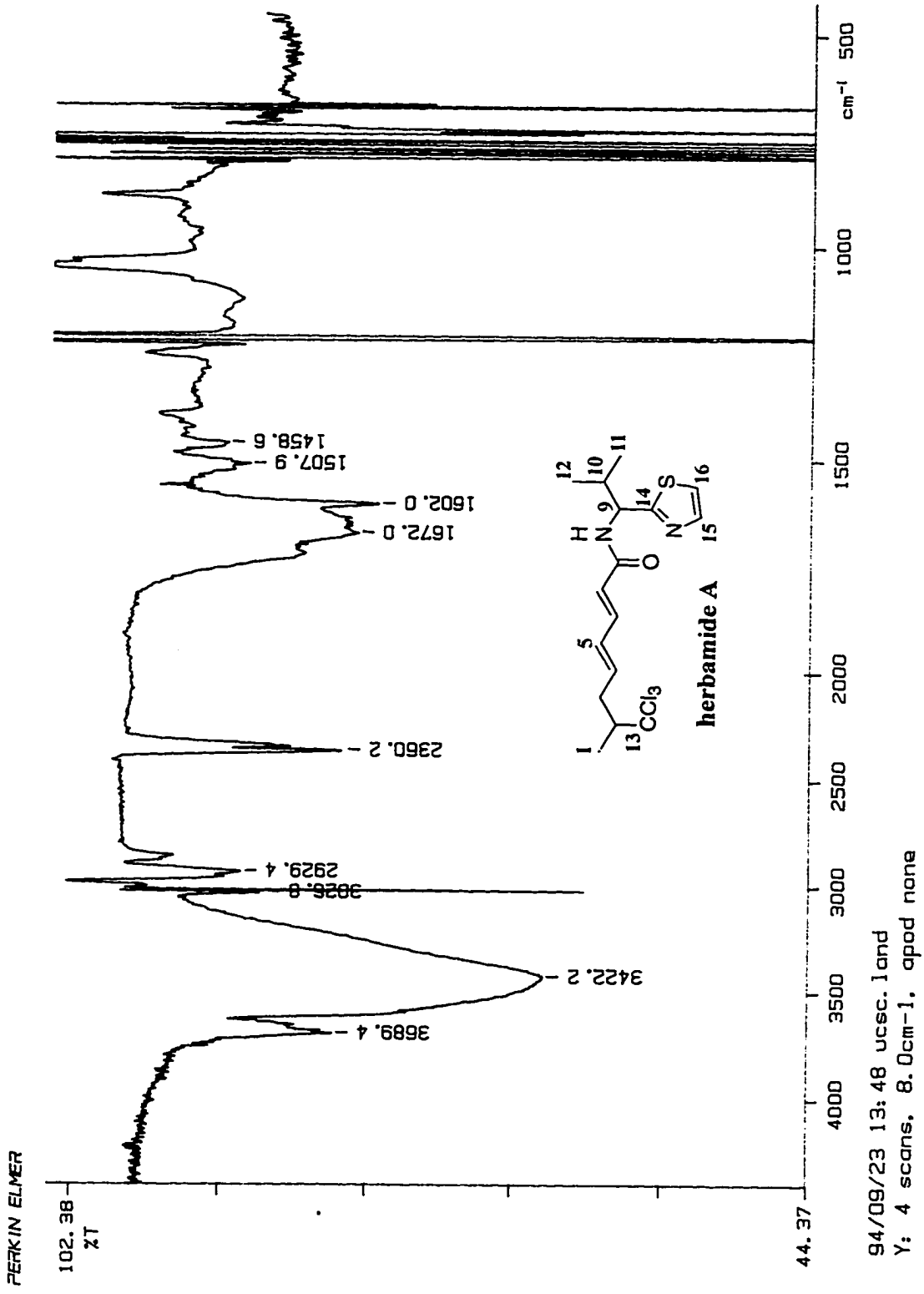


Figure 3.15. LRFAB Mass Spectra-herbamide A.

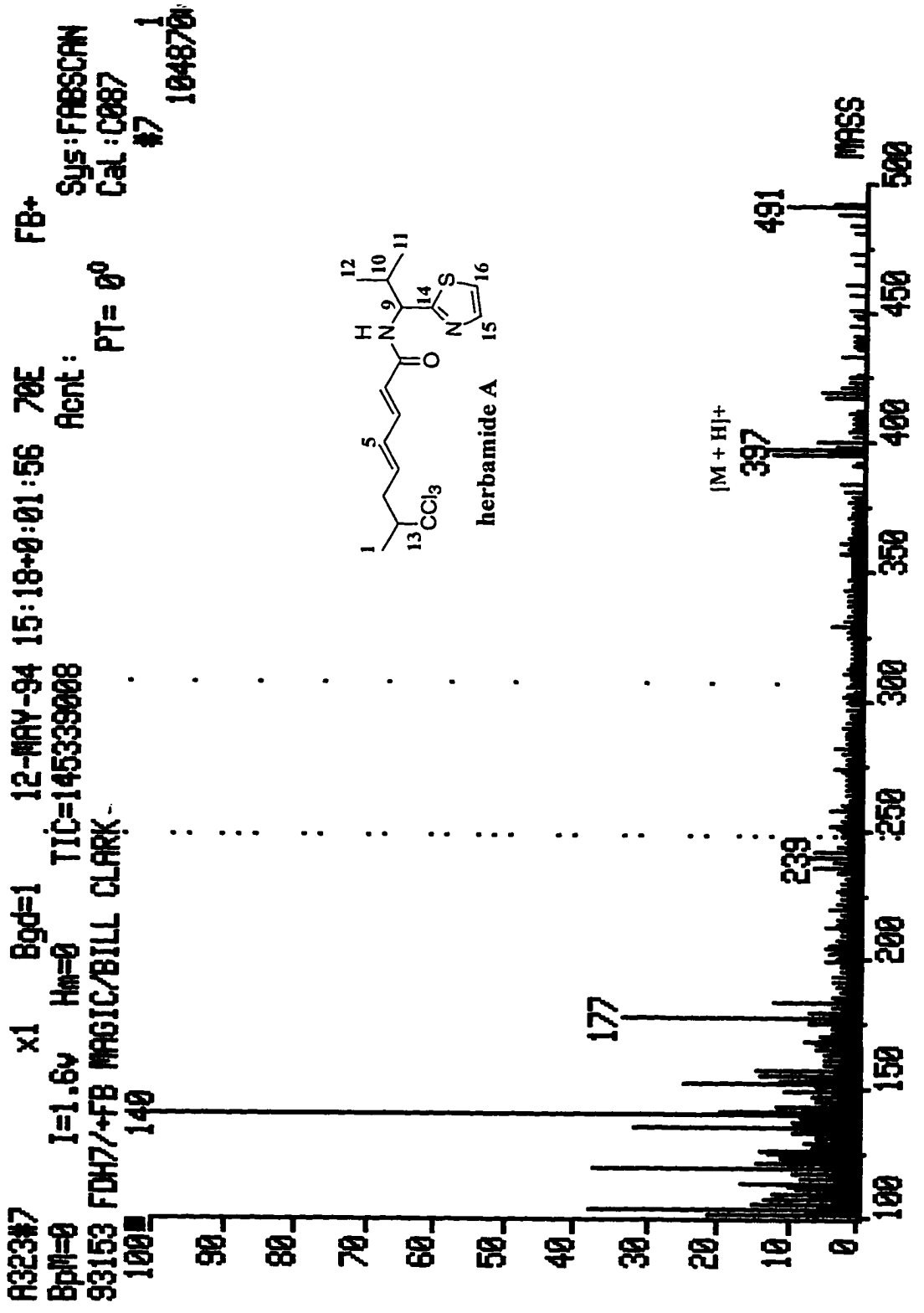
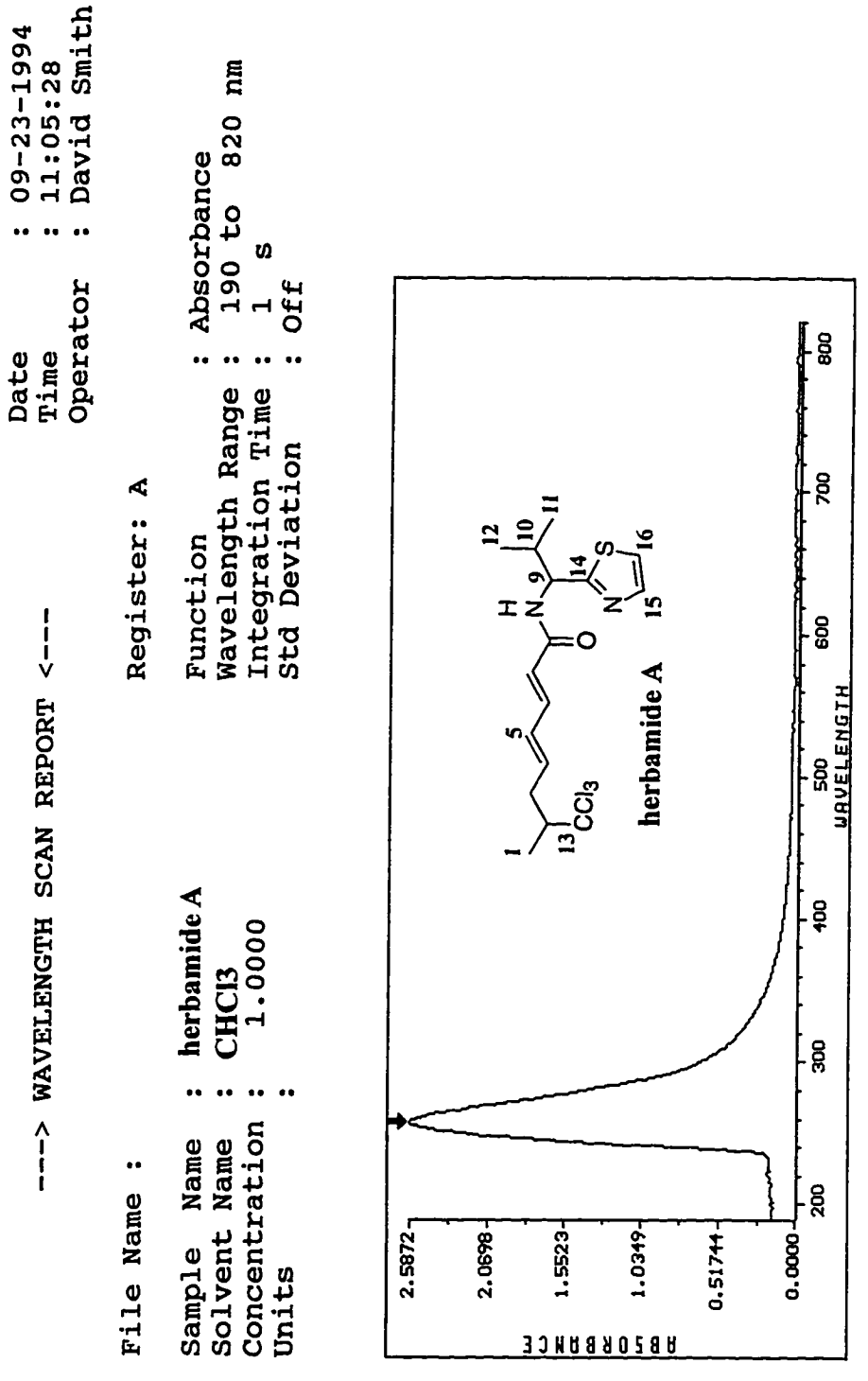


Figure 3.16. UV Spectra-herbamide A ($CHCl_3$).



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10. (a) Pretsch, E; Clerc, T; Seibl, J; Simon, W *Spectral Data for Structure Determination of Organic Compounds*; Fresenius, W; Huber, J. F. K.; Pungor, E; Rechnitz, G. A.; Simon, W; West, Th. S. Eds.; Springer-Verlag: Berlin, 1989, 2nd ed.; pp. C10-C25. (b) Isaacs, S.; Berman, R.; Kashman, Y. *J. Nat. Prod.* **1991**, *54*, 83-91. (c) Lee, G. M.; Molinski, T. F. *Tetrahedron Lett.* **1992**, *33*, 7671-7674.
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17. A sample (1.5 mg) of herbamide A was sent to Bill Gerwick at Oregon State University for testing in the mollusk assay on September 23, 1994. In about two weeks Gerwick responded by phone to say that herbamide A was inactive in their assay.

Chapter 4-New Halogenated Constituents from the Indo-Pacific Sponge *Dysidea*

Marine Sponge-Papua New Guinea Photo Courtesy of Jay Burreson, Ph.D.

Abstract

The Indo-Pacific marine sponge *Dysidea* continues to be a rich source of both brominated diphenyl ether and chlorinated peptide constituents. During the chemical analysis of forty-three specimens, by electrospray mass spectrometry, five new halogenated compounds were identified. Three new chlorinated peptides continue a theme represented by the known polychlorinated tetrapeptides dysidenin and isodysidenin. Dysidenins C (**1**) and D (**2**) contain a proline residue in place of alanine. The absolute stereochemistry of (**1**) was determined by X-ray crystallography as 2*S*, 5*R*, 7*S*, 13*S*, analogous to that reported for isodysidenin. Another specimen yielded the tetrachloro peptide 9, 11-didechlorodysidenin (**3**). Brominated dioxins, tetrabromodioxin (**4**) and tribromodioxin (**5**), were identified from another specimen. They represent the first ever naturally-occurring dioxins isolated from *Dysidea*. The structure of the previously unreported ketide amino acid, herbamide B (**6**), is also elucidated.

Background

As discussed in Chapter 3, I was investigating the chemistry of *Dysidea* sponges from new areas of Papua New Guinea (PNG) in search of chlorinated peptides and to evaluate the chemotaxonomy of this genus. Later on in the chemotaxonomy study the UCSC marine natural products program began to investigate new areas in Indonesia as well as in PNG. These collections (three expeditions) took place over a two year period (Spring 1994-Fall 1995) from both Papua New Guinea and Indonesia. It was during the chemotaxonomic analysis of ~50 specimens from these new areas which identified the presence of new halogenated, bromo and chloro, metabolites of the chemotypes normally identified with this sponge; the chlorinated peptides[1] and brominated diphenyl ethers[2].

The chemotaxonomic analysis of the specimens collected involved the use of electrospray ionization mass spectrometry (ESIMS).[3] ESIMS was chosen over thin layer chromatography as the method for identification of the constituents present in the specimens after the following events. First, the UCSC marine natural products program had begun to use ESIMS in the routine screening of fungal extracts as a dereplication tool. ESIMS is a soft-ionization technique that allows visualization of only molecular ion peaks with little or no fragmentation.[4] The goal was to grow up only those fungal cultures that appeared to produce new constituents by examination of their respective masses. This method had been traditionally used in the examination of large peptides (> 10,000 Dalton)[5] and not so much with small organic molecules[6]. Since only molecular ions are visualized during this technique, I obtained ESI mass spectra of dysidenin and a hexabrominated diphenyl ether, that were previously isolated, to make sure my standards for TLC were pure. It was discovered at this time that both compounds ionized extremely well due to their polyhalogenation (It should be mentioned that not all small

organic compounds ionize well in electrospray. For example, cyclocinamide A gave only a small peak in positive mode even though it is a peptide with available *N*H's for ionization.). Additionally, the bromophenol ionized only in negative mode while the chlorinated peptide ionized only in positive mode. This was very interesting and therefore it was decided that ESIMS would prove to be a much better tool than TLC in the chemotaxonomy study. Not only could it easily distinguish between a chloro or bromo compound, but it could also unequivocally identify *which* chloro or bromo compound was present due to the fact that there was no fragmentation.

Table 4.1. Masses of Known Polychlorinated Peptides Reported from *Dysidea.*

Exact Mass	Molar Mass	Formula	Reference (Chapter 1)	Name
253.1	254.2	C10 H17 Cl2 N O2	85	Dysidamide C
390	392.2	C14 H22 Cl4 N2 O2	87	Dysamide B
394	395.8	C16 H21 Cl3 N2 O S	59	Herbamide A
397.1	398.7	C16 H22 Cl3 N O4	79	Dysidin
404	406.2	C14 H20 Cl4 N2 O S	88	9,10-Didechloro-N-methyldysideathiazole
405	407.2	C15 H23 Cl4 N 03	85	Dysidamide B
424	426.6	C13 H17 Cl5 N2 O S	88	10-Dechloro-dysideathiazole
438	440.6	C14 H19 Cl5 N2 O S	88	10-Dechloro-N-methyldysideathiazole
453.9	457	C14 H16 Cl6 N2 O2	87	Dysamide C
455.9	459	C14 H18 Cl6 N2 O2	82	Diketopiperazine
457.9	461.1	C13 H16 Cl6 N2 O S	88	Dysideathiazole
458	461	C14 H20 Cl6 N2 O2	87	Dysamide A
471.9	475.1	C14 H18 Cl6 N2 O S	88	N-Methyldysideathiazole
473	476.1	C15 H21 Cl6 N O3	86	Herbaceamide
473	476.1	C15 H21 Cl6 N 03	84	Dysidamide
495	497.7	C16 H22 Cl5 N3 O2 S	83	9-Monodechloro-13-Demethylisodysidenin
495	497.7	C16 H22 Cl5 N3 O2 S	83	11-Monodechloro-13-Demethylisodysidenin
528	531.1	C17 H22 Cl6 N2 O4	88	Dysideapyrrolidone
528.9	532.1	C16 H21 Cl6 N3 O2 S	83	13-Demethyldysidenin
528.9	532.1	C16 H21 Cl6 N3 O2 S	83	13-Demethylisodysidenin
543	546.2	C17 H23 Cl6 N3 O2 S	81	(+)-Isodysidenin
543	546.2	C17 H23 Cl6 N3 O2 S	80	(-)-Dysidenin

Table 4.2. Masses of Known Polybrominated Diphenyl Ethers Reported from *Dysidea.*

Exact Mass	Molar Mass	Formula	Reference (Chapter 1)
265.9	267.9	C6 H4 Br2 O2	61
265.9	267.9	C6 H4 Br2 O2	61
341.9	344	C12 H8 Br2 O2	62
419.8	422.9	C12 H7 Br3 O2	62
435.8	438.9	C12 H7 Br3 O3	63
449.8	452.9	C13 H9 Br3 O3	63
497.7	501.8	C12 H6 Br4 O2	64
497.7	501.8	C12 H6 Br4 O2	65
497.7	501.8	C12 H6 Br4 O2	66
511.7	515.7	C13 H8 Br4 O2	67
513.7	517.8	C12 H6 Br4 O3	63
527.7	531.8	C13 H8 Br4 O3	61
527.7	531.8	C13 H8 Br4 O3	68
531.7	536.2	C12 H5 Br4 Cl O2	65
541.7	545.9	C14 H10 Br4 O3	64
575.6	580.7	C12 H5 Br5 O2	69
575.6	580.7	C12 H5 Br5 O2	64
575.6	580.7	C12 H5 Br5 O2	62
591.6	596.7	C12 H5 Br5 O3	61
591.6	596.7	C12 H5 Br5 O3	68
591.6	596.7	C12 H5 Br5 O3	61
605.6	610.7	C13 H7 Br5 O3	61
605.6	610.7	C13 H7 Br5 O3	63
605.6	610.7	C13 H7 Br5 O3	68
619.6	624.7	C14 H9 Br5 O3	68
653.5	659.6	C12 H4 Br6 O2	70
669.5	675.6	C12 H4 Br6 O3	68

Since I had planned to use ESIMS in the chemotaxonomic analysis of the sponge specimens it became necessary to construct mass spec tables comprising the masses of all the known chlorinated peptides and brominated diphenyl ethers (Tables 4.1 and 4.2). The information in these tables was to serve a twofold purpose; it would allow identification of the type of metabolites present in the sponge extracts and hopefully identify new halogenated constituents as well. Consequently, the serendipitous nature of all the above events lead to the discovery of five new halogenated compounds from two collections of *Dysidea.* I will now discuss the isolation and structure elucidation of all these new compounds and another ketide amino acid isolated along with herbamide A. This work has been submitted for publication in the *Journal of Natural Products.*

Collection and Isolation of dysidenins C, D and 9, 11-didechlorodysidenin

During the expedition to Milne Bay, Papua New Guinea in the Spring of 1995 fourteen specimens of *Dysidea* were collected that fit the general morphology described by Bergquist.[7] They were all identified as containing cyanobacteria due to their greenish color as is characteristic of the sponge (Figure 4.1; no photograph was available of 95153). The screening of these specimens by electrospray ionization mass spectroscopy allowed identification of two collections, 95078 and 95153, as different due to mass peaks of *m/z* 569.1 and 476.1 visualized in the mass spectra, respectively (Figures 4.16, 4.35 in Supp. Mat.). These masses were not represented in the mass spec databases and appeared to be highly chlorinated upon examination of their isotope distribution patterns (Tables 4.1, 4.2).[8] The *m/z* 569.1 peak suggested six chlorine atoms while the *m/z* 476.1 peak was indicative of four chlorines. This was very interesting because dysidenin represented the highest molecular weight chlorinated peptide known at *m/z* 543.

This information in hand I began the workup of both 95078 and 95153. The crude methanol extracts were subjected to our standard liquid-liquid partitioning scheme to yield the usual semi-pure fractions (Schemes 4.1, 4.2; Appendix A).[9] Examination of the ^{1}H-NMR spectra of the FD fractions of both sponge collections revealed the presence of a thiazole ring with the characteristic shifts at ~ δ 7.74 and 7.26. Subsequent purification of the 95078 FD fraction by HPLC (ODS, MeOH:THF:MeCN:H_2O, 51:9:12:28; Appendix B) yielded unexpectantly two fractions, H4-dysidenin C (**1**) and H6-dysidenin D (**2**), that both looked identical in their ^{1}H-NMR spectra and had the same mass of *m/z* 569.1. Additionally, they eluted 25 minutes apart indicating that they might be diastereomeric in nature. Also isolated was the known sterol hebasterol, H2, based on

comparison to NMR data of previously isolated material of mine from another collection and to the literature.[10] Purification of 95153 FD by HPLC (ODS, 35 → 100% MeOH in 90 minutes; λ 215) easily afforded the tetrachloropeptide 9,11-didechlorodysidenin (**3**). I will now discuss the structure elucidation of these three compounds.

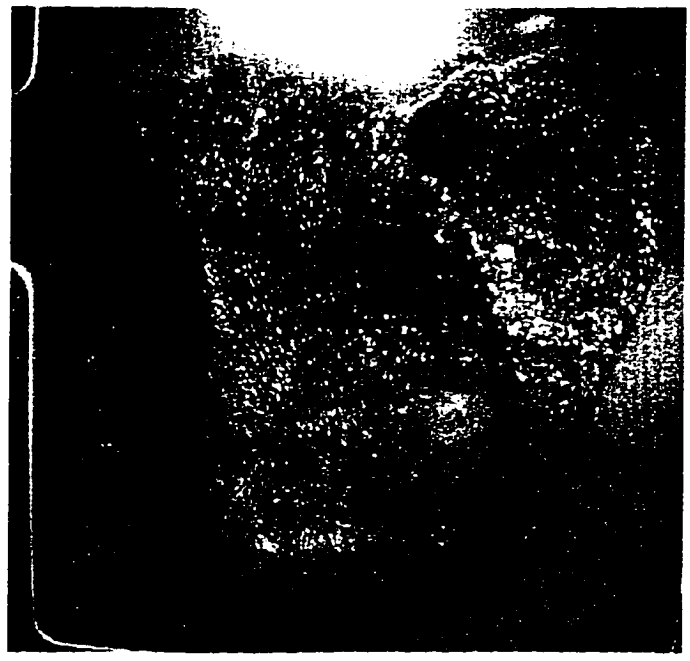
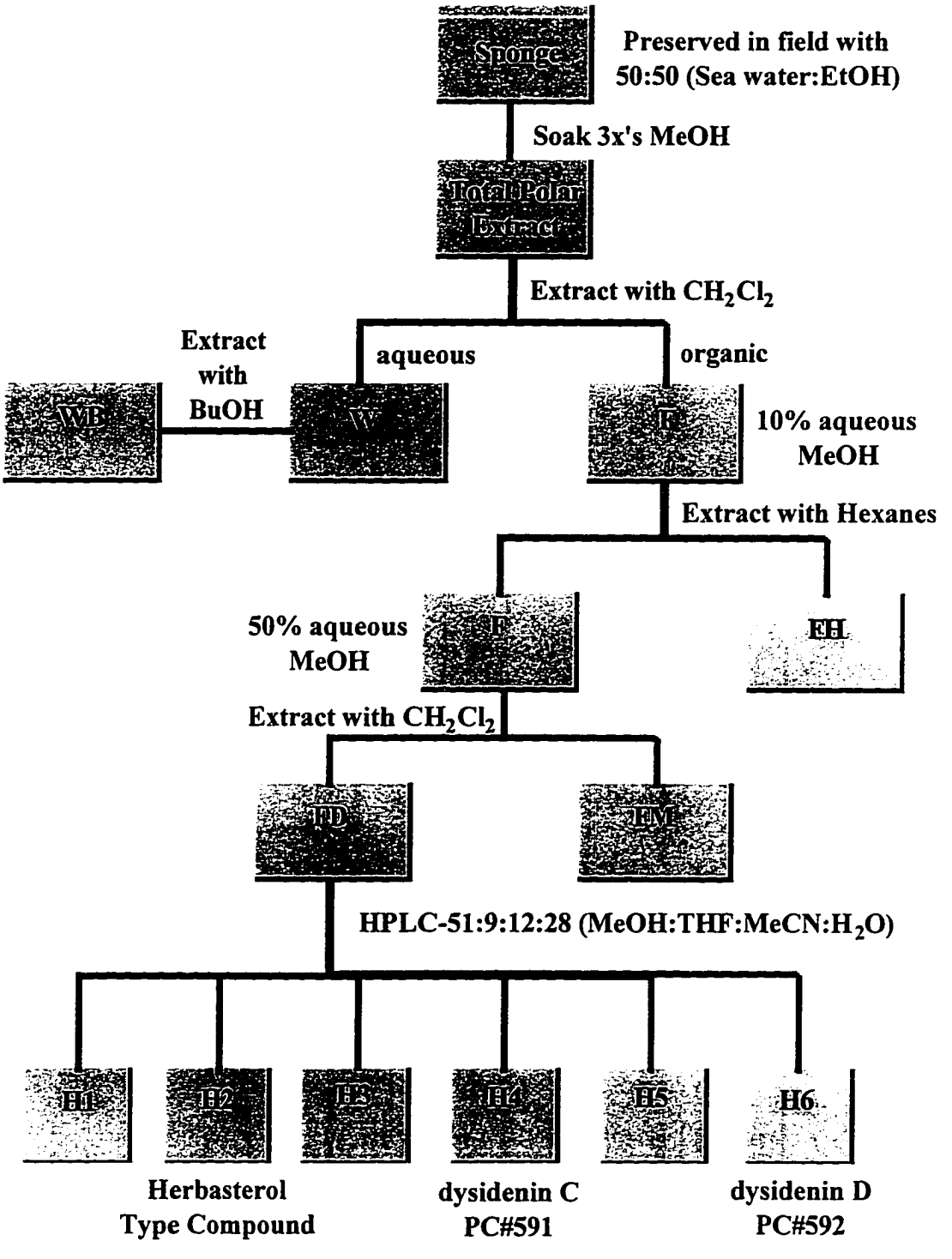
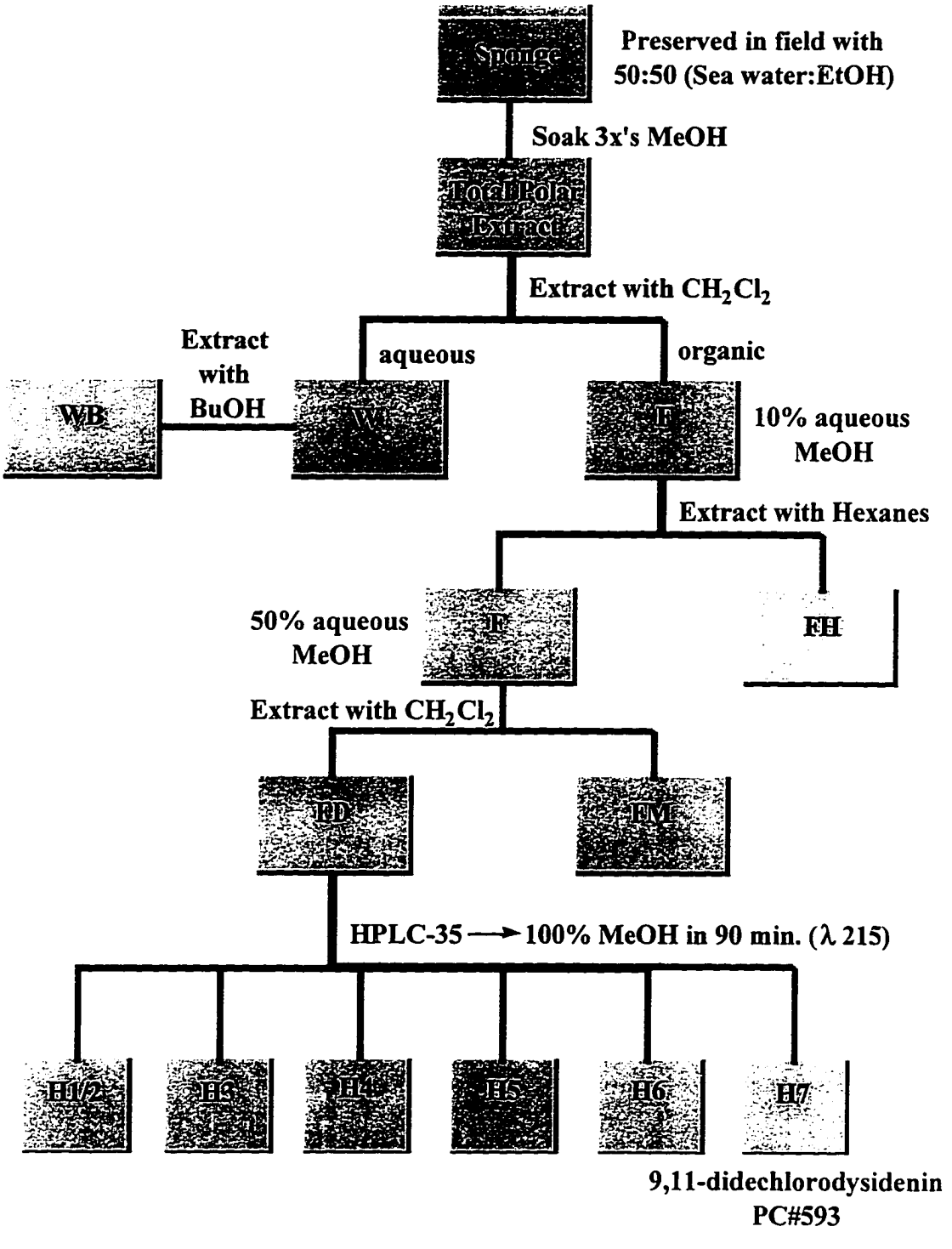


Figure 4.1. Topside Photograph of 95078.



Scheme 4.1. Extraction Scheme of 95078.



Scheme 4.2. Extraction Scheme of 95153.

Structure Elucidation of dysidenins C and D.

The elucidation of dysidenin C (**1**) was undertaken first realizing that this was another hexachloro tetrapeptide like dysidenin. However, it probably contained a different amino acid due to the larger molecular weight as evidence by LRFAB and ESI mass spec (positive mode) (Figures 4.16, 4.17 in Supp. Mat.). The difference in molecular weight between dysidenin and (**1**) was only 26 a.m.u.'s. This indicated to me that there was probably not a large difference in their structures. Another curious observation was the apparent doubling of some of the proton resonances in the NMR spectra of not only (**1**) but also (**2**) even though their peaks were quite resolved in the HPLC trace. I believed the compounds to be pure and concluded that the doubling was due to some conformational behavior of the molecule. The ^{1}H and ^{13}C resonances of the thiazole ring exhibited the most noticeable doubling which appeared to be in a 2:1 ratio (Figures 4.13, 4.14 in Supp. Mat.). To investigate this further I obtained a ^{1}H-NMR spectra in dimethylformamide-d7 at 150°C (DMF-d7, 500 MHz). The high boiling point of DMF-d7 allowed me to raise the temperature enough (25°C increments) to observe the peaks coalescing confirming that there was indeed conformational mobility in the molecule (Figures 4.30, 4.31 Supp. Mat.).

A molecular formula of $C_{19}H_{25}Cl_6N_3O_2S$ was established by HRFABMS with a $[MH]^+$ peak at *m/z* 568.9877 (Δ 0.0 ppm) indicating an unsaturation of six, one more than dysidenin. Comparison of the ^{1}H, ^{13}C, APT and HMQC NMR spectra suggested an APT formula of $C_{19}H_{25}$ with no protons attached to heteroatoms (Table 4.3; Figures 4.13-15, 4.22-24 in Supp. Mat.). Five ^{13}C resonances at δ 171.5, 170.2, 168.0, 142.4 and 118.9 were evidence of two amide carbonyls (IR, 1724, 1645 cm^{-1}) and a thiazole ring

leaving one ring to satisfy the unsaturation number (Table 4.3; Figures 4.14, 4.18 in Supp. Mat.).

Table 4.3. ^{1}H, ^{13}C, COSY and HMBC NMR Data ($CDCl_3$, 125/500 MHz) of dysidenin C (**1**).

Position	^{13}C δ	^{1}H δ, mult., (*J*= Hz)	COSY (H → H)	HMBC (C → H)
1	17.5	1.39, d, (6.5)	H2	H3, 3'
2	51.6	3.35, ddq, (9.0, 6.5, 2.0)	H1, H3, 3'	H1, H3 ,3'
3	37.4	3.07, dd, (16.5, 2.0)	H2, H3'	H1
		2.50, dd, (16.5, 9.0)	H2, H3	
4	170.2	-	-	H3, 3', H10
5	58.7	5.56, t, (5.5, 5.5)	H6, 6'	-
6	32.3	2.85, ddd, (11.5, 9.5, 5.5)	H5, H7	H8
		1.44, ddd, (11.5, 5.5, 2.5)	H5, H7	
7	51.4	2.66, ddq, (9.5, 6.5, 2.5)	H6, 6', H8	H6, 6', H8
8	16.6	1.42, d, (6.5)	H7	H6
9	105.8	-	-	H6, 6'
10	31.1	2.94, s	-	H5
11	105.3	-	-	H3, 3'
12	168.0	-	-	H6, 6', H13
13	53.2	5.60, dd, (10.0, 5.0)	H14, 14'	-
14	31.8	2.32, m	H13, H19, 19'	H19
15	171.5	-	-	H13, H14, 14', H16, H17
16	142.4	7.69, d, (3.0)	H17	H17
17	118.9	7.24, d, (3.0)	H16	H16
18	47.0	3.64, m	H19, 19'	-
		3.59, m		
19	24.7	2.18, m	H14,14', H18, 18'	H14, 14'
		2.07, m		

The elucidation of (**1**) was challenging due to the doubling of resonances in both the ^{1}H and ^{13}C NMR spectra and was performed using the cross-peaks of the major conformer (see Supp. Mat.). Analysis of the ^{1}H-^{1}H COSY NMR data suggested six substructures (**A-F**); two highly chlorinated acyclic chains (**A**, **C**) which appeared to be the derived trichloroleucine and trichlorovaline residues normally found in dysidenin, a thiazole ring (**D**), two amide carbonyl functionalities (**B**, **E**) and a C_4H_7 acyclic chain (**F**) as shown in Figure 4.2 (Table 4.3; Figures 4.19-21 in Supp. Mat.). Substructure (**F**) was something that I had not seen before and suggested the presence of proline subunit in

place of an alanine residue due to the lack of a methyl doublet at δ 1.66 and a proton attached to the amide nitrogen on C12 (δ 168.0).

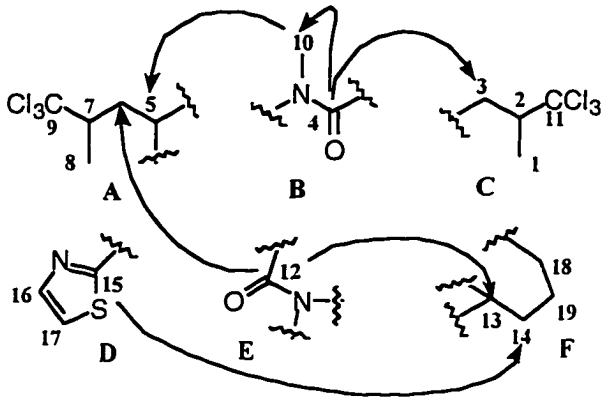
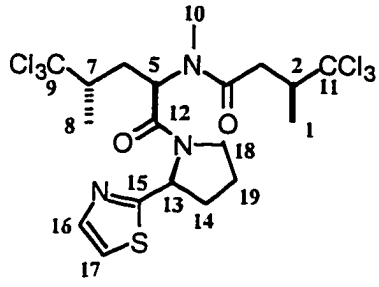


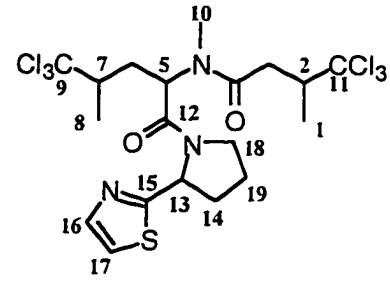
Figure 4.2. Substructures (**A-F**) Established after Analysis of ^{1}H, ^{13}C, APT and ^{1}H-^{1}H COSY NMR Data ($CDCl_3$, 500 MHz). Arrows Indicate Diagnostic HMBC ($CDCl_3$, 500 MHz) Correlations.

The planar structure and sequence of substructures (**A-F**) of (**1**) was rapidly deduced after examination of HMBC NMR data ($CDCl_3$, 500 MHz) (Figure 4.2, Table 4.3; Figures 4.25-29 in Supp. Mat.). Key HMBC correlations from C4 (δ 170.2) to H_23 (δ 3.07, 2.50) and H_310 (δ 2.94) established the connection of the *N*-methyl to carbonyl C4 (**B**) and to the trichloro residue (**C**). A subsequent correlation from C10 (δ 31.1) to H5 (δ 5.56) connected subunit (**A**) to (**B-C**) to give the sequence C1-C8 normally found in dysidenin. The thiazole ring (**D**) was connected to C13 (δ 53.2) through a correlation from C15 (δ 171.5) to H_214 (δ 2.32) on (**F**). Likewise, two HMBC correlations from C12 (δ 168.0) (**E**) to H_26 (δ 2.85, 1.44) and H13 (δ 5.60) nearly completed the structure of this molecule. The only remaining point of attachment was between the amide nitrogen on C12 (**E**) and CH_218 (δ 47.0, 3.64, 3.59) (**F**) to complete the proline residue. This final connection was based on the downfield chemical shift C18 and by comparison to the ^{13}C

shifts found on proline (δ 47.4) as no HMBC correlations were observed.[11] This completed the planar structure of dysidenin C (**1**).



dysidenin C **1**
$[\alpha]_D = +10°$ (c 3.2, $CHCl_3$)



dysidenin D **2**
$[\alpha]_D = +58°$ (c 0.1, $CHCl_3$)

The planar structure of dysidenin D (**2**) was identical to dysidenin (**1**) based on the NMR data obtained for (**2**) and compared to that of (**1**) (Figures 4.33-37 in Supp. Mat.). The only difference being in their respective optical rotations and by the fact that they were resolved by HPLC. This indicated that they might be diastereomers of one another. To determine the stereochemistry of both (**1**) and (**2**) several attempts at recrystallization were carried out, successfully recrystallizing (**1**) from methanol. All attempts at recrystallization of (**2**) failed and was probably due to the lack of material. I only obtained one-third the amount I isolated of (**1**). A cluster of large crystals of (**1**) was sent to Prof. Clardy (Cornell U.) for X-ray crystallographic analysis[12] where the absolute stereochemistry of (**1**) was determined to be 2*S*, 5*R*, 7*S*, 13*S*; identical to that reported for isodysidenin (Figure 4.3; Figure 32 in Supp. Mat.).

After obtaining the absolute stereochemistry of (**1**), I began to think that the stereochemistry of (**2**) might differ only by being epimeric at C5 with *S* configuration; analogous to dysidenin. This was feasible considering the previous biogenesis of both dysidenin and isodysidenin.[13] Therefore, I began to compare the proton and carbon

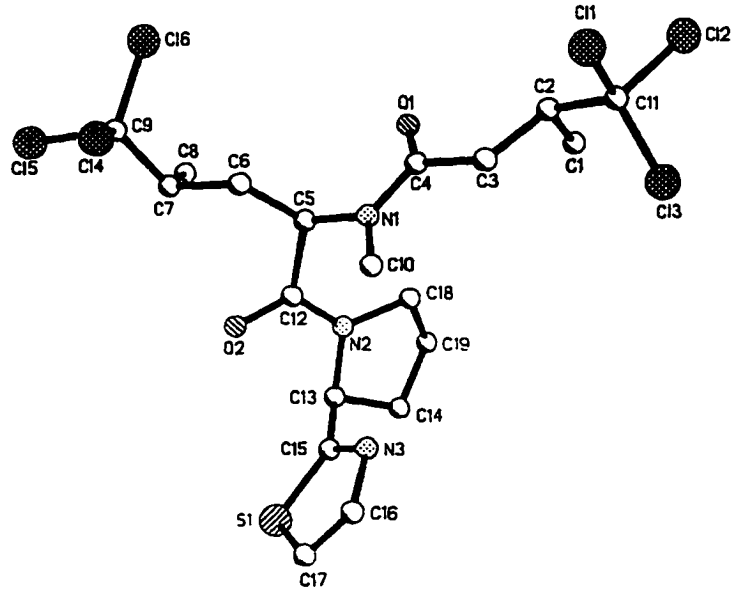


Figure 4.3. X-Ray Crystal Structure of dysidenin C (**1**).

Table 4.4. Comparison of ^{1}H and ^{13}C-NMR Data Between dysidenin C (**1**) and Literature.

Position	^{13}C δ	^{1}H δ, mult., (*J* = Hz)	^{13}C δ Lit.*	^{1}H δ Lit*	^{1}H δ Lit‡
1	17.5	1.39, d, (6.5)	17.3	1.36	1.39
2	51.6	3.35, ddq, (9.0, 6.5, 2.0)	51.4	3.30	3.30
3	37.4	3.07, dd, (16.5, 2.0)	37.4	3.15	3.08
		2.50, dd, (16.5, 9.0)		2.51	2.51
4	170.2	-	171.9	-	-
5	58.7	5.56, t, (5.5, 5.5)	54.0	5.27	5.34
6	32.3	2.85, ddd, (11.5, 9.5, 5.5)	31.0	2.64	2.94
		1.44, ddd, (11.5, 5.5, 2.5)		1.94	1.49
7	51.4	2.66, ddq, (9.5, 6.5, 2.5)	51.9	2.20	2.68
8	16.6	1.42, d, (6.5)	16.2	1.33	1.36
9	105.8	-	105.5	-	-
10	31.1	2.94, s	30.8	3.04	2.98
11	105.3	-	105.1	-	-
12	168.0	-	168.2	-	-
13	53.2	5.60, dd, (10.0, 5.0)	47.3	5.20	5.43
14	31.8	2.32, m	21.8	1.56	1.65
15	171.5	-	171.2	-	-
16	142.4	7.69, d, (3.0)	142.3	7.60	7.70
17	118.9	7.24, d, (3.0)	118.9	7.26	7.31
18	47.0	3.64, m	-	-	-
		3.59, m			
19	24.7	2.18, m	-	-	-
		2.07, m			
NH	-	6.84, bd, (7.0)	-	6.86	6.97

* dysidenin; ‡ isodysidenin.

chemical shifts between (**1**) and (**2**), which are identical, to dysidenin and isodysidenin in an attempt to confirm this hypothesis (Table 4.4). The proton chemical shifts at C5, C6 and C7 differ most noticeably between dysidenin and isodysidenin and were the basis of the analysis that these compounds were epimeric at C5.[14] Since I knew the stereochemistry of (**1**) to be 2*S*, 5*R*, 7*S*, 13*S*, like isodysidenin, I expected the proton chemical shifts of C5, C6 and C7 to be nearly identical. However, this was not the case. The resonances at H_26 (δ 2.85, 1.44) and H7 (δ 2.66) of (**1**) matched closely to those reported for isodysidenin, but the ^1H and ^{13}C shifts at CH5 (δ 58.7, 5.56) were quite different. I can only conclude that this is due to the proline residue; the only difference between these molecules. As expected the chemical shift information at CH13 is also quite different and not comparable since there is no precedent in this class of compounds. Considering the above information along with identical chemical shift information of (**1**) and (**2**) and the NMR data of (**1**) not entirely matching that reported for isodysidenin at position C5, it is difficult to ascertain the final stereochemistry of (**2**). Additionally, the conformational nature of the proline in both (**1**) and (**2**) adds a new variable to this type of analysis further hindering the process. I believe the stereochemistry at positions C2 and C7 to be of *S* configuration in (**2**) because the NMR data in all compounds is nearly identical. Therefore, I conclude that the diastereomeric difference between (**1**) and (**2**) is occurring at either C5 or C13 or both.

Structure Elucidation of 9, 11-didechlorodysidenin

The structure elucidation of 9, 11-didechlorodysidenin (**3**) proceeded efficiently once it was recognized that this molecule was very similar to dysidenin. The only precedence for dechlorination on dysidenin was reported by Wells[15] where two pentachlorodysidenins were isolated. The electrospray ionization mass spectra from the chemotaxonomic analysis indicated a molecular ion of *m/z* 476.1 that exhibited a pattern typical of four chlorine atoms. This was further supported by LRFABMS and the presence of two doublet ^{1}H NMR resonances at δ 5.94 and 6.04 believed to be methine protons on dichlorocarbons (Figure 4.38, 4.40 in Supp. Mat.). Examination of both the ^{1}H and ^{13}C NMR spectra revealed a number of overlapping resonances. Consequently, a full structure elucidation was necessary to confirm the structure of a didechlorodysidenin.

A molecular formula of $C_{17}H_{25}Cl_4N_3O_2S$ was established by HRFABMS with a $[MH]^+$ peak at *m/z* 476.0500 (Δ 0.4 ppm) that differed from the molecular formula of dysidenin by only two chlorine atoms. Comparison of the ^{1}H, ^{13}C, HMQC, HRFABMS to that of dysidenin that I had previously isolated suggested an APT formula of $C_{17}H_{25}$ giving an unsaturation number of five with one proton attached to nitrogen (Table 4.5; Figures 4.38, 4.39, 4.46-48 in Supp. Mat.). Five ^{13}C resonances were attributed to two amide carbonyls (IR, 1636.1 cm^{-1}) at δ 172.5 and 169.0 and the representative carbons of a thiazole ring at δ 172.2, 142.5 and 119.0 to satisfy all five unsaturations (Table 4.5; Figure 4.41 in Supp. Mat.).

Analysis of the ^{1}H-^{1}H COSY NMR spectra produced the similar array of substructures (**A-E**) expected for this class of compound; two acyclic dichlorinated residues (**A**) and (**C**) which represented the chloroleucine and chlorovaline subunits of dysidenin, a thiazole ring (**D**) and two amide carbonyl resonances (**B**, **C**) (Figure 4.4,

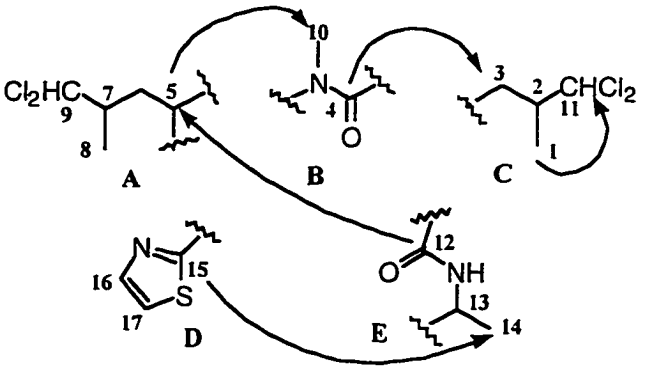


Figure 4.4. Substructures (**A-E**) Established after Analysis of ^{1}H, ^{13}C, HMQC and ^{1}H-^{1}H COSY NMR Data ($CDCl_3$, 500 MHz). Arrows Indicate Diagnostic HMBC ($CDCl_3$, 500 MHz) Correlations.

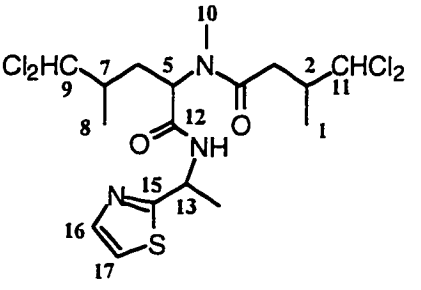
Table 4.5. ^{1}H, ^{13}C, COSY and HMBC NMR Data ($CDCl_3$, 125/500 MHz) of 9, 11-didechlorodysidenin (**3**).

Position	^{13}C δ	^{1}H δ, mult., (*J* = Hz)	COSY (H → H)	HMBC (C → H)
1	15.5	1.22, d, (6.5)	H2	H3, H11
2	40.6	2.85, dddq, (7.0, 6.5, 6.5, 3.0)	H1, H3, 3', H11	H1, H3
3	36.6	2.70, dd, (16.5, 6.5)	H2, H3'	H1
		2.43, dd, (16.5, 7.0)	H2, H3	
4	172.5	-	-	H3, 3', H10
5	53.5	5.26, t, (7.5, 7.5)	H6, 6'	H6', H10
6	31.0	2.29, ddd, (14.0, 7.5, 6.5)	H5, H6', H7	H8
		1.62, m	H5, H6, H7	
7	40.6	2.12, m	H6, 6', H8, H9	H8
8	14.4	1.22, d, (6.5)	H7	
9	78.0	5.94, d, (3.0)	H7	H6', H8
10	31.0	2.91, s	-	H5
11	78.0	6.03, d, (3.0)	H2	H1, H3, 3'
12	169.0	-	-	H5, H6', H13
13	47.2	5.40, m	NH, H14	H14
14	21.8	1.63, d, (7.0)	H13	
15	172.2	-	-	H13, H14, H16, H17
16	142.5	7.69, d, (3.0)	H17	H17
17	119.0	7.26, d, (3.0)	H16	H16
NH	-	6.82, bd, (8.0)	H13	-

Table 4.5; Figures 4.42-4.45 in Supp. Mat.). The presence of two dichloro functionalities on substructures (**A**) and (**C**) was confirmed by the ^{13}C chemical shifts of δ 78.0 for a dichlorocarbon and comparison to the analysis presented in Chapter 3 for the

chloroisopropyl group. The chemical shifts of C2 and C7 at δ 40.6 closely matched this analysis and that reported in the literature.[16]

The completion of the planar structure of (**3**) was deduced by HMBC NMR correlations in the following manner (Table 4.5, Figure 4.4; Figures 4.49-54 in Supp. Mat.). Key correlations from C4 (δ 172.5) to $H_2$3 (δ 2.70, 2.43) and $H_3$10 (δ 2.91) along with a correlation from C5 (δ 53.5) to $H_3$10 (δ 2.91) confirmed the sequence (**A-B-C**) to give the familiar *N*-methyl chloroleucine-chlorovaline connection of dysidenin. Final correlations from C12 (δ 169.0) (**E**) to H5 (δ 5.26) (**A**) and C15 (δ 172.2) (**D**) to $H_3$14 (δ 1.63) (**E**) completed the planar structure of 9, 11-didechlorodysidenin (**3**). No attempt was made to deduce the stereochemistry as only a few milligrams of material was isolated. Many of the sponge specimens collected for the chemotaxonomic analysis were very small. Based on comparison of the ^{1}H NMR data at positions C5, C6 and C7 of (**3**) to that of dysidenin and isodysidenin I believe that (**3**) has the same configuration as dysidenin where all stereocenters are *S*.

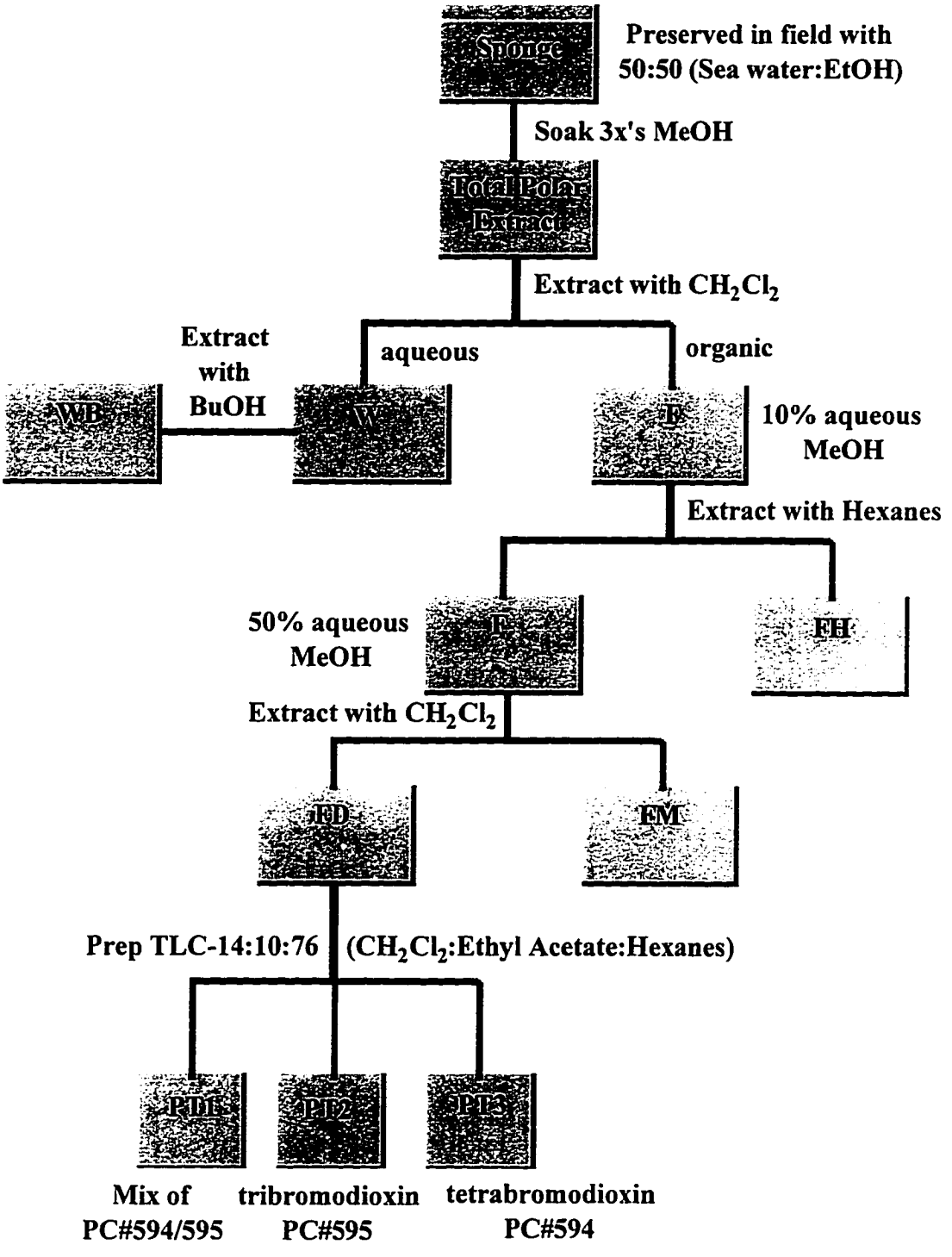


9,11-didechlorodysidenin **3**
$[\alpha]_D$ = +39° (c 0.23, $CHCl_3$)

Collection and Isolation of tetra- and tribromodioxin

During a different expedition to Southern Sulawesi, Indonesia a total of fifteen specimens fitting the description of *Dysidea* and all appearing to contain cyanobacteria were analyzed by electrospray ionization mass spectrometry (ESIMS). After screening by ESIMS (negative mode) one specimen (coll. no. 95595) was identified as containing a new brominated diphenyl ether, *m/z* 511.8, by comparison to the mass spec tables (Tables 4.1, 4.2). The isotope distribution pattern suggested that this metabolite was polybrominated with four bromines. Interestingly, this collection, 95595, was yellow in color; not the usual greenish color typically associated with a sponge containing cyanobacteria (Figure 4.5). Also noteworthy was the fact that 95595 was collected in very shallow water (6-10 ft) as compared to most of the other specimens which were collected in depths >20 ft. Two other specimens (coll. no. 95594, 95596) were collected in nearby shallow water, but were greenish in color. I proceeded to workup this sponge by the usual procedure expecting to find the bromophenol in the FD fraction (Appendix A). The FD fraction was purified by preparative TLC (14:10:76, dichloromethane:ethyl acetate:hexanes) to yield not only the tetrabromodioxin (**4**) of *m/z* 511.8 but also a minor component, tribromodioxin (**5**) (Scheme 4.3). I will now discuss the structure elucidation of both of these compounds.

Figure 4.5. Topside Photograph of 95595.



Scheme 4.3. Extraction Scheme of 95595.

Structure Elucidation of tetra- and tribromodioxin

The structure elucidation of tetrabromodioxin (**4**) began with the assumption that this was one of many brominated diphenyl ethers isolated from *Dysidea*. However, after examination of the ^{1}H-NMR spectra it was revealed that there were only three aromatic proton resonances instead of four as would be expected of a tetrabrominated diphenyl ether (Figure 4.55 in Supp. Mat.). This suggested that another ether linkage had formed to give a dioxin compound. This was very exciting because no brominated dioxins have ever been isolated from *Dysidea*. In 1981, Faulkner investigated the possibility that polybrominated dioxins could be produced by *Dysidea* sponges.[17] They synthesized a bromodioxin and performed a TLC analysis against crude extracts, but found no evidence of their existence.

A molecular formula of $C_{12}H_4Br_4O_3$ was established by HREIMS to give a $[M]^+$ peak of *m/z* 511.6894 (18) (calcd for $C_{12}H_4{}^{79}Br_4O_3$, Δ 0.4 ppm) indicating an unsaturation of nine which was satisfied by twelve highly brominated and oxygenated ^{13}C aromatic resonances (IR, 1465.9, 1429.7, 1072.6 cm^{-1}) and three rings (Table 4.6; Figure 4.56-4.58 in Supp. Mat.). Examination of the ^{1}H-NMR spectra (CD_3OD, 500 MHz) showed one singlet aromatic proton resonance at δ 6.91 and two doublet resonances at δ 7.10 and 7.31 (J = 2 Hz) (Figure 4.55 in Supp. Mat.). This indicated that the singlet proton was on one ring and the two doublet protons were meta substituted on the other ring. Based on this information I deduced there were five possible structures (**A-E**) and their respective regioisomers depending on the bromine substitution pattern (Figure 4.6).

The determination of the correct structure was based on the analysis of a combination of chemical shift data from the literature, calculated ^{13}C resonances, HMQC

Table 4.6. ^{1}H, ^{13}C and HMBC NMR Data (CD_3OD, 125/500 MHz) for tetrabromodioxin (**4**) and tribromodioxin (**5**).

Position	^{13}C δ (**4**)	^{1}H δ, mult., (*J* = Hz)	HMBC (C → H)	^{13}C δ (**5**)	^{1}H δ, mult., (*J* = Hz)	HMBC (C → H)
1	145.2	-	H6	146.5	-	-
2	129.3	-	H6	129.4	-	H4, H6
3	140.5	-	-	142.4	-	H4
4	102.2	-	H6	110.0	6.64, d, (2.5)	H6
5	118.3	-	H6	115.0	-	H4, H6
6	116.3	6.91, s	-	115.5	6.71, d, (2.5)	-
1'	142.7	-	H6'	143.0	-	H6'
2'	138.9	-	H4', H6'	138.7	-	H4', H6'
3'	110.5	-	H4'	110.0	-	-
4'	129.3	7.31, d, (2.0)	H6'	129.6	7.36, d, (2.0)	H6'
5'	115.9	-	H4', H6'	115.2	-	H4', H6'
6'	118.6	7.10, d, (2.0)	H4'	118.7	7.14, d, (2.0)	H4'
OH	-	3.44 (CD_2Cl_2)	-	-	-	-

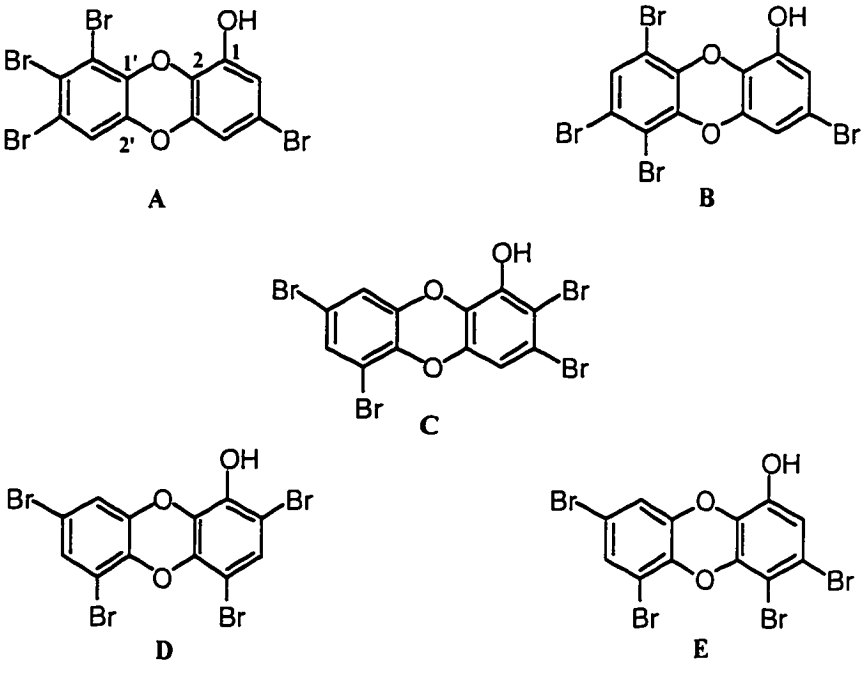
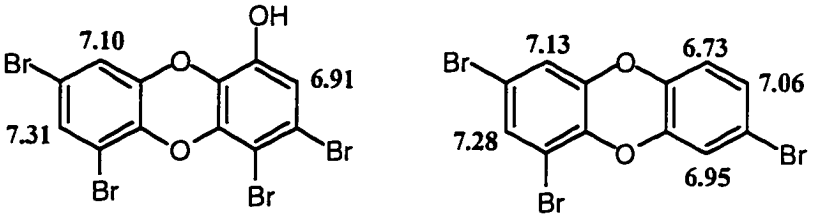


Figure 4.6. Possible Structures of tetrabromodioxin (**4**).

and HMBC data. Structures (**B**) and (**D**) were easily eliminated based on evaluation of the ^{13}C chemical shift where the singlet proton was attached. Data reported in the literature

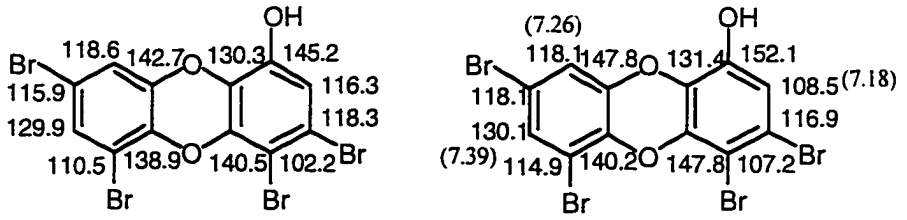
shows that this carbon (between two bromines and beta to the ether linkage) normally occurs at a chemical shift of ~ δ 126.0-128.5.[18] The HMQC data of (**4**) showed that the singlet proton was attached to a carbon with a shift at δ 116.3 (Figure 4.59). The same analysis could not be used for structures (**A**, **C**, and **E**) because all three show a singlet proton attached to a carbon between bromine and an oxygen.



E **synthetic dioxin**

Figure 4.7. ^{1}H Chemical Shifts of Synthetic Dioxin.[17]

Structure (**A**) was eliminated based on comparison of ^{1}H-NMR data obtained for (**4**) to that of synthetic dioxin discussed earlier. The chemical shifts expected for the meta-coupled protons of structures (**C**) and (**E**) would be nearly identical to those reported for synthetic dioxin (Figure 4.7). Unfortunately, no carbon data was reported for the synthetic dioxin. The final deduction to eliminate structure (**C**) over (**E**) was based on evaluation of the HMBC data and comparison to ^{13}C NMR chemical shifts. This was necessary due to the lack of data on highly oxygenated and brominated phenols. All the known bromophenols only have two oxygenated positions. The ^{13}C chemical shifts were calculated using ACD's NMR simulation program[19] and were found to match closely to structure (**E**) (Figure 4.8). Further support of structure (**E**) came from HMBC NMR data which showed all two and three bond correlations (Figure 4.9; Figure 4.60 in Supp. Mat.). A key correlation from C2' (δ 138.9) to H4' (δ 7.31) established the ether carbon next to a bromine at ~ δ 140.0. which was also predicted nicely in the calculated data. This



E (exp) **E** (calc)

Figure 4.8. Calculated ^{13}C-NMR Chemical Shifts for tetrabromodioxin (**4**).

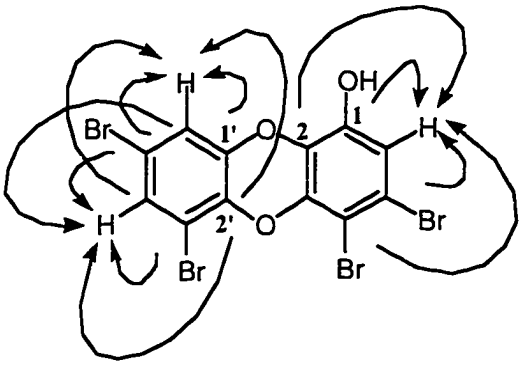
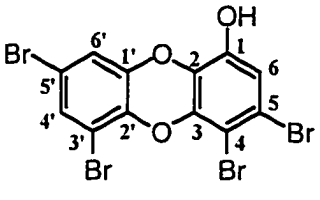


Figure 4.9. HMBC Correlations for tetrabromodioxin (**4**) (CD_3OD, 500 MHz).

established the δ 140.5 shift on the phenol ring at position C3 rather than C1. The slight downfield shift of C3 over C2' (δ140.5 vs. 138.9) can be explained be the slight additivity effects of the hydroxy and bromine beta to C3.[20] Additionally, the hydroxy aromatic carbon is typically furthest downfield in chemical shift value with the substitution pattern of (**E**).[15] The above information coupled with the fact no HMBC correlations were observed from C3 to H6 confirmed (**E**) as the structure of tetrabromodioxin (**4**).



tetrabromodioxin **4**

The regiochemistry of (**4**) was determined by the analysis of a NOESY NMR experiment (CD_2Cl_2, 500 MHz) (Figures 4.61-4.65 in Supp. Mat.). I had hoped to see a nOe correlation between the hydroxy proton and the doublet resonance at δ 7.10 in one of the two possible regioisomers (Figure 4.10). To see the correlation would be difficult

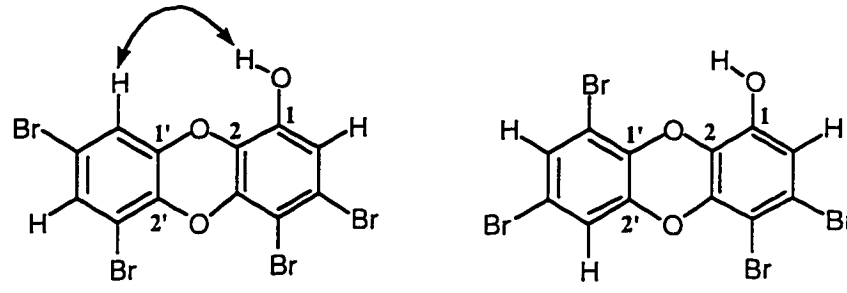


Figure 4.10. The Two Possible Regioisomers of tetrabromodioxin (**4**).

due to the distance measured of 3.8 Å.[21] This would place the correlation at the limits of detection and would probably be weak.[22] To enhance the possibility of detection I decided to conduct a T_1 analysis (CD_2Cl_2, 500 MHz) of the hydroxy and aromatic protons so that I might optimize the NOESY NMR experiment (Figures 4.62, 4.63 in Supp. Mat.). The T_1 analysis allows measurement of the relaxation time of the proton in question which is necessary to determine the delay time of the NOESY experiment; the longer the delay, the more time the protons have to relax and consequently the better the chance of seeing a correlation. As I expected the aromatic protons had long relaxation times and to fully relax would require a delay time of 10.0 seconds in the NOESY experiment (the default delay time is normally run at 1.0 sec.). Therefore, I ran my NOESY NMR experiment at a delay of 8.0 seconds at two different mixing times (0.300 and 0.800 msec.). The experiment conducted at a mixing time of 0.800 msec proved to be successful and a nOe correlation was observed between the hydroxy proton (δ 3.44) and

H6' (δ 7.10) to confirm the regiochemistry shown above for tetrabromodioxin (**4**) (Figures 4.64, 4.65 in Supp. Mat.).

The structure elucidation of tribromodioxin (**5**) was quite easily deduced based on comparison to (**4**) (Table 4.6; Figures 4.66-4.73 in Supp. Mat.). A molecular formula of $C_{12}H_4Br_3O_3$ was established by HREIMS to give a $[MH]^+$ peak of *m/z* 433.7789 (41) (calcd for $C_{12}H_4{}^{79}Br_3O_3$, Δ 1.3 ppm) (Figure 4.68 in Supp. Mat.). The HREIMS indicated that there was no phenolic proton. An additional aromatic proton resonance at δ 6.64 was visible in the ^{1}H-NMR spectra and was placed at position C4 due to the meta coupling (J = 2.5 Hz) (Figure 4.66 in Supp. Mat.). This information coupled with the COSY, HMQC and HMBC NMR data confirmed the structure of tribromodioxin (**5**) as shown below (Figure 4.11; Figures 4.71-4.73 in Supp. Mat.). A HMBC correlation from C3 to H4 reconfirmed placement of the ~ δ 140 carbon at C3 (Table 4.6). The regiochemistry of (**5**) was not determined but is believed to be the same as (**4**).

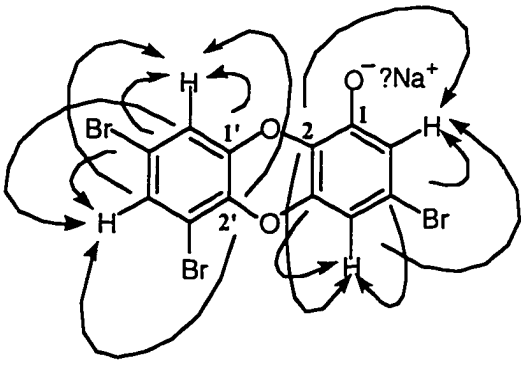
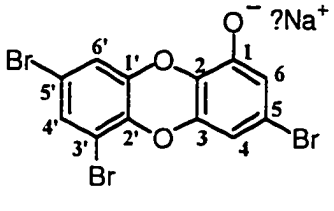


Figure 4.11. HMBC Correlations of tribromodioxin (**5**).



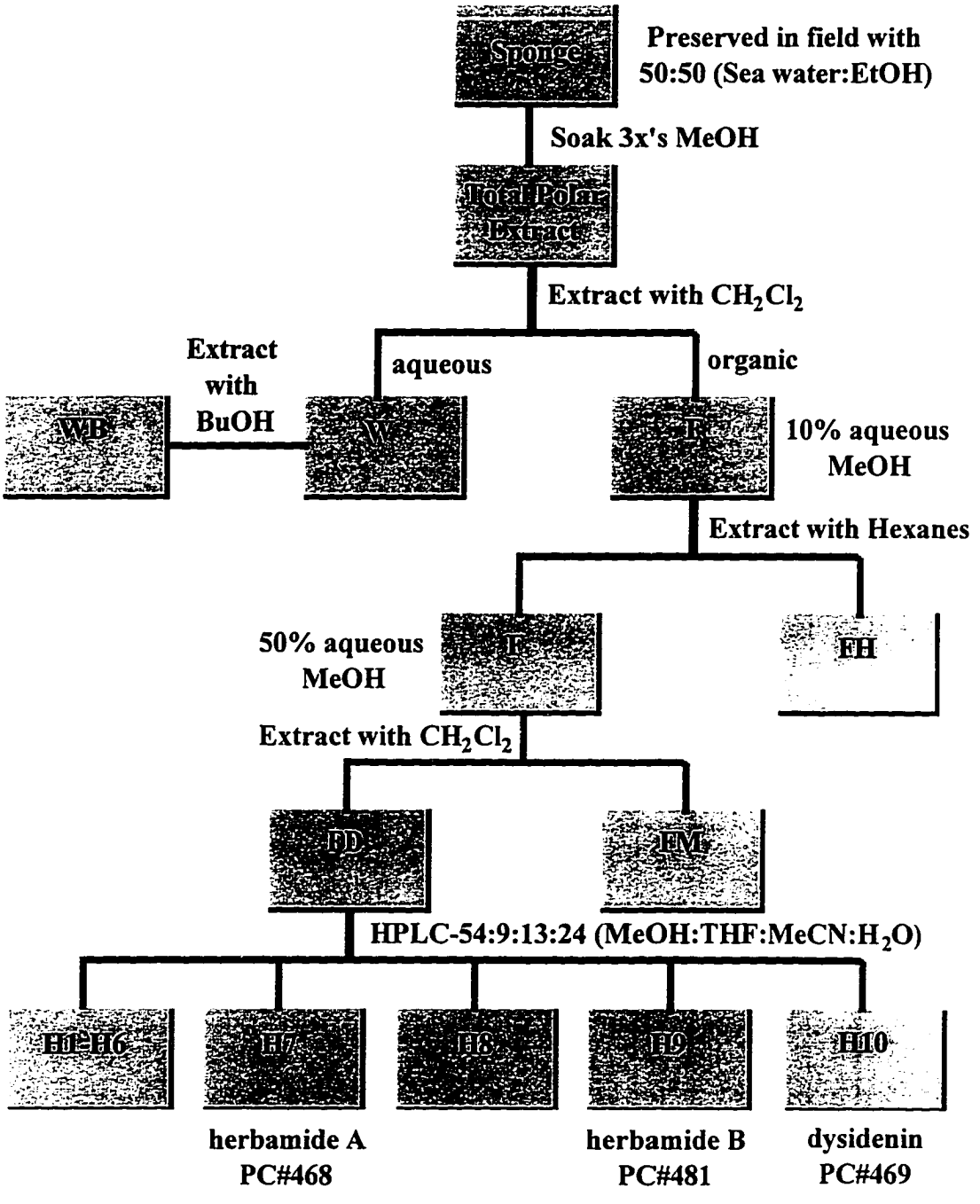
tribromodioxin **5**

Structure Elucidation of herbamide B

During the purification of the FD fraction of the Papua New Guinea sponge (coll. no. 93153), where herbamide A was isolated, another HPLC fraction was discovered to have additional resonances in the ^{1}H and ^{13}C-NMR spectra that were absent in the spectra of herbamide A (Scheme 4.4). Further investigation by LRFABMS revealed a molecular ion peak (*m/z* 408) that exhibited an isotope distribution pattern characteristic of three chlorines and differed by only 14 a.m.u.'s from herbamide A (Figure 4.77 in Supp. Mat.). This suggested the addition of a methyl group somewhere on the structure of herbamide A. Based on the change in the coupling pattern of the vinylic protons and the lack of one resonance in this region, I believed the methyl to be attached to one of the four positions on the diene portion of herbamide A (Table 4.7; Figure 4.74 in Supp. Mat.).

Table 4.7. ^{1}H, ^{13}C and COSY NMR Data ($CDCl_3$, 75.5/300 MHz) of herbamide B (**6**) Compared with herbamide A.

Position	^{13}C δ (**6**)	^{1}H δ, mult., (*J* = Hz) (**6**)	COSY (H → H) (**6**)	^{13}C δ	^{1}H δ, mult., (*J* = Hz)
1	16.2	1.30, d, (6.0)	H2	16.2	1.29, d, (6.0)
2	54.8	2.61, m	H1	54.6	2.58, (m)
3	36.8	3.00, m	H3', H4	36.6	2.96, (m);
		2.24, m			2.19, (m)
4	137.1	5.98, m	H3, H5	138.8	6.02, ddd, (14.7, 8.4, 2.4)
5	128.5	6.42, dd, (13.5, 11.0)	H4, H6	131.1	6.25, dd, (14.7, 11.0)
6	133.7	6.94, d, (11.0)	H5	141.2	7.24, dd, (14.7, 11.0)
7	129.1	-	-	122.8	5.90, d, (14.7)
8	168.5	-	-	165.6	–
9	56.2	5.33, m	NH, H10	56.0	5.33, dd, (8.7, 6.3)
10	34.0	2.36, m	H9, H11, H12	34.1	2.33, (m)
11	18.3	0.97, d, (6.9)	H10	18.2	0.97, d, (6.9)
12	19.2	0.93, d, (6.9)	H10	19.2	0.93, d, (6.9)
13	105.5	-	-	105.7	–
14	170.1	-	-	170.0	–
15	142.4	7.74, d, (3.0)	H16	142.3	7.74, d, (3.0)
16	118.7	7.26, d, (3.0)	H15	118.8	7.26, d, (3.0)
17	13.1	2.01, s	-	–	6.46, bd, (8.4)
NH	-	6.68, bd, (8)	H9		



Scheme 4.4. Extraction Scheme of 93153.

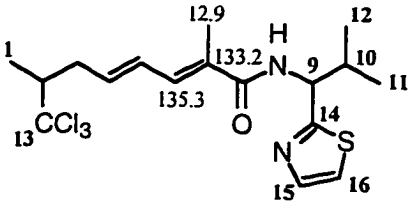
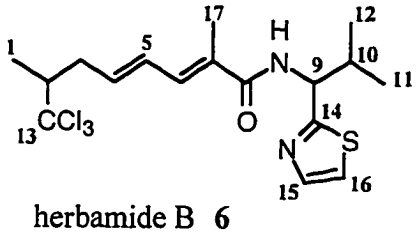


Figure 4.12. Calculated ^{13}C Chemical Shifts for herbamide B (**6**).

A molecular formula of $C_{17}H_{23}Cl_3N_2OS$ was determined by HRFABMS which gave a $[MH]^+$ peak of *m/z* 408.0675 (Δ -0.3 mmu of calcd.). Analysis of the ^1H, ^{13}C and APT NMR spectra showed the addition of a methyl resonance CH (δ 13.0, 2.01) along with a downfield chemical shift at position C7 (δ 129.1) and upfield shift of C6 (δ 133.7) (Table 4.7; Figures 4.74-4.77 in Supp. Mat.). This suggested that the methyl group was located in the alpha position of the dieneamide functionality. Further support of this came from the analysis of a ^1H-^1H COSY NMR spectra and calculated ^{13}C chemical shifts[23] at positions C6 and C7 (Figure 4.12; Figure 4.78 in Supp. Mat.). Additionally, H6 was a doublet lacking the large trans vinylic coupling of $J = \sim 15$ Hz (Table 4.7; Figure 4.74 in Supp. Mat.). All of the above considered, the final structure of herbamide B (**6**) was established as shown below. Based on biogenetic arguments I would speculate the configuration at C2 to be *S* in both herbamide compounds.

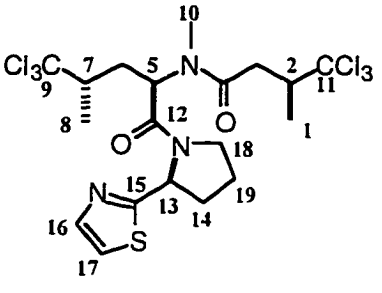


herbamide B **6**

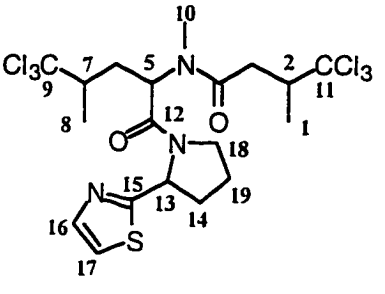
$[\alpha]_D = -8°$ (c 0.89, $CHCl_3$)

Discussion

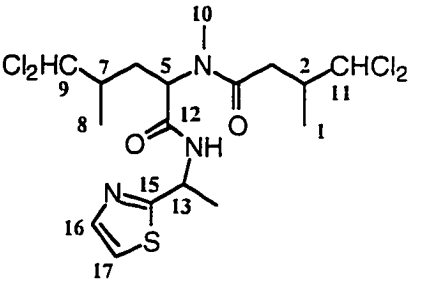
In summary, four new polychlorinated peptides and two brominated dioxins were isolated from three sponge collections of *Dysidea* demonstrating that this sponge is still a viable source of new and interesting compounds. It would not surprise me if more of these types of metabolites are isolated in future collections. The substitution of a proline residue for alanine in dysidenins C (**1**) and D (**2**) adds a new variable to this subclass of tetrapeptides. However, there still seems to be a consistent biogenetic theme of the presence of a trichlorovaline, a trichloroleucine and a thiazole ring.



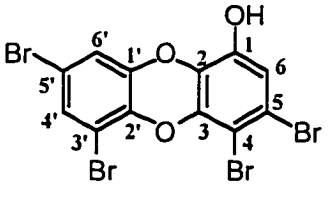
dysidenin C **1**



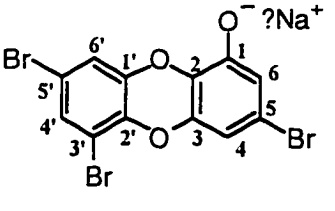
dysidenin D **2**



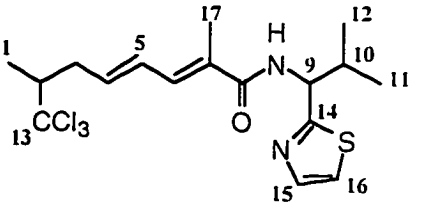
9,11-didechlorodysidenin **3**



tetrabromodioxin **4**



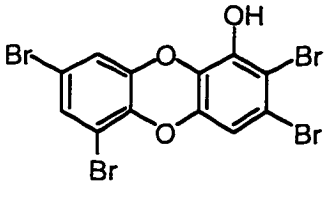
tribromodioxin **5**



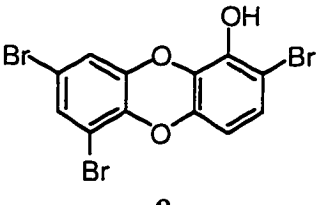
herbamide B **6**

As for the brominated dioxins, they introduce a second subclass of brominated diphenyl ethers. Their speculated existence is now a reality. During the course of my investigations for this thesis work I discovered that in Gribble's review[24] of naturally occurring organohalogen compounds reference to another dissertation where two

brominated dioxins (**7**, **8**), tetra- and tribromo, were isolated from the Caribbean sponge *Tedania ignis*[25]. However, to my knowledge the structure elucidation of these metabolites has not been published. They only differ in their bromine substitution patterns. It is interesting to note that *T. ignis* does not contain filamentous cyanobacteria like *Dysidea*[26] which has been reported to be the producer of these bromophenolic metabolites.[27] Consequently, it is difficult to explain this observation based on the available information.



7



8

Supplementary Material-
dysidenin C

Figure 4.13. ^{1}H-NMR Spectra ($CDCl_3$, 500 MHz).

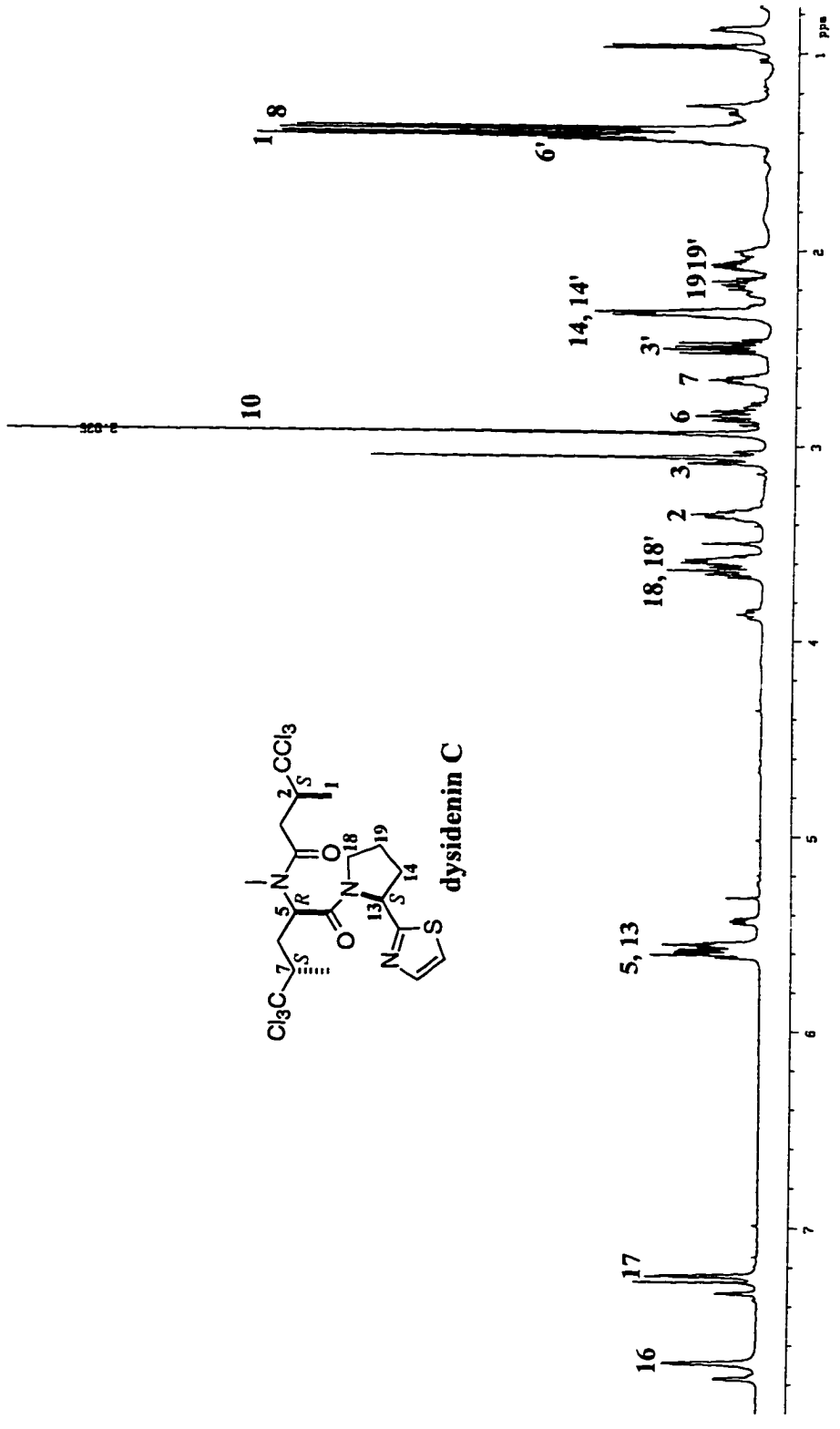


Figure 4.14. ^{13}C-NMR Spectra ($CDCl_3$, 125 MHz).

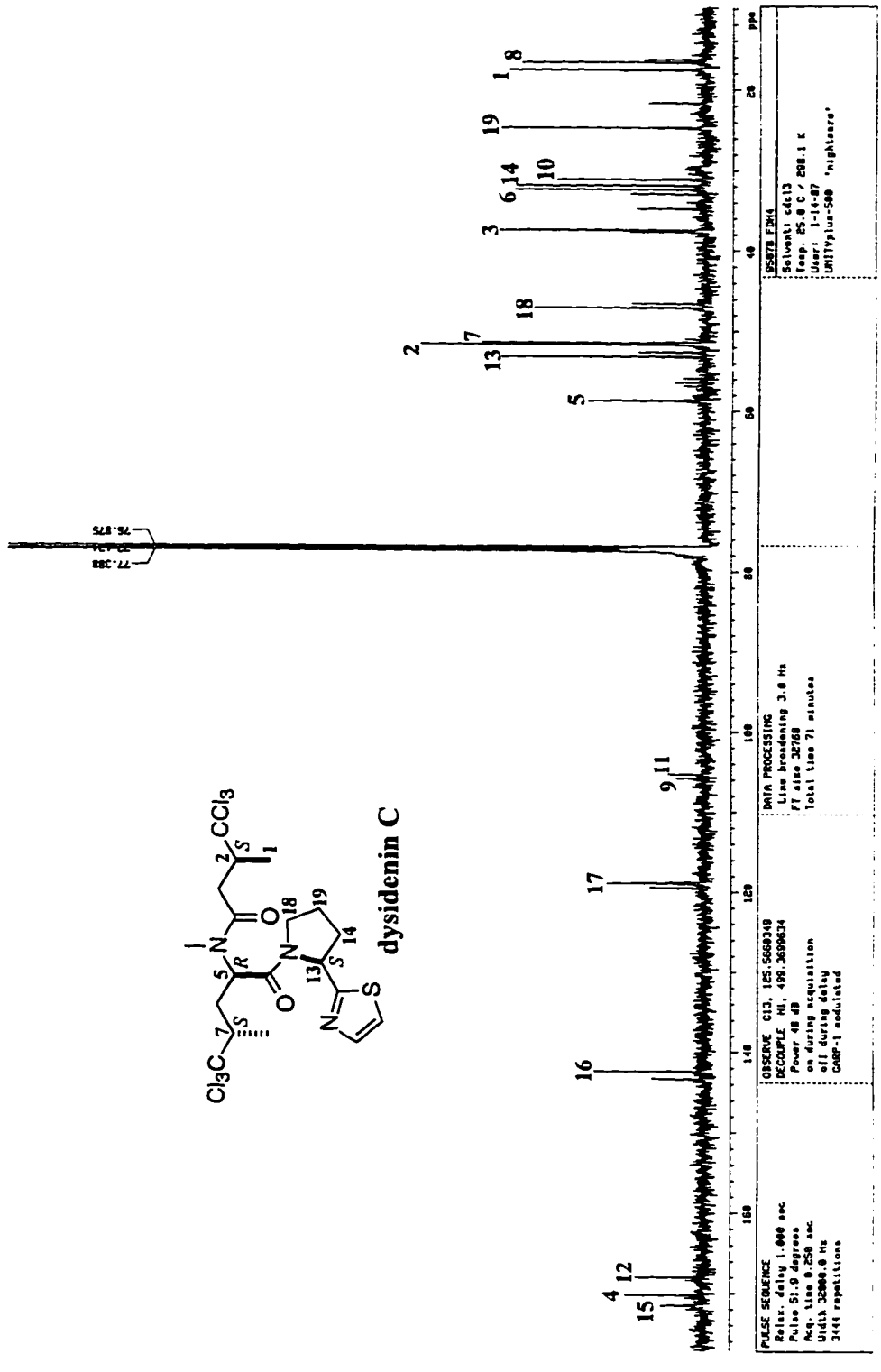


Figure 4.15. APT-NMR Spectra ($CDCl_3$, 125 MHz).

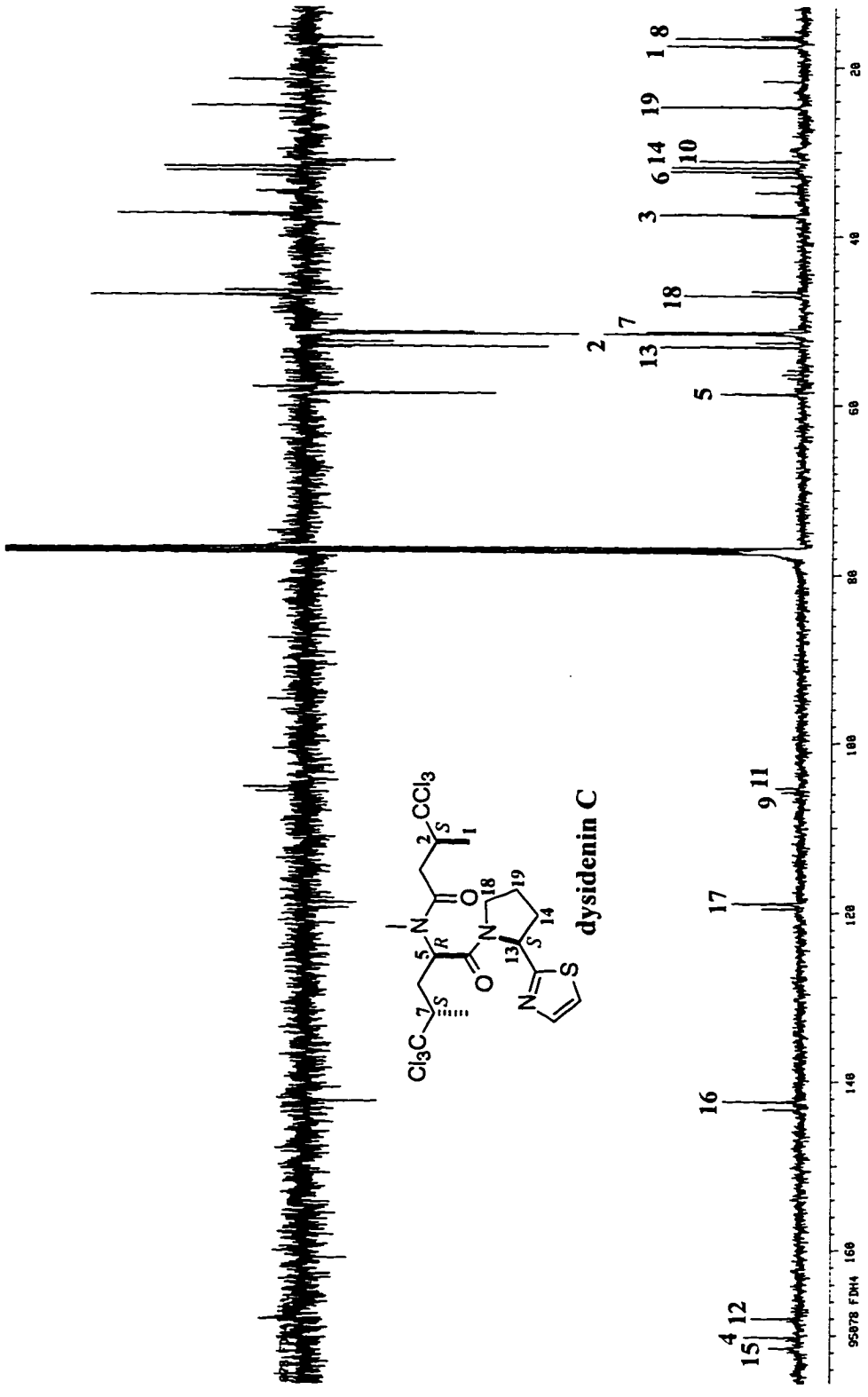


Figure 4.16. Electrospray Ionization Mass Spectra.

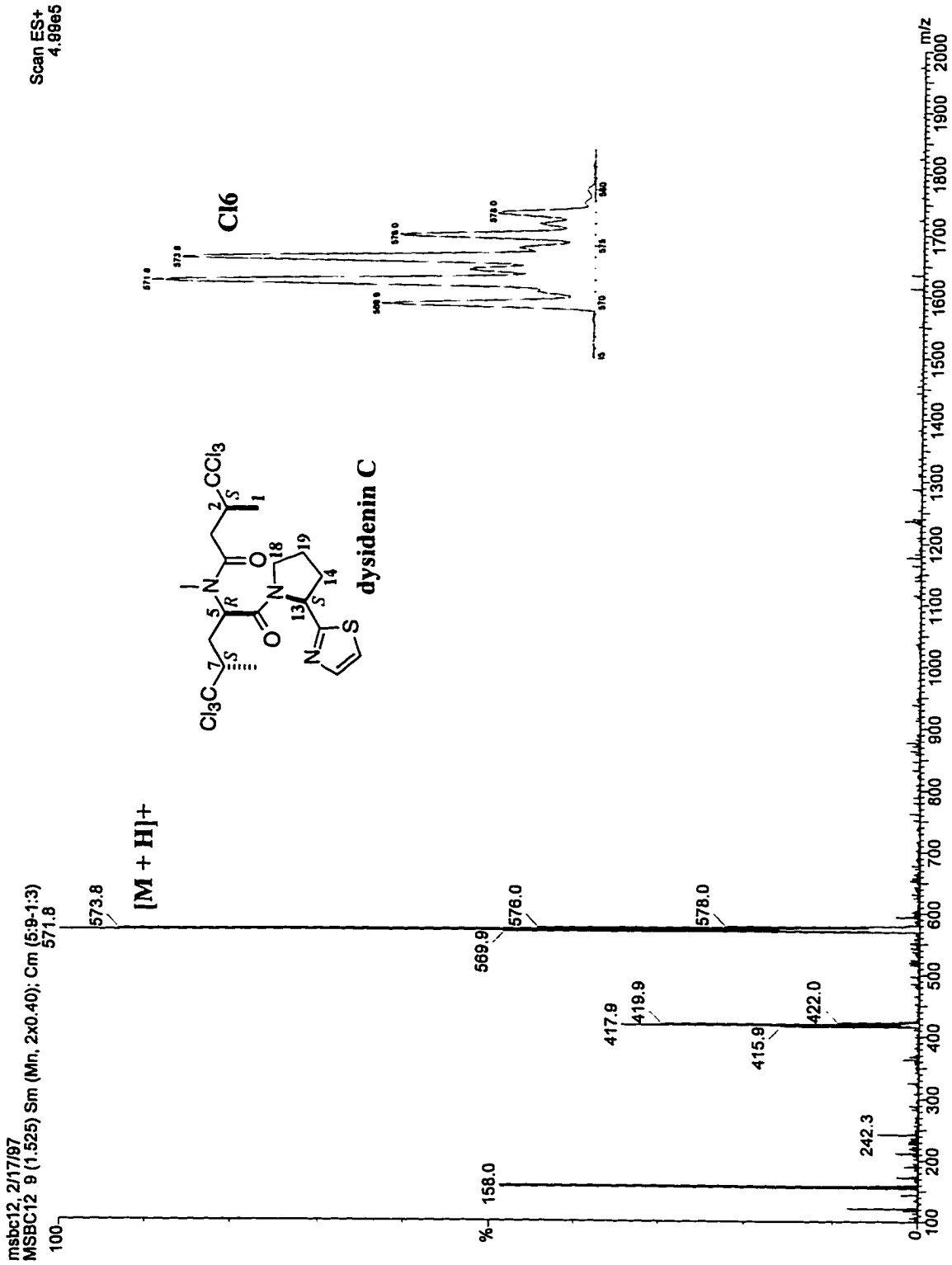


Figure 4.17. LRFAB Mass Spectra.

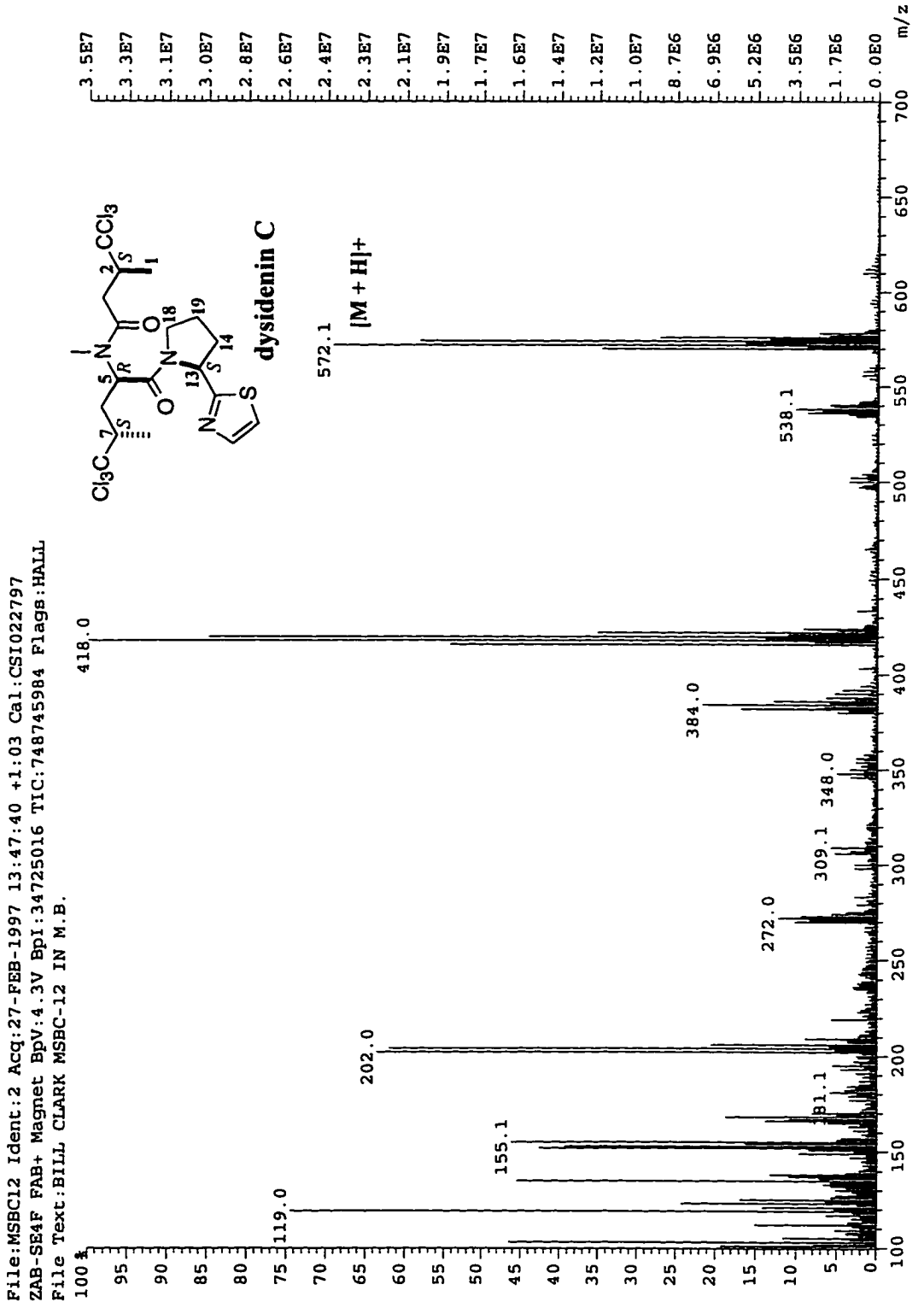


Figure 4.18. IR Spectra.

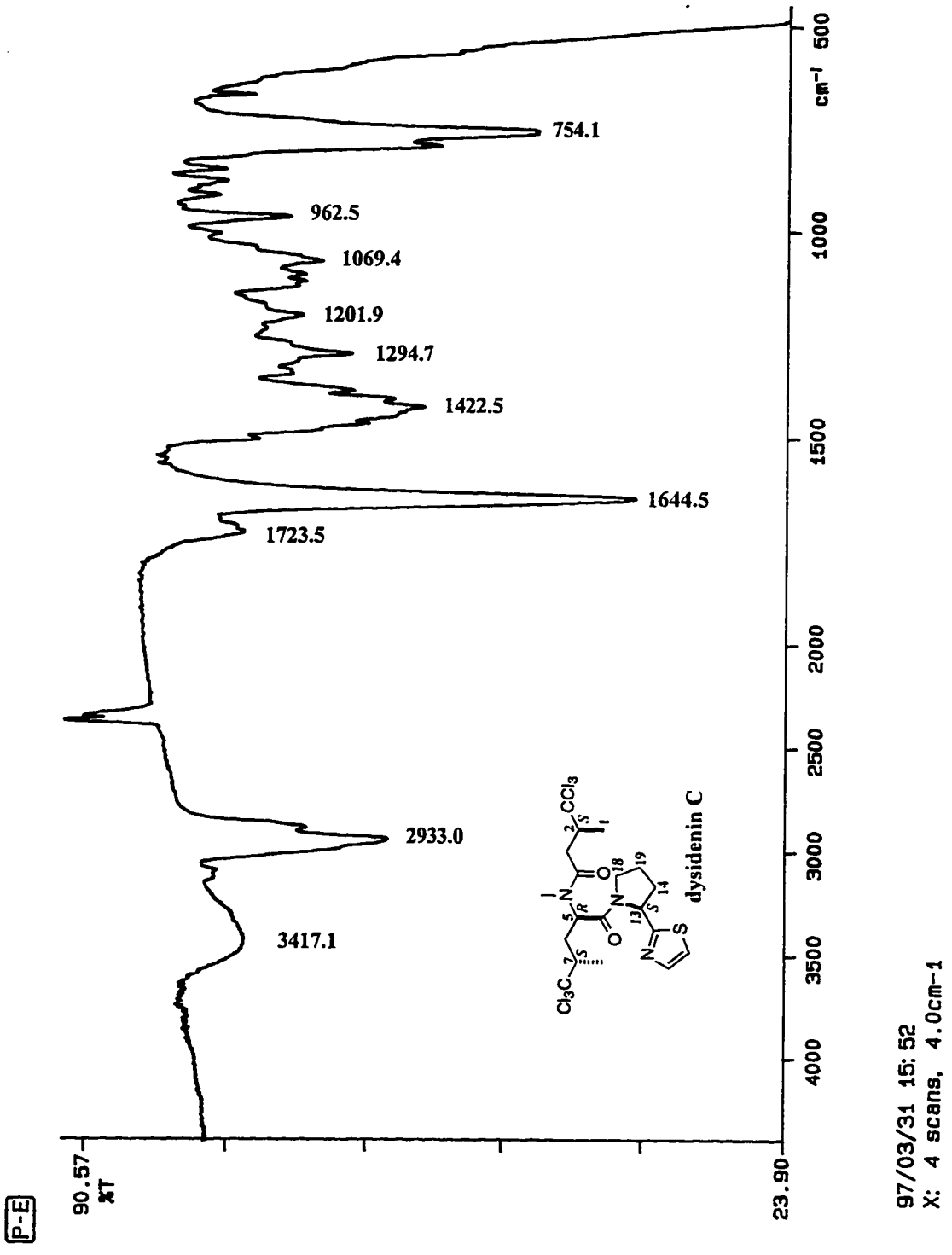


Figure 4.19. COSY Spectra ($CDCl_3$, 500 MHz).

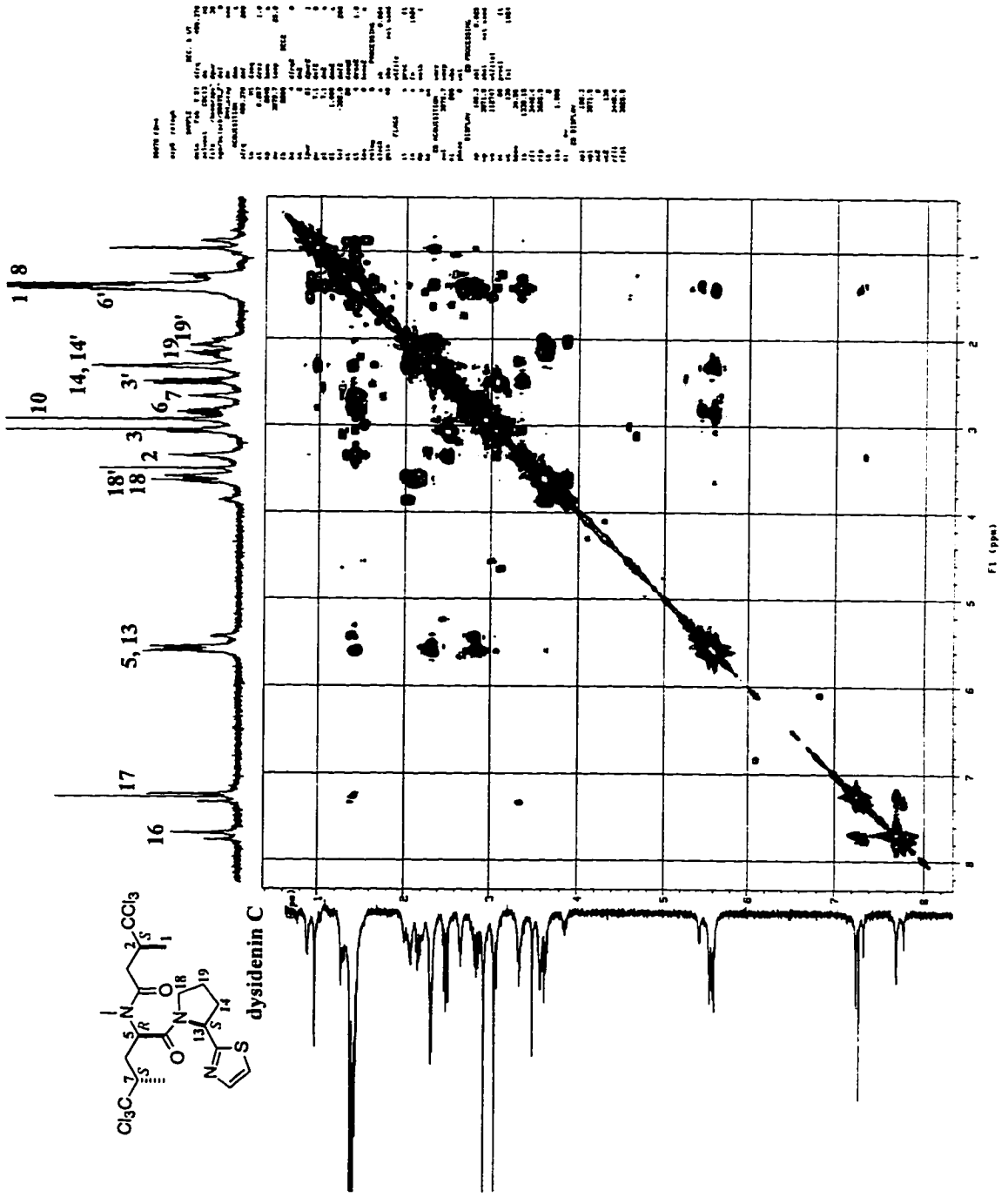


Figure 4.20. COSY Expansion Spectra ($CDCl_3$, 500 MHz).

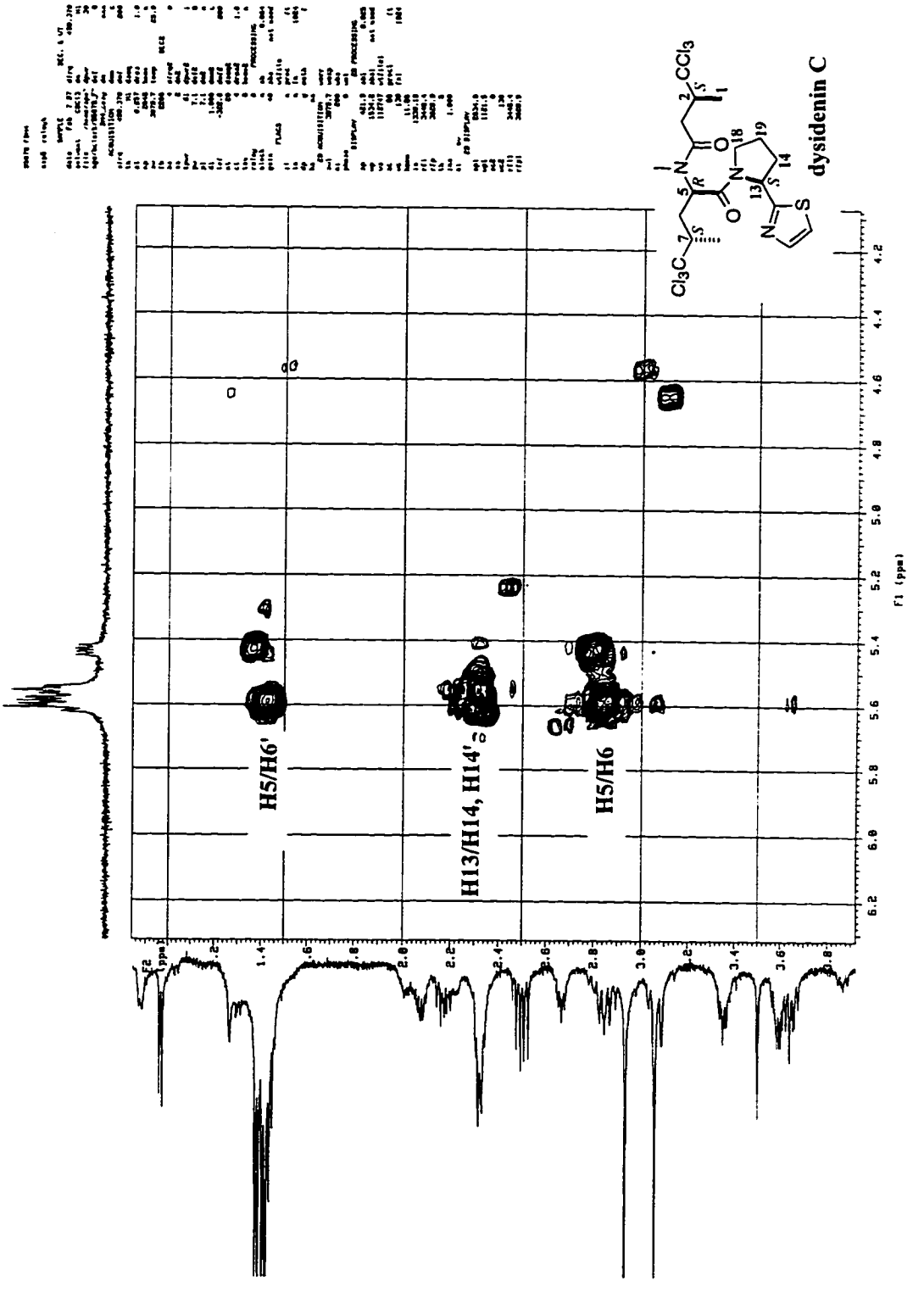


Figure 4.21. COSY Expansion Spectra ($CDCl_3$, 500 MHz).

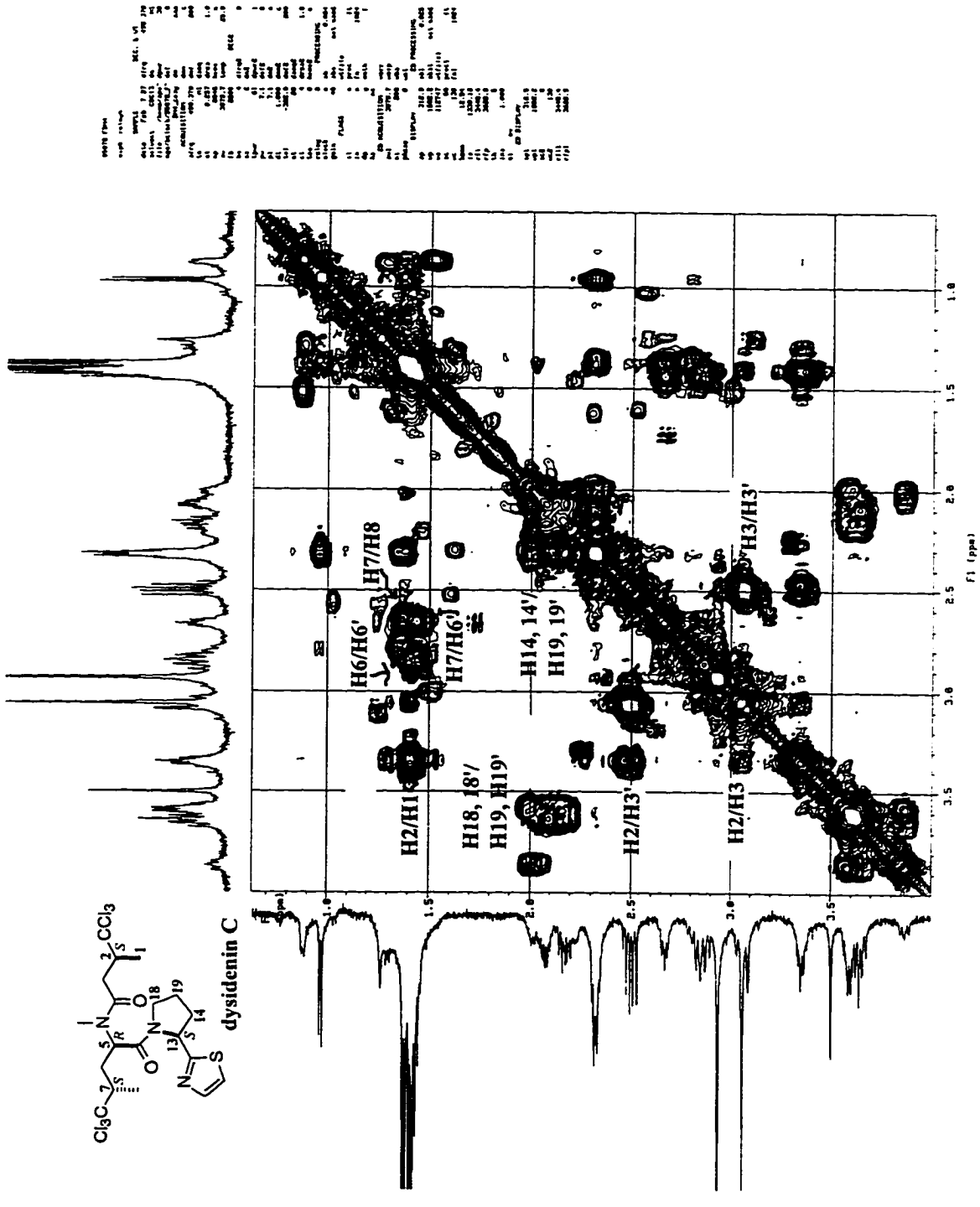


Figure 4.22. HMQC Spectra ($CDCl_3$, 500 MHz).

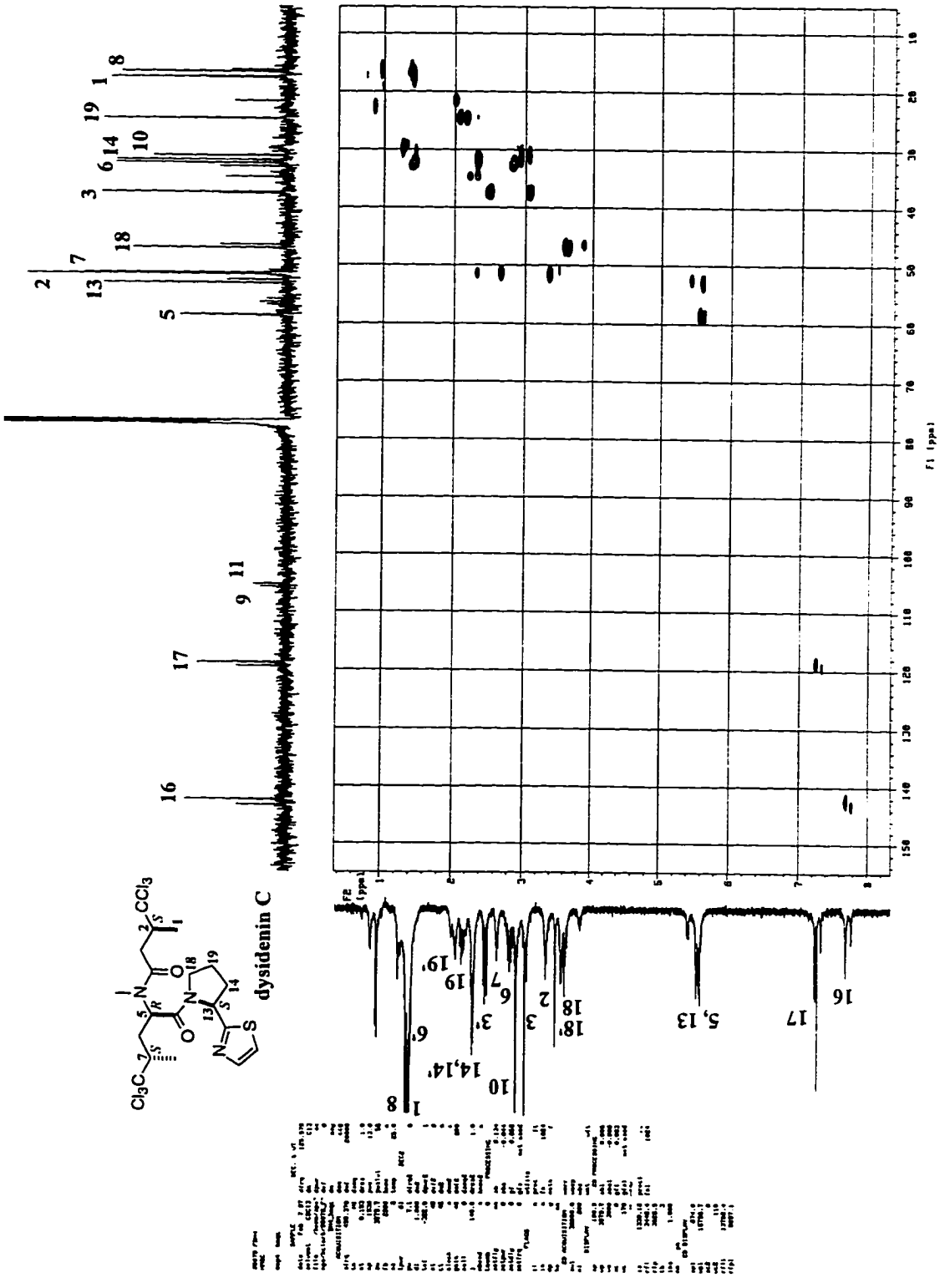


Figure 4.23. HMQC Expansion Spectra ($CDCl_3$, 500 MHz).

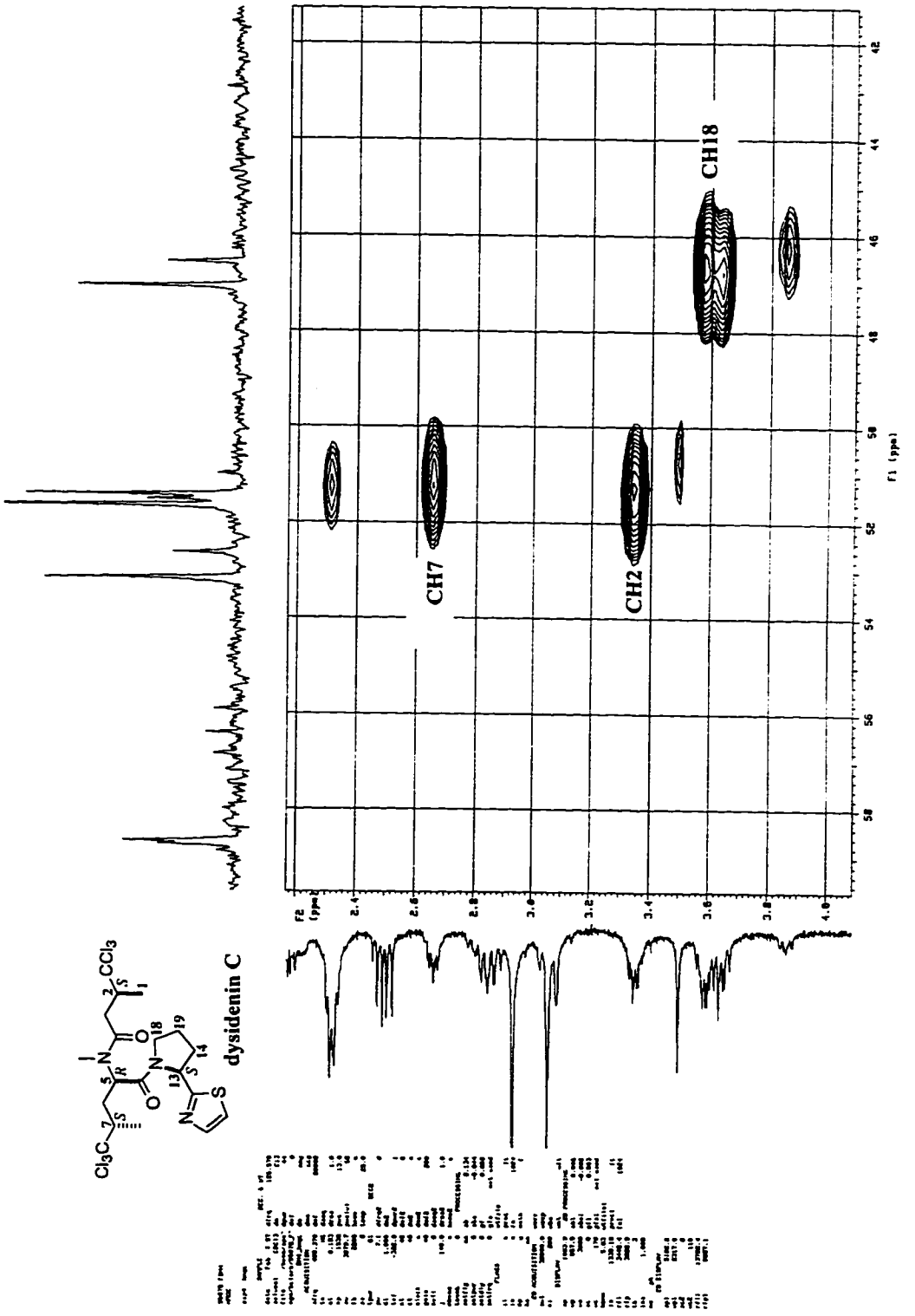


Figure 4.24. HMQC Expansion Spectra ($CDCl_3$, 500 MHz).

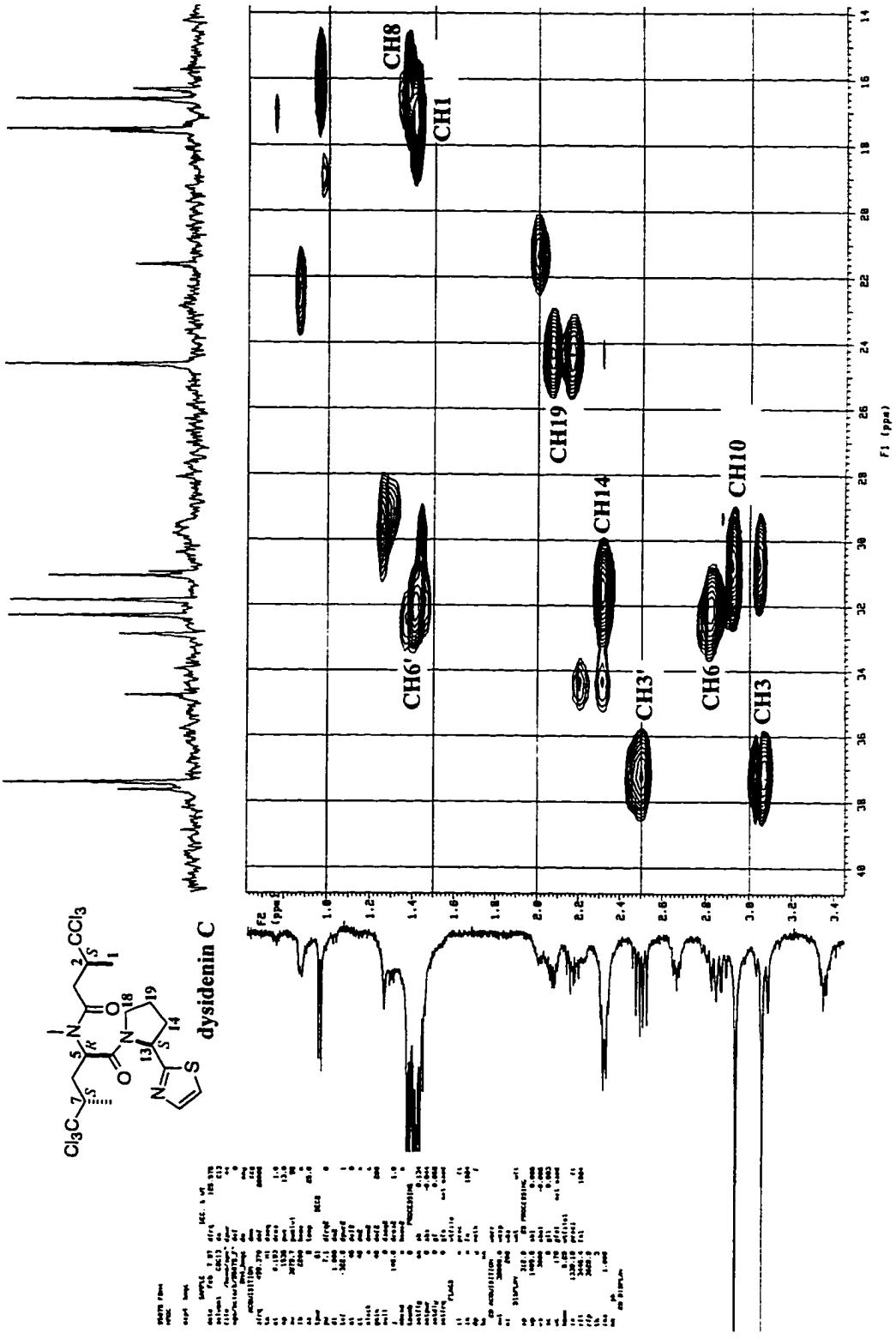


Figure 4.25. HMBC Spectra ($CDCl_3$, 500 MHz).

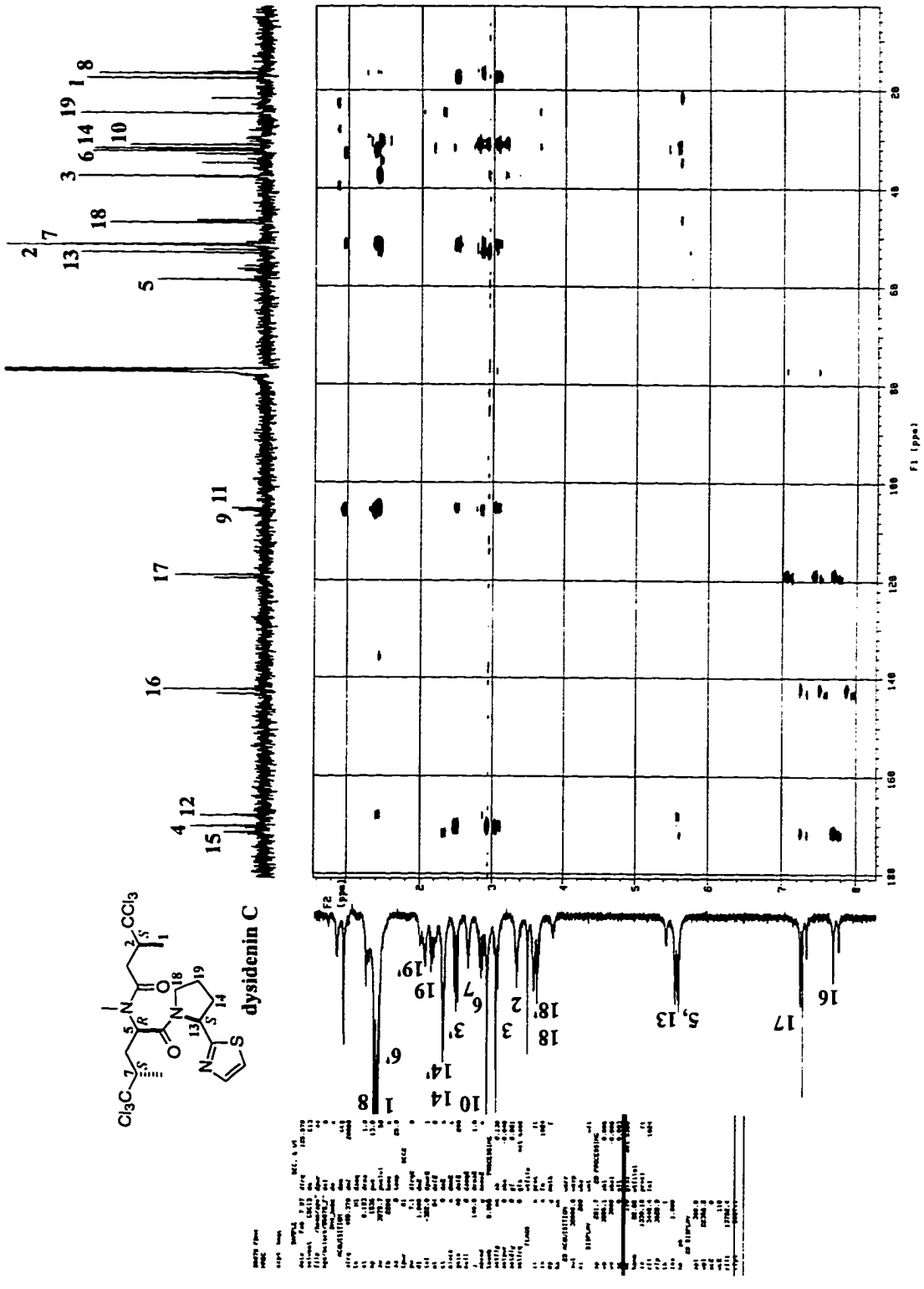


Figure 4.26. HMBC Expansion Spectra ($CDCl_3$, 500 MHz).

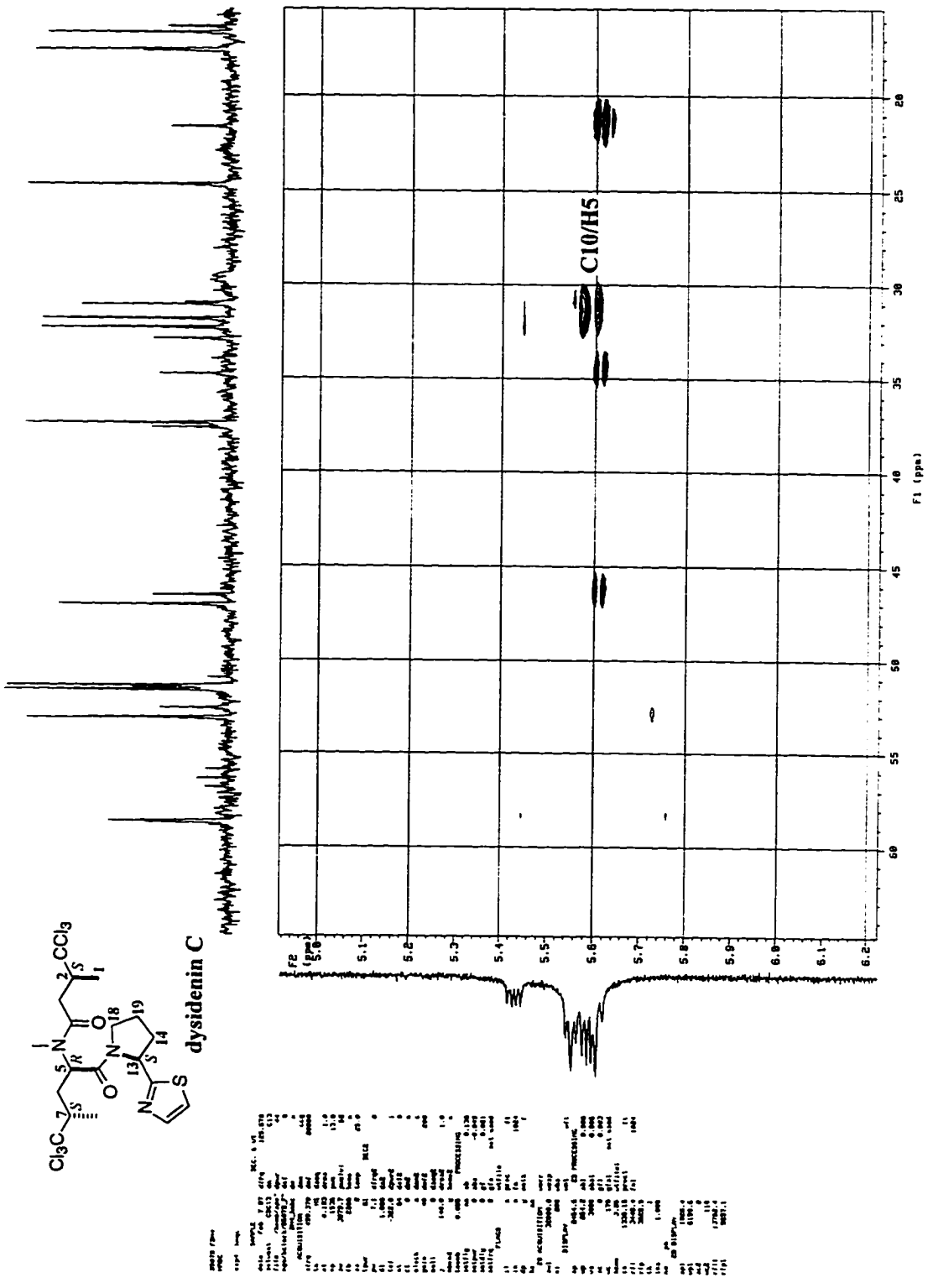


Figure 4.27. HMBC Expansion Spectra ($CDCl_3$, 500 MHz).

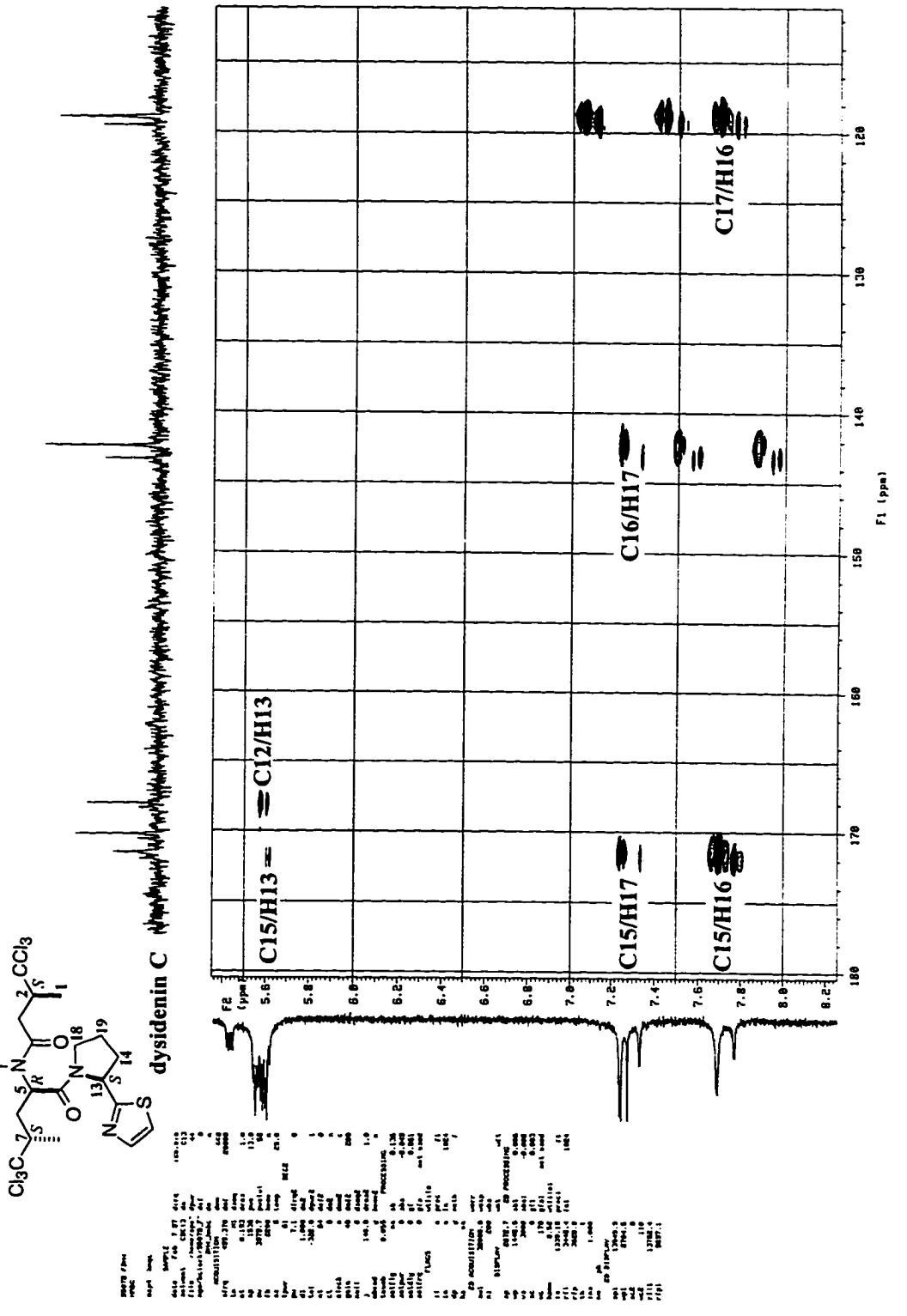


Figure 4.28. HMBC Expansion Spectra ($CDCl_3$, 500 MHz).

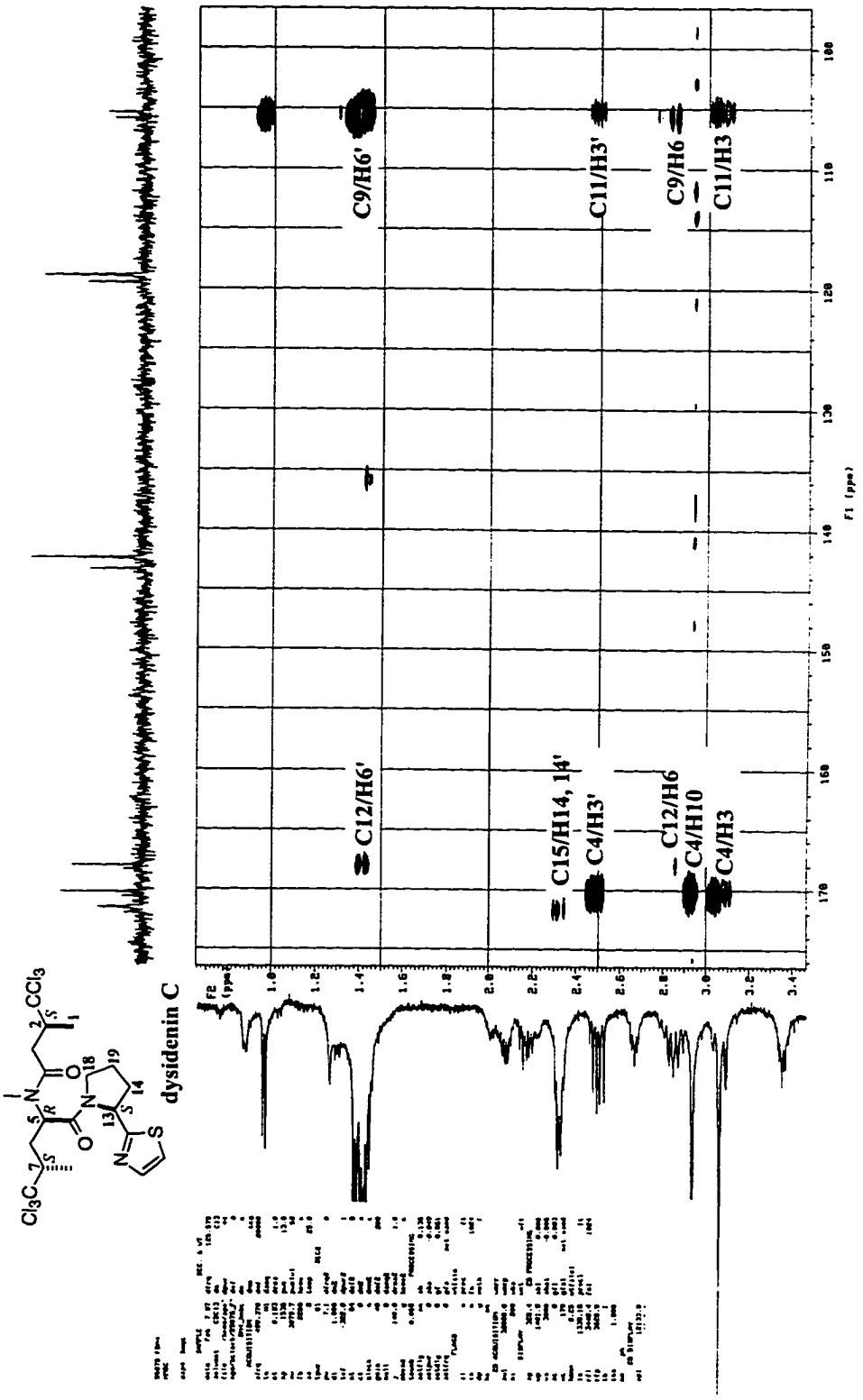


Figure 4.29. HMBC Expansion Spectra ($CDCl_3$, 500 MHz).

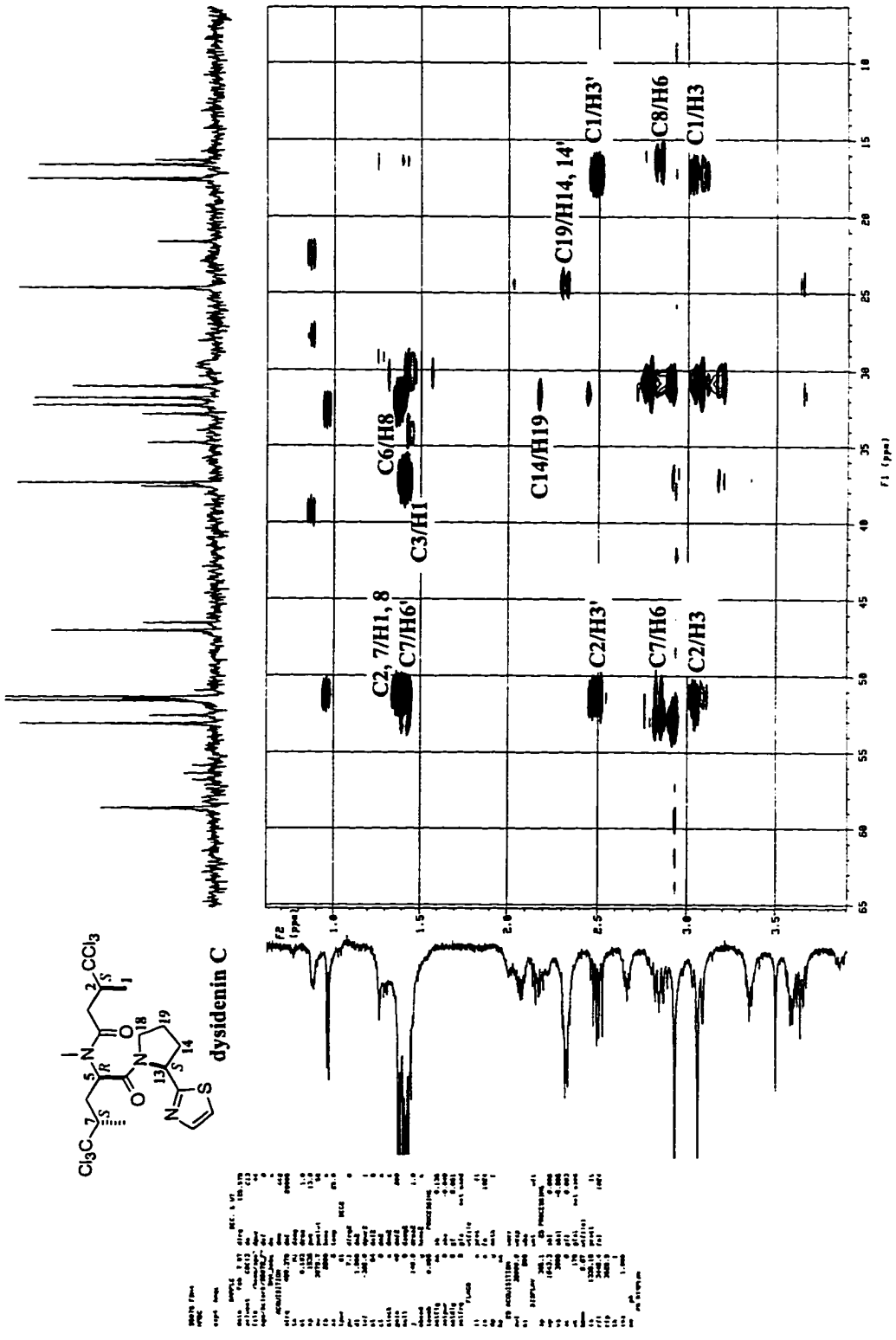


Figure 4.30. ^{1}H-NMR Spectra (DMF-d7, 150°C, 500 MHz).

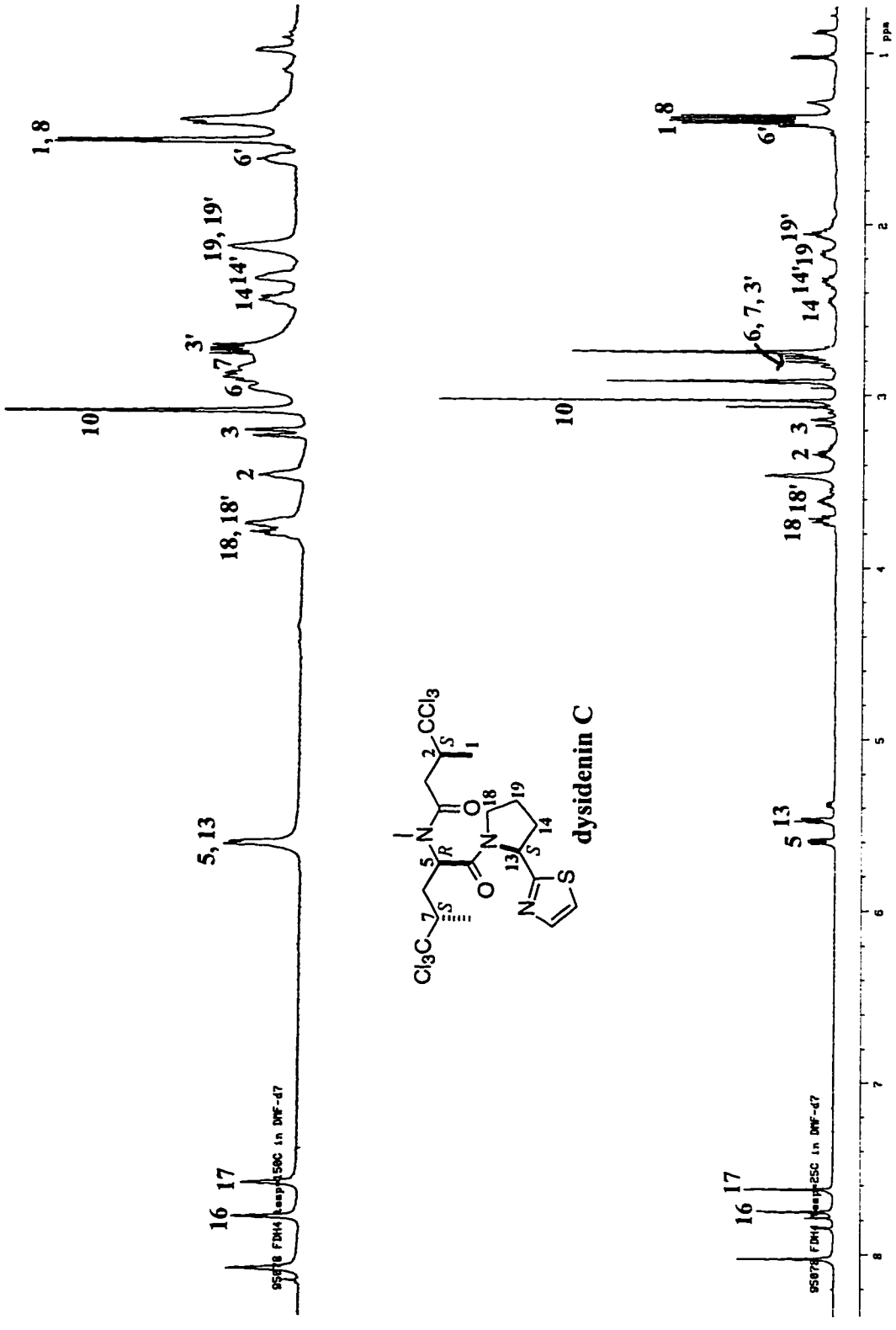


Figure 4.31. ^{1}H-NMR Expansion Spectra (DMF-d7, 150°C, 500 MHz).

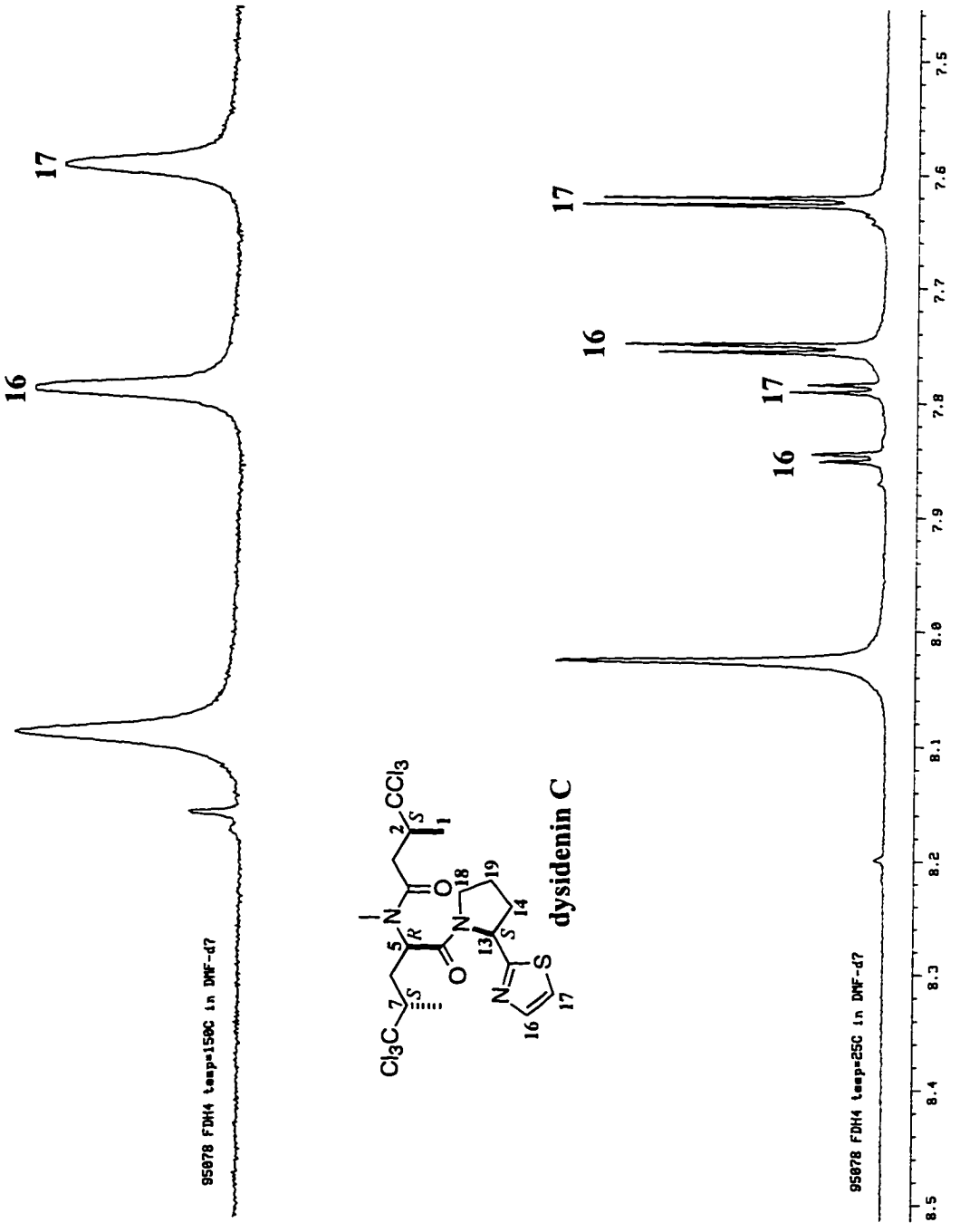
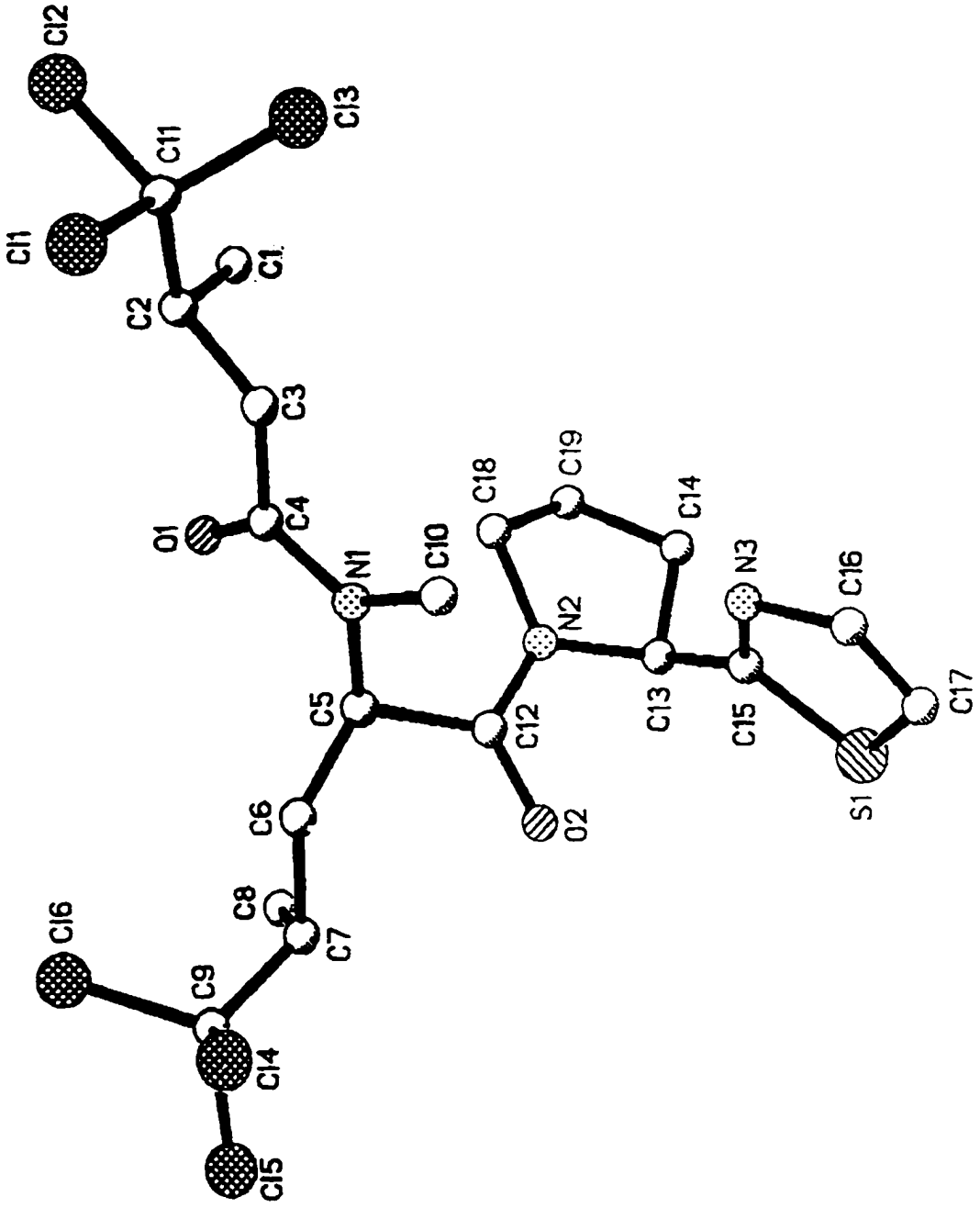


Figure 4.32..X-Ray Structure.



Supplementary Material-

dysidenin D

Figure 4.33. ^{1}H-NMR Spectra ($CDCl_3$, 500 MHz).

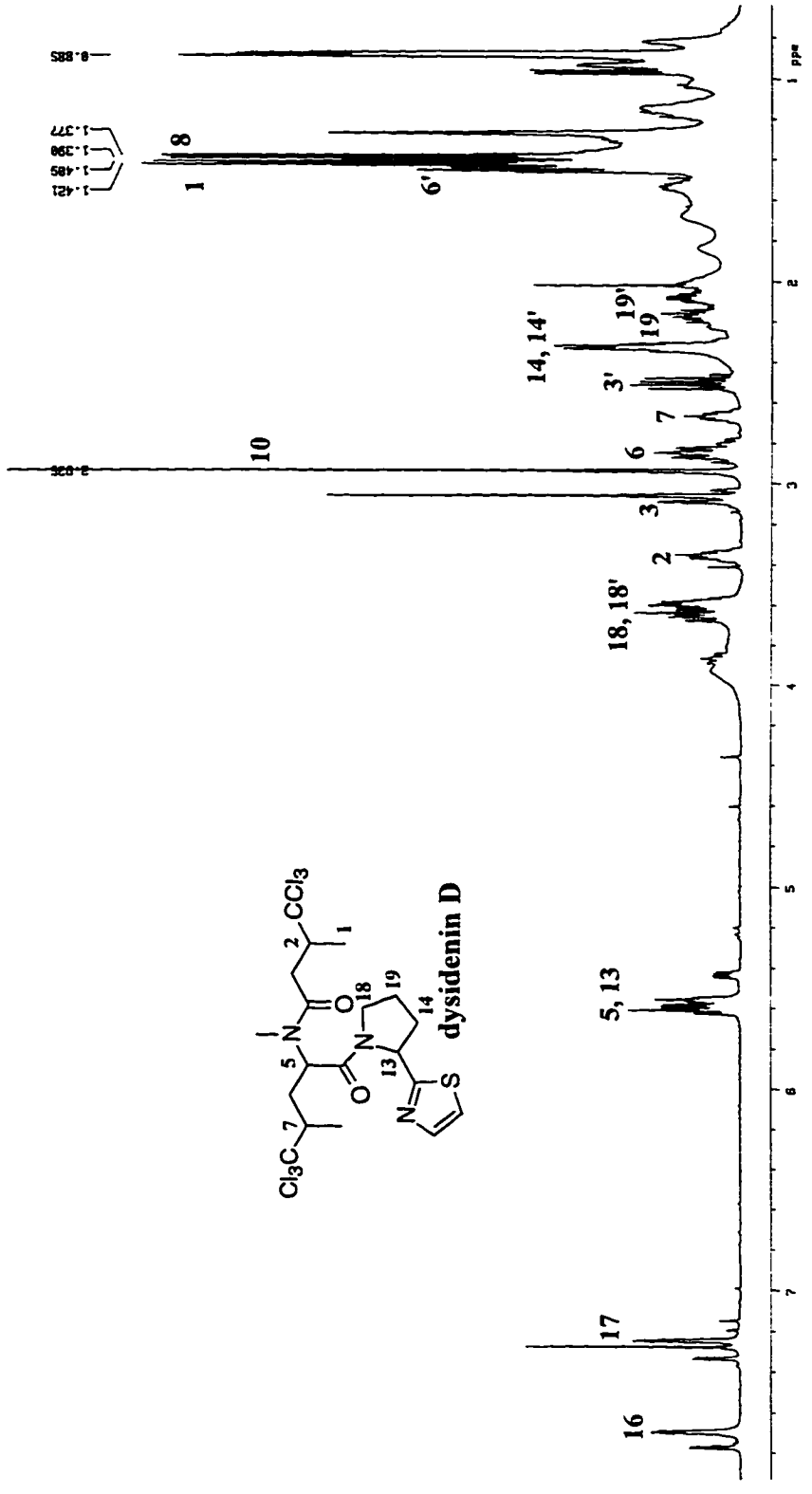


Figure 4.34. ^{13}C-NMR Spectra ($CDCl_3$, 125 MHz).

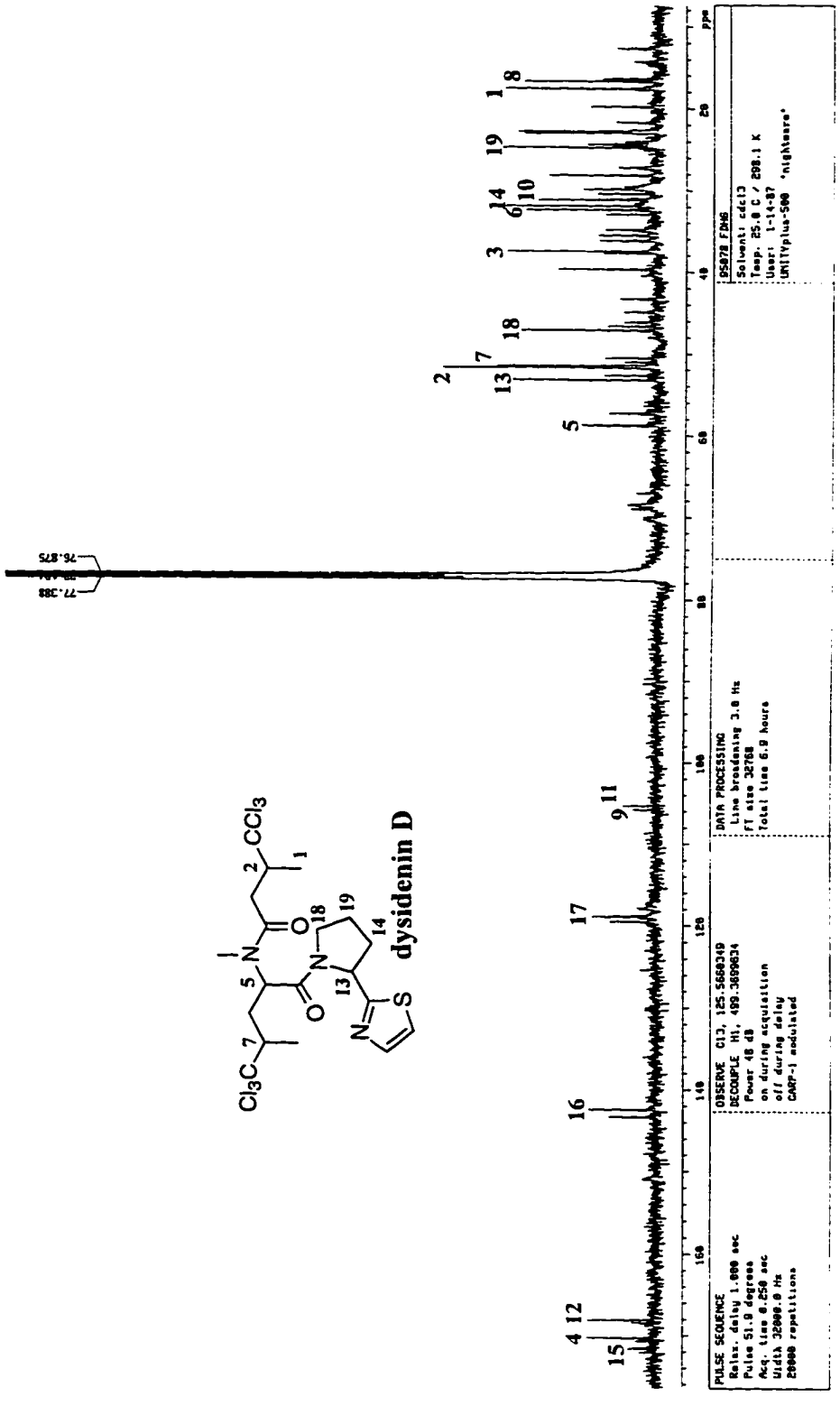


Figure 4.35. Electrospray Ionization Mass Spectra.

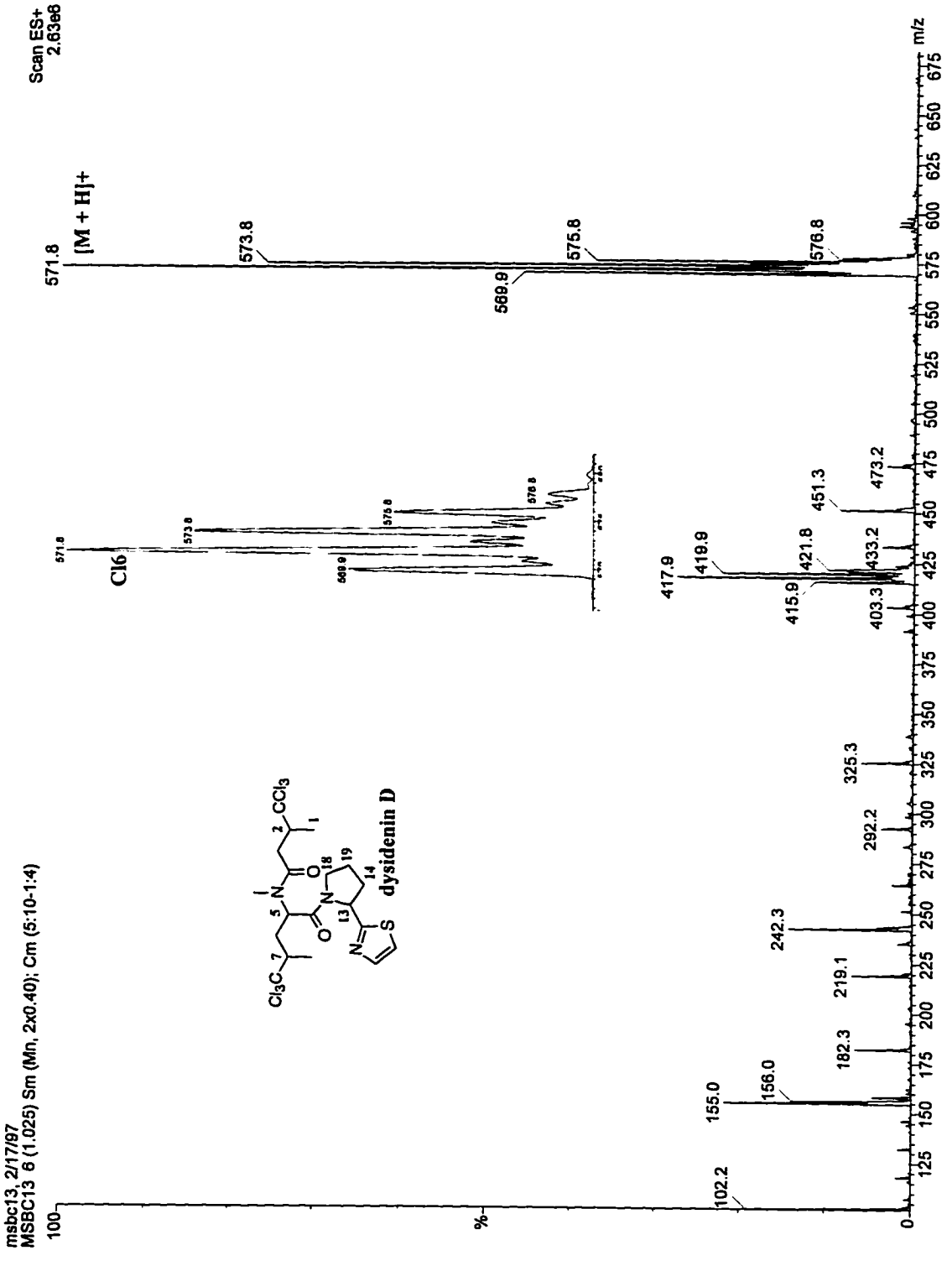


Figure 4.36. LRFAB Mass Spectra.

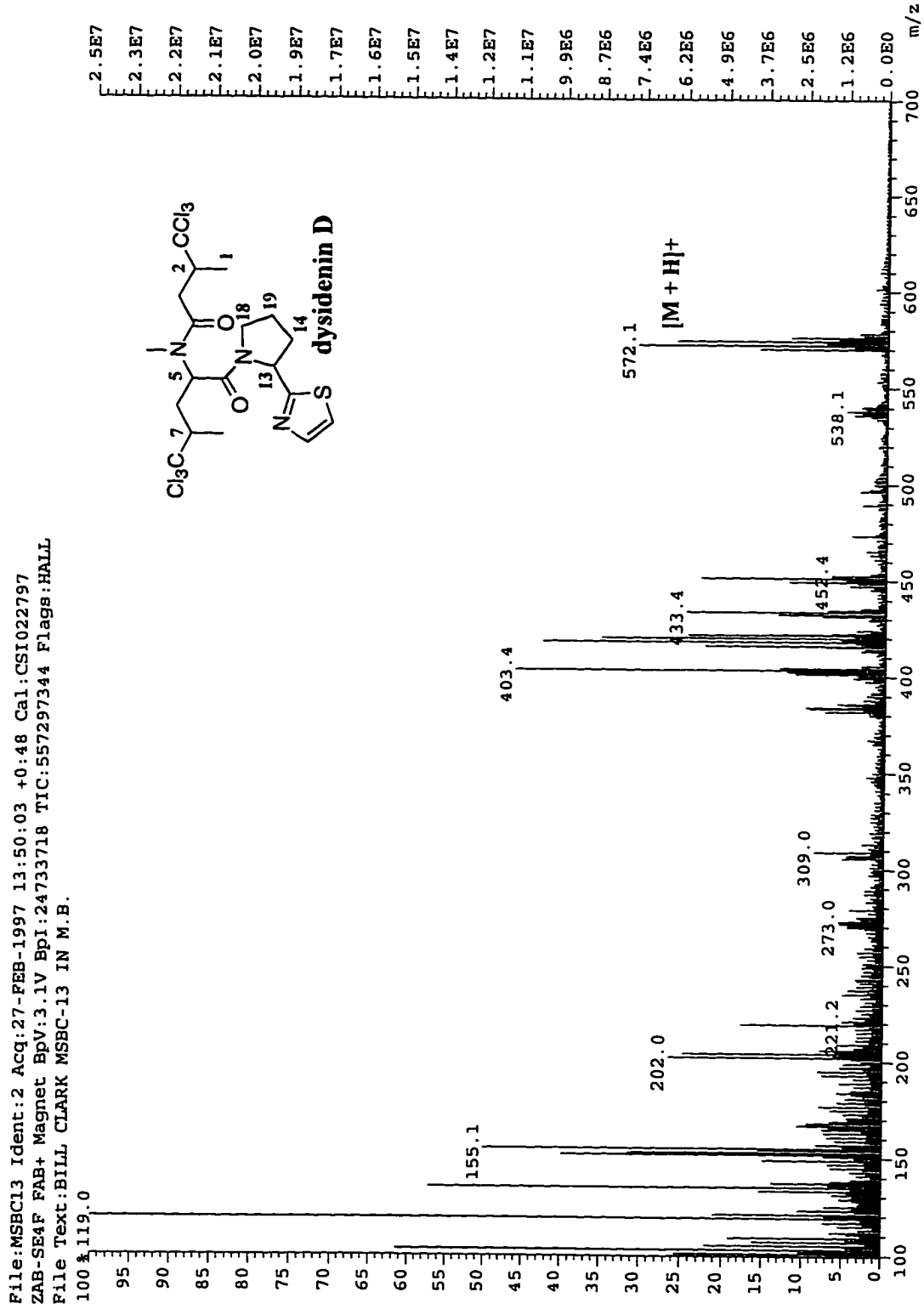
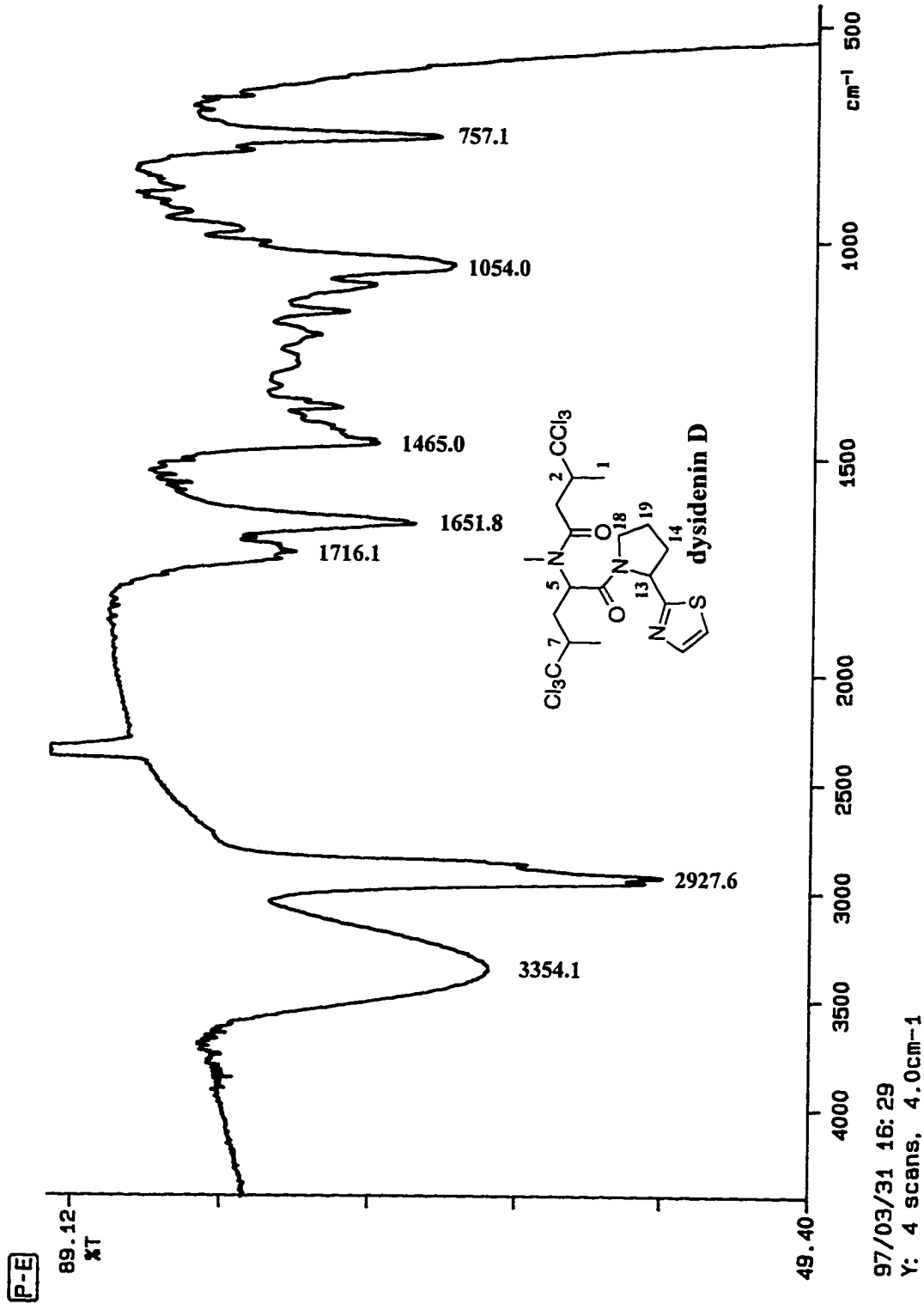


Figure 4.37. IR Spectra.



Supplementary Material-
9, 11-didechlorodysidenin

Figure 4.38. ^{1}H-NMR Spectra ($CDCl_3$, 500 MHz).

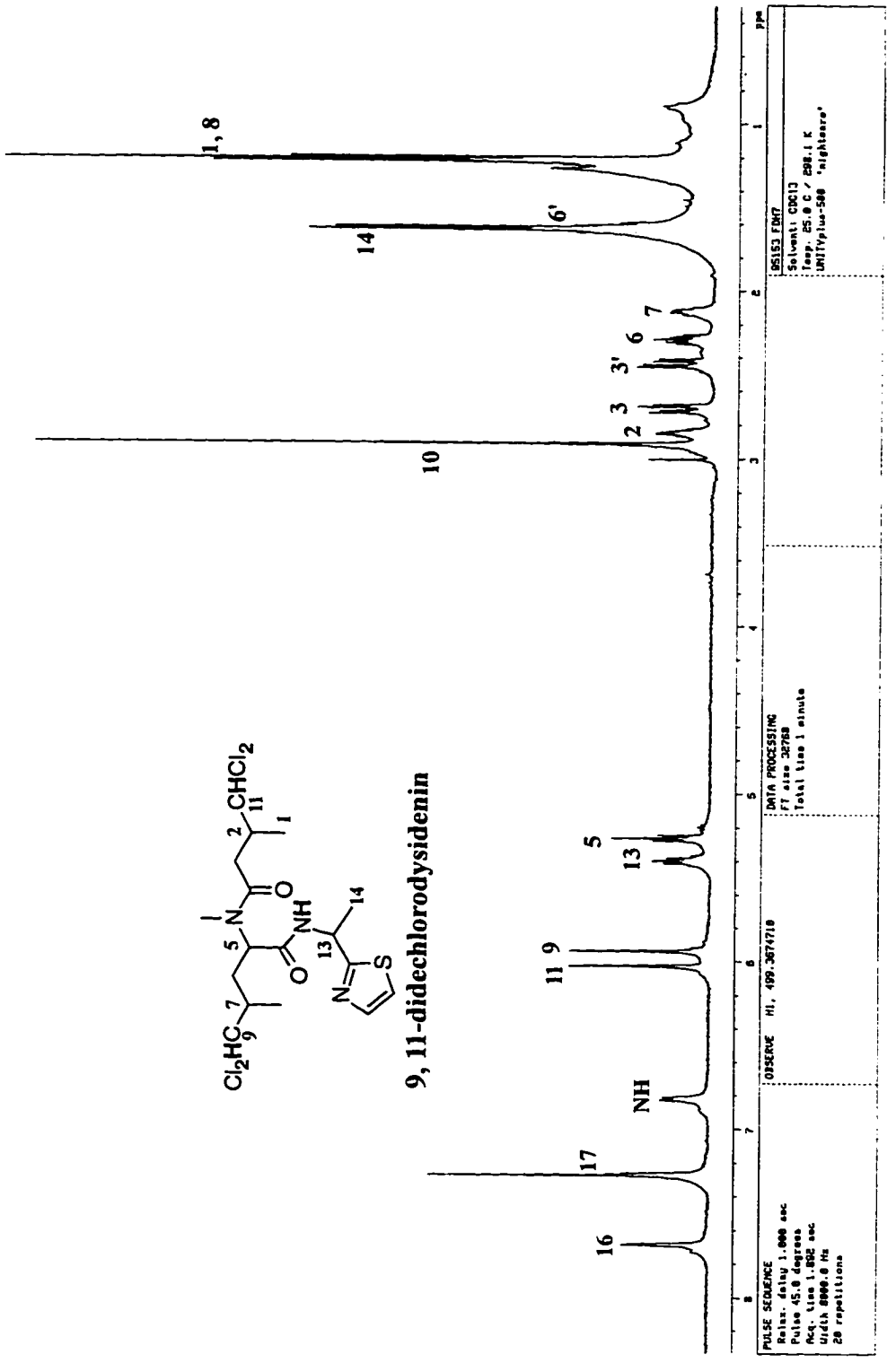


Figure 4.39. ^{13}C-NMR Spectra ($CDCl_3$, 125 MHz).

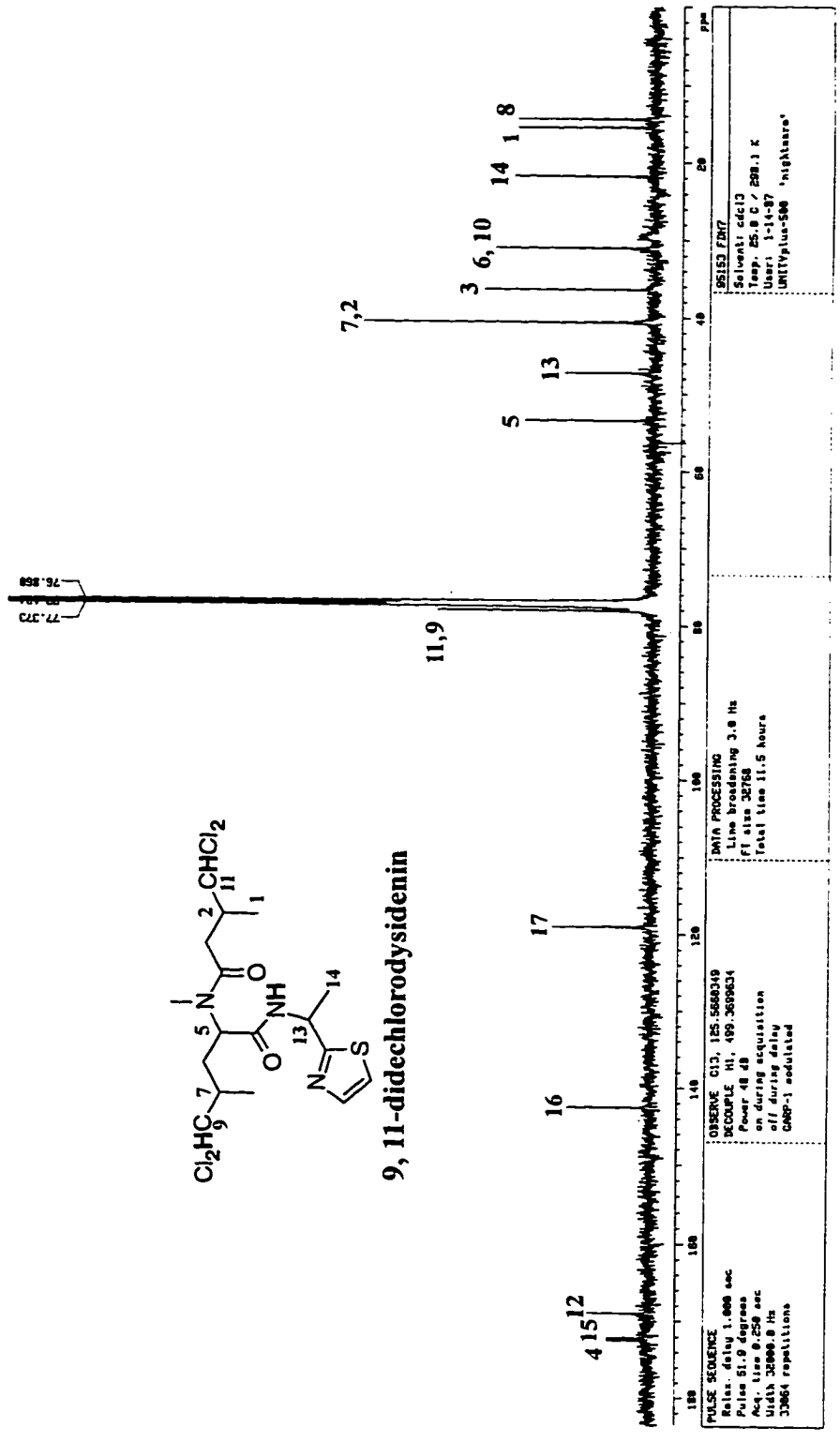


Figure 4.40. LRFAB Mass Spectra.

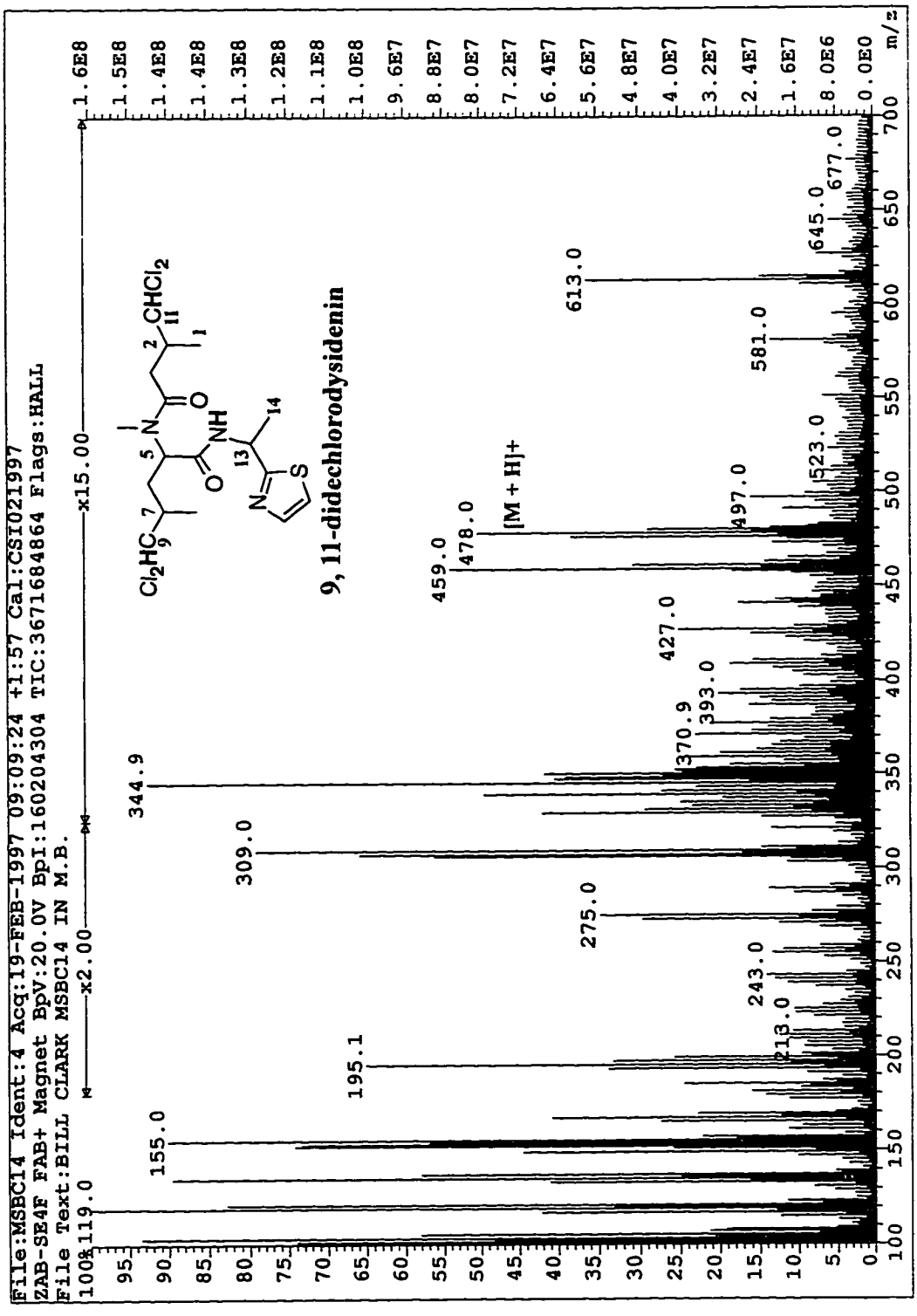


Figure 4.41. IR Spectra.

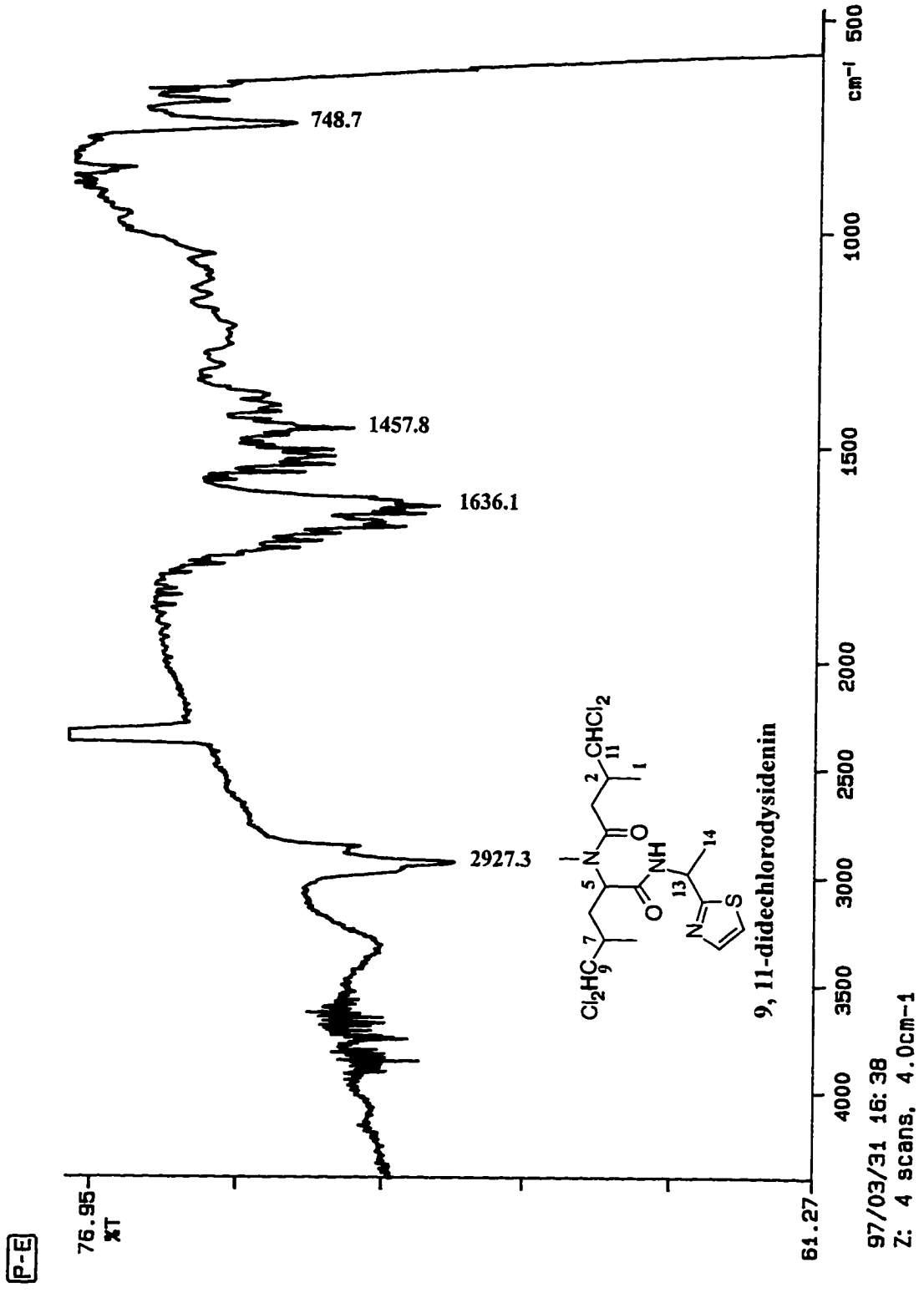


Figure 4.42. COSY NMR Spectra ($CDCl_3$, 500 MHz).

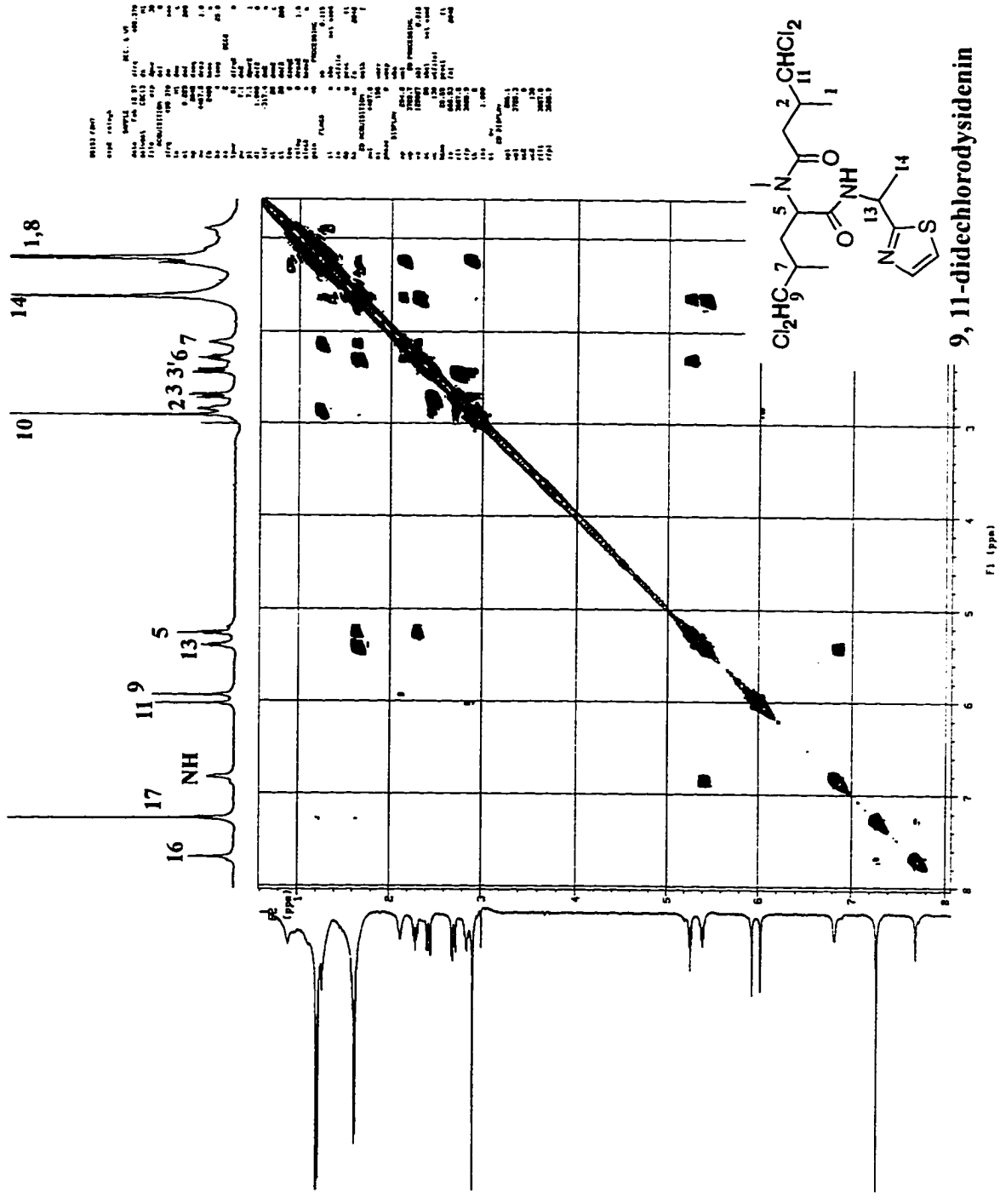


Figure 4.43. COSY NMR Expansion Spectra ($CDCl_3$, 500 MHz).

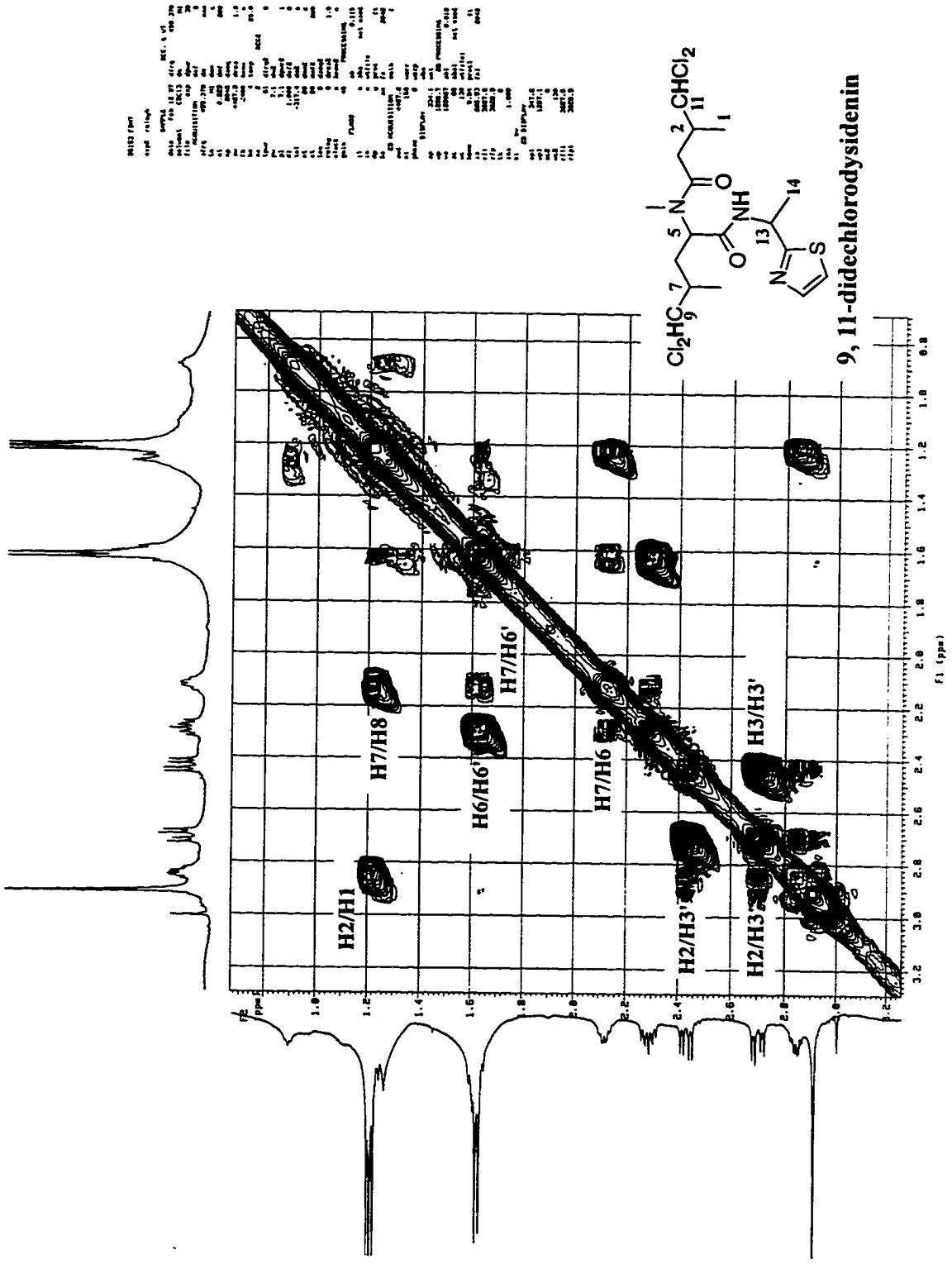


Figure 4.44. COSY NMR Expansion Spectra ($CDCl_3$, 500 MHz).

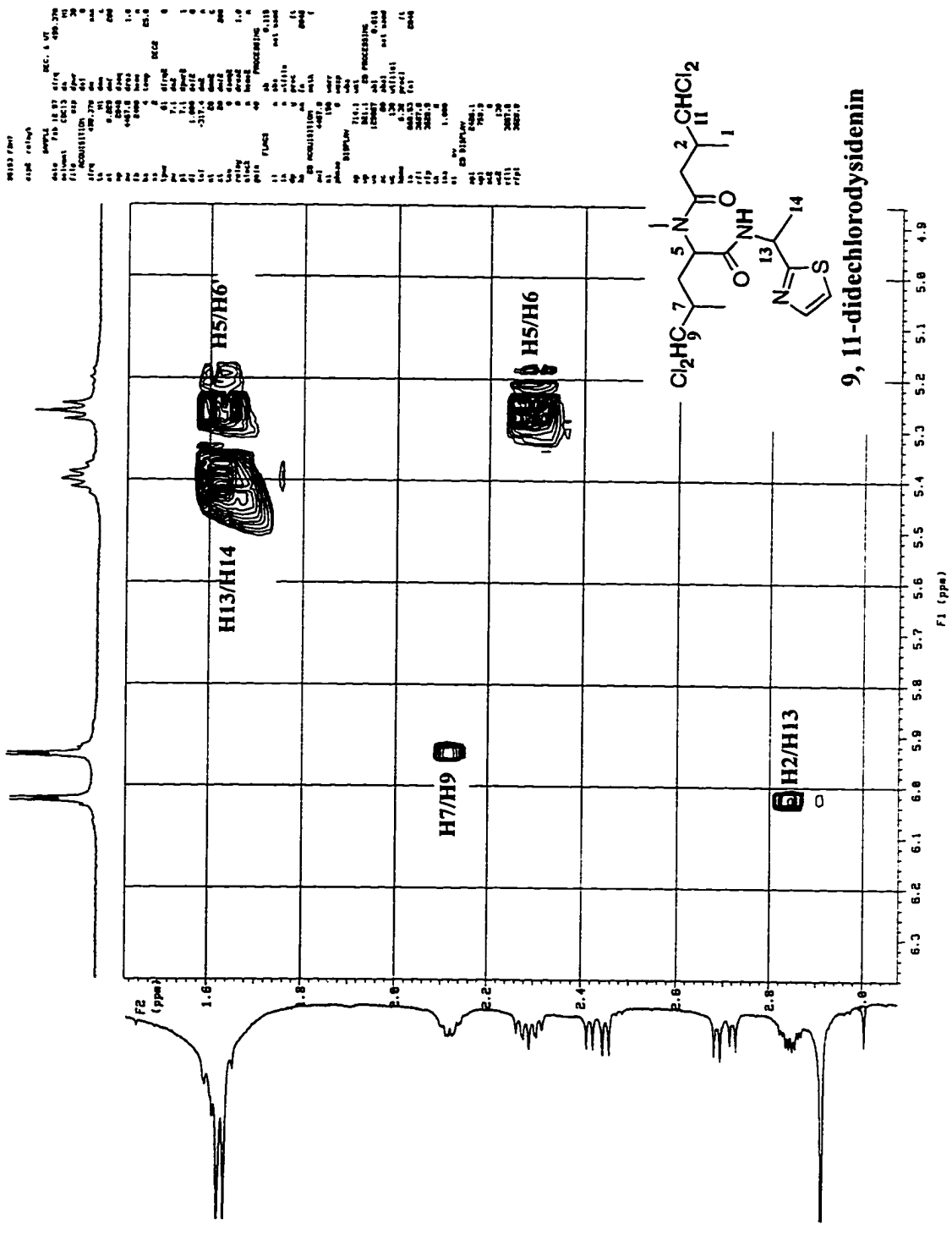


Figure 4.45. COSY NMR Expansion Spectra ($CDCl_3$, 500 MHz).

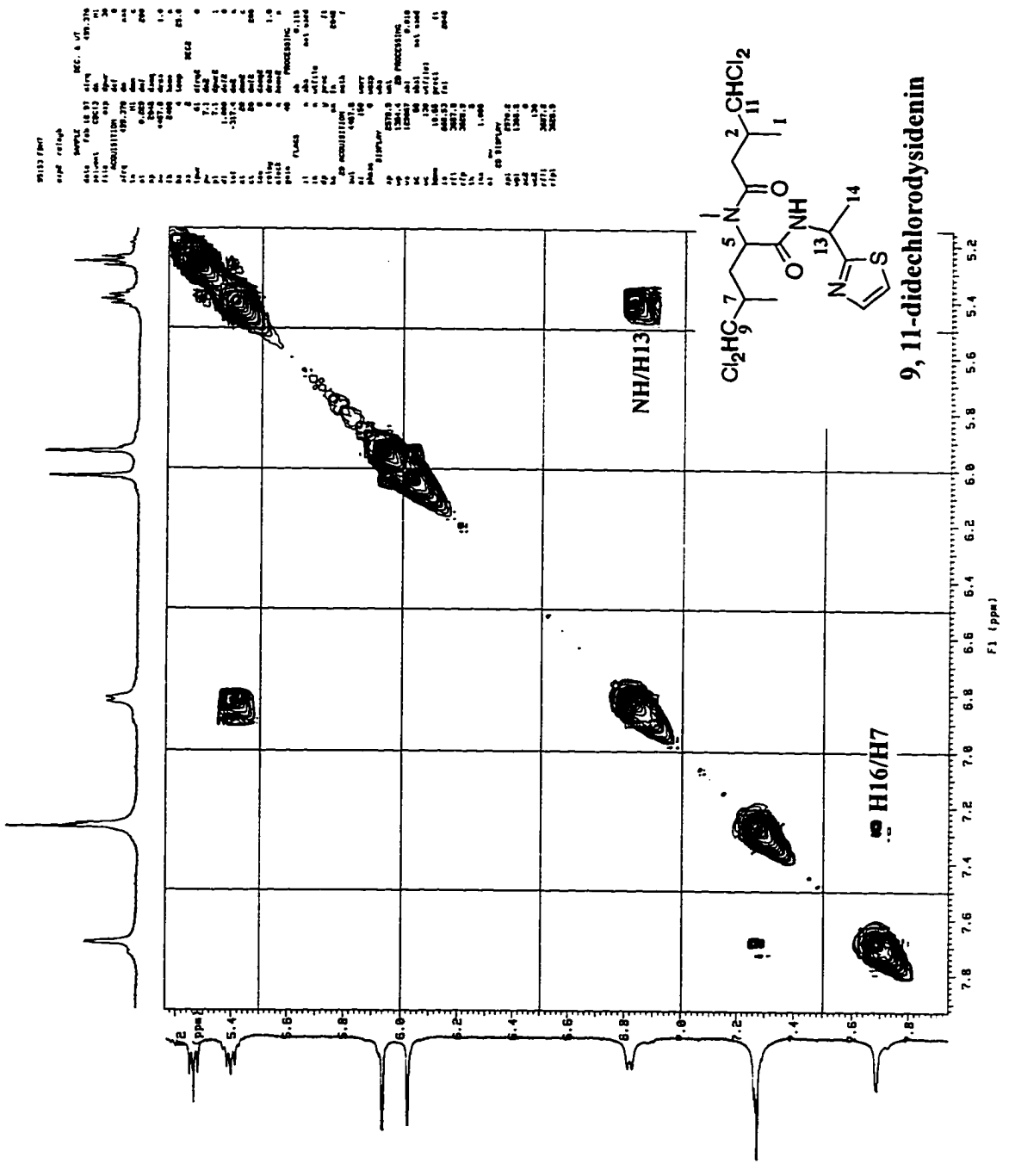


Figure 4.46. HMQC NMR Spectra ($CDCl_3$, 500 MHz).

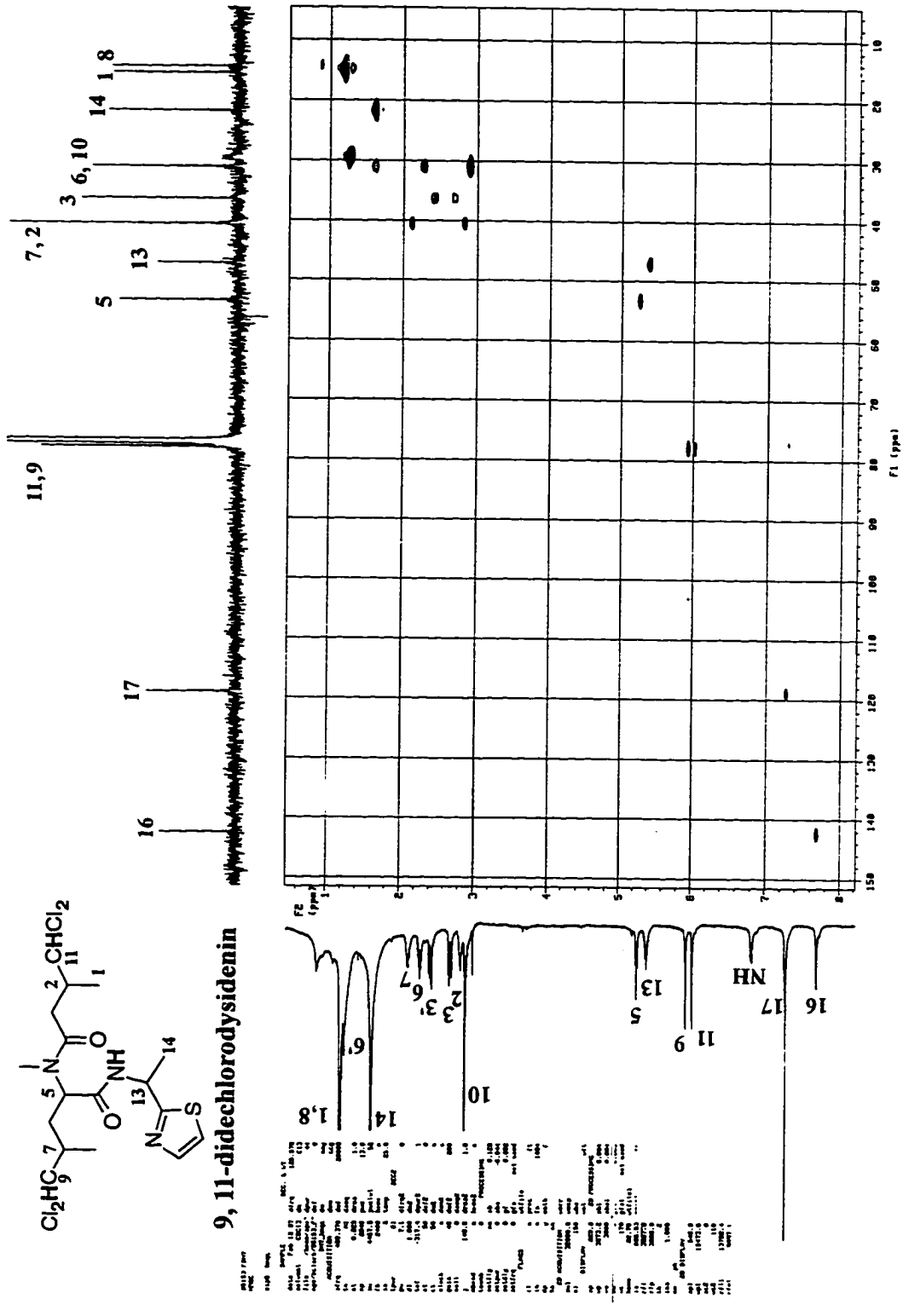


Figure 4.47. HMQC NMR Expansion Spectra ($CDCl_3$, 500 MHz).

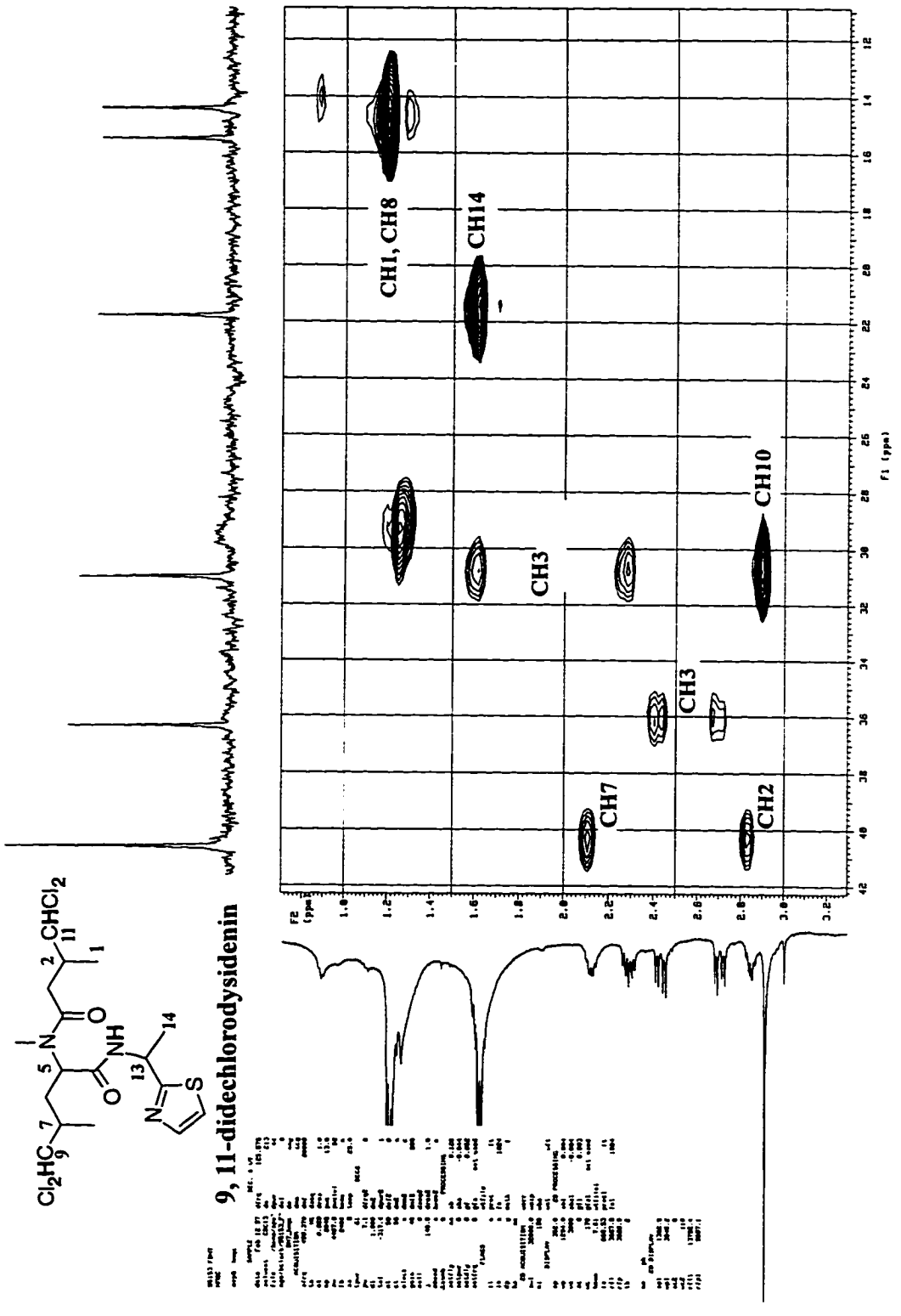


Figure 4.48. HMQC NMR Expansion Spectra ($CDCl_3$, 500 MHz).

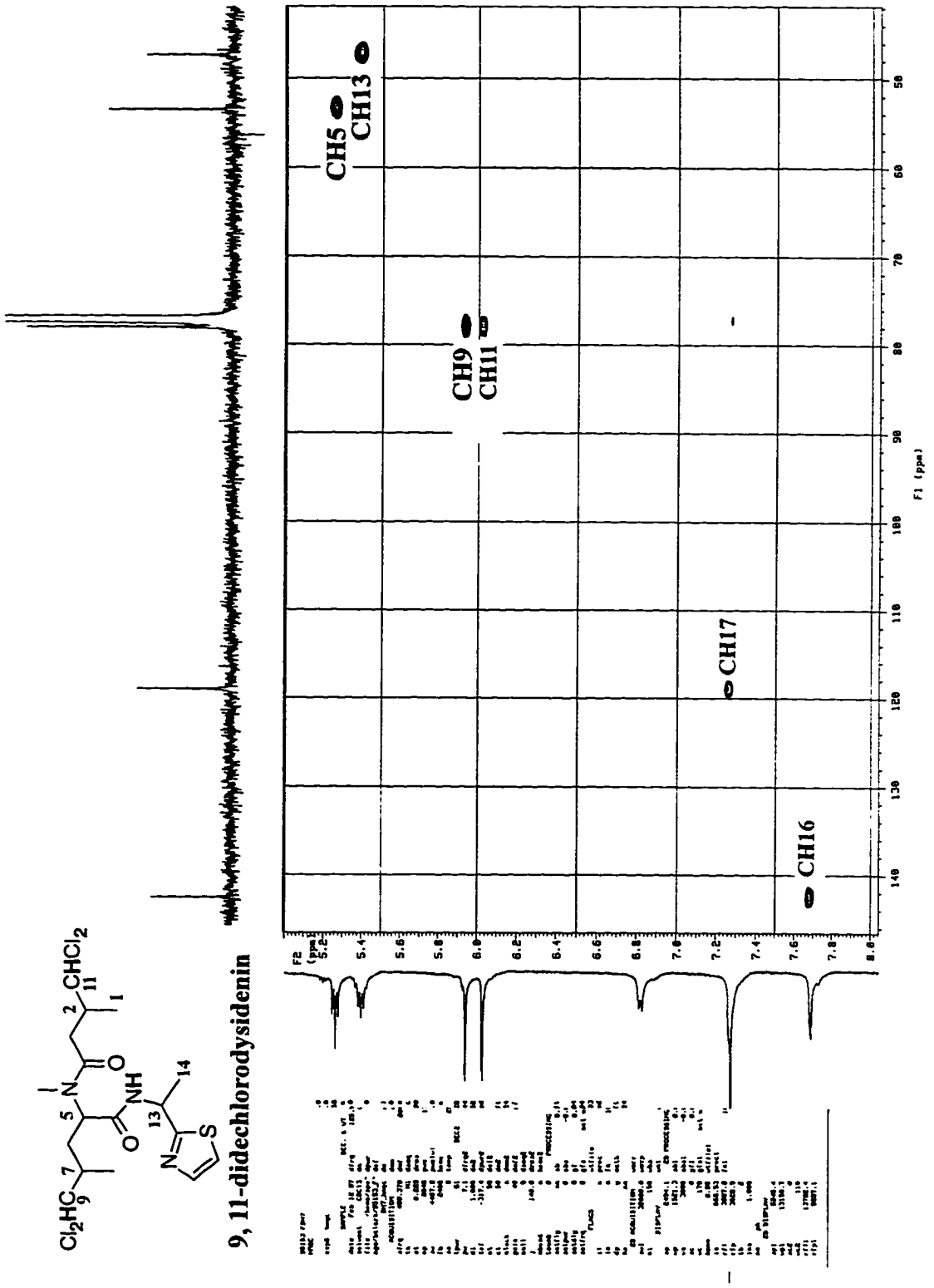


Figure 4.49. HMBC NMR Spectra ($CDCl_3$, 500 MHz).

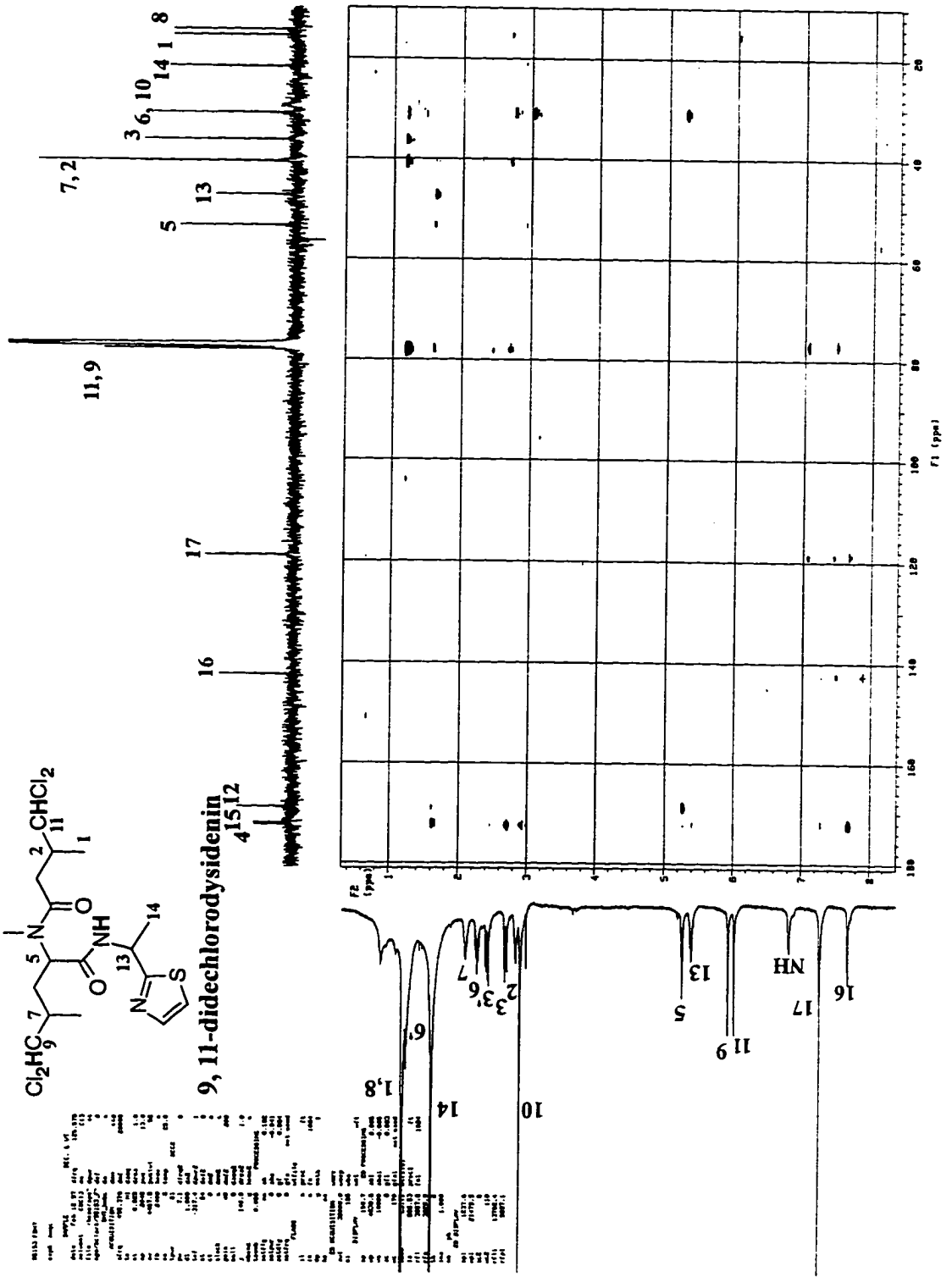


Figure 4.50. HMBC Expansion NMR Spectra ($CDCl_3$, 500 MHz).

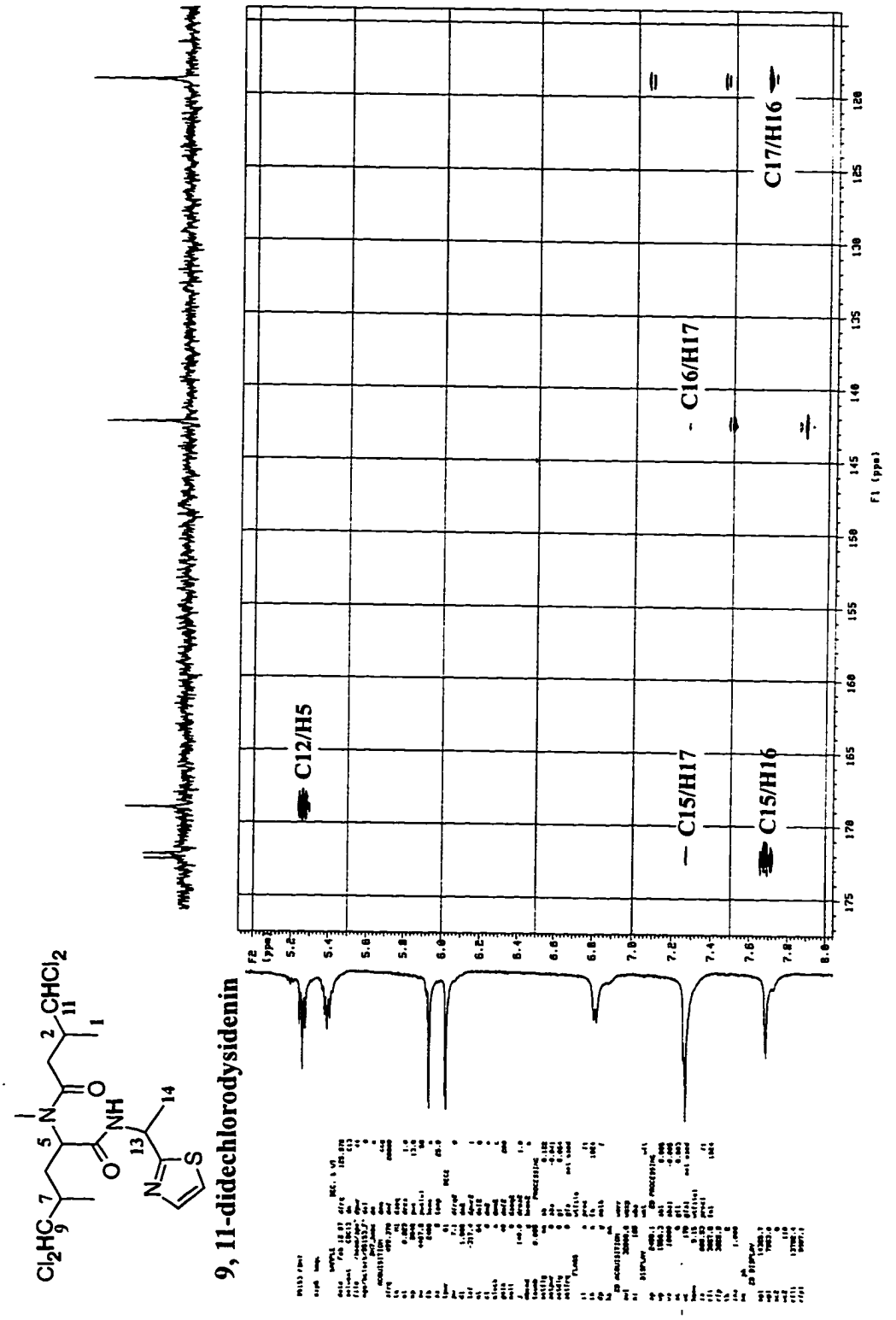


Figure 4.51. HMBC Expansion NMR Spectra ($CDCl_3$, 500 MHz).

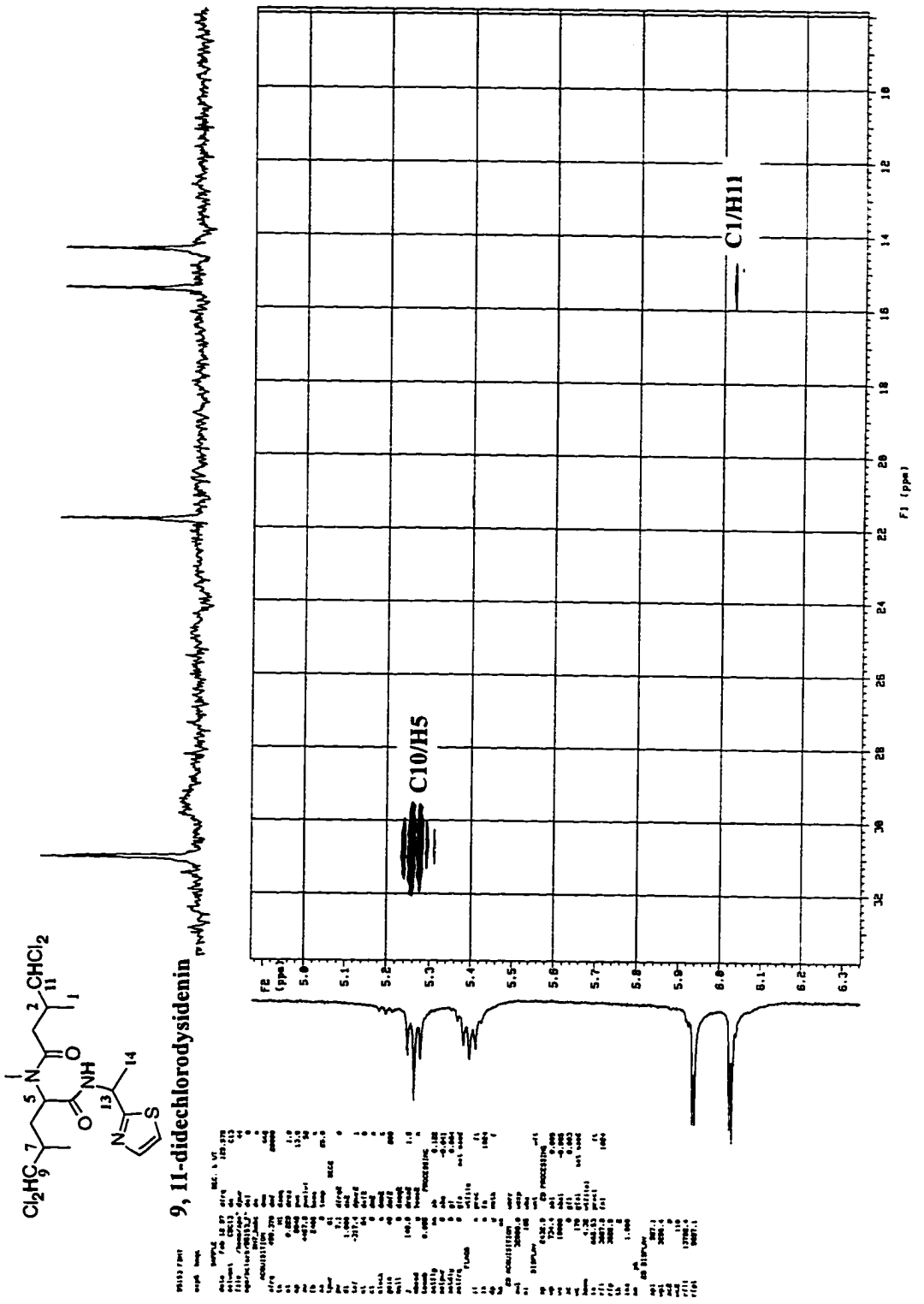


Figure 4.52. HMBC Expansion NMR Spectra ($CDCl_3$, 500 MHz).

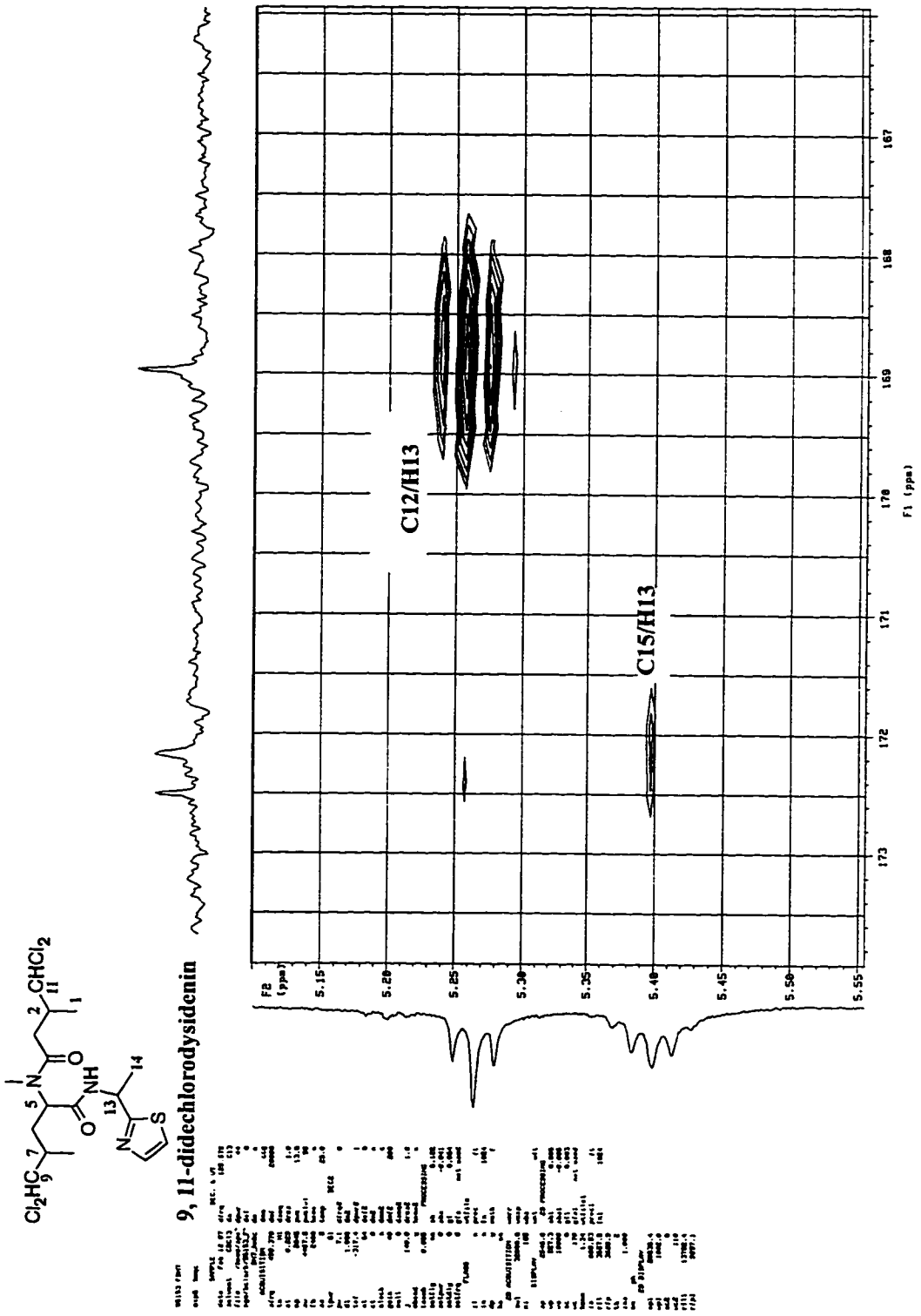


Figure 4.53. HMBC Expansion NMR Spectra ($CDCl_3$, 500 MHz).

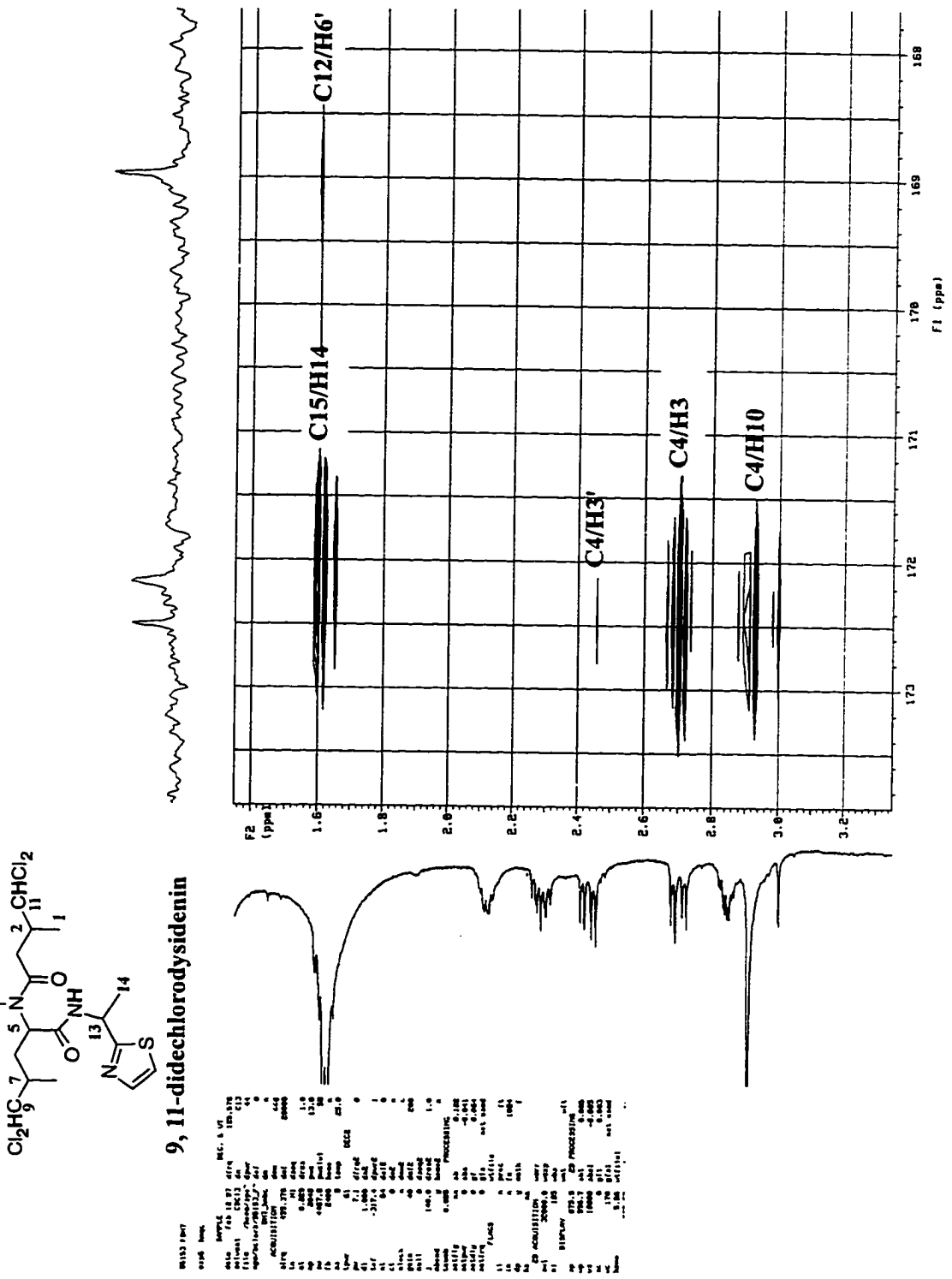
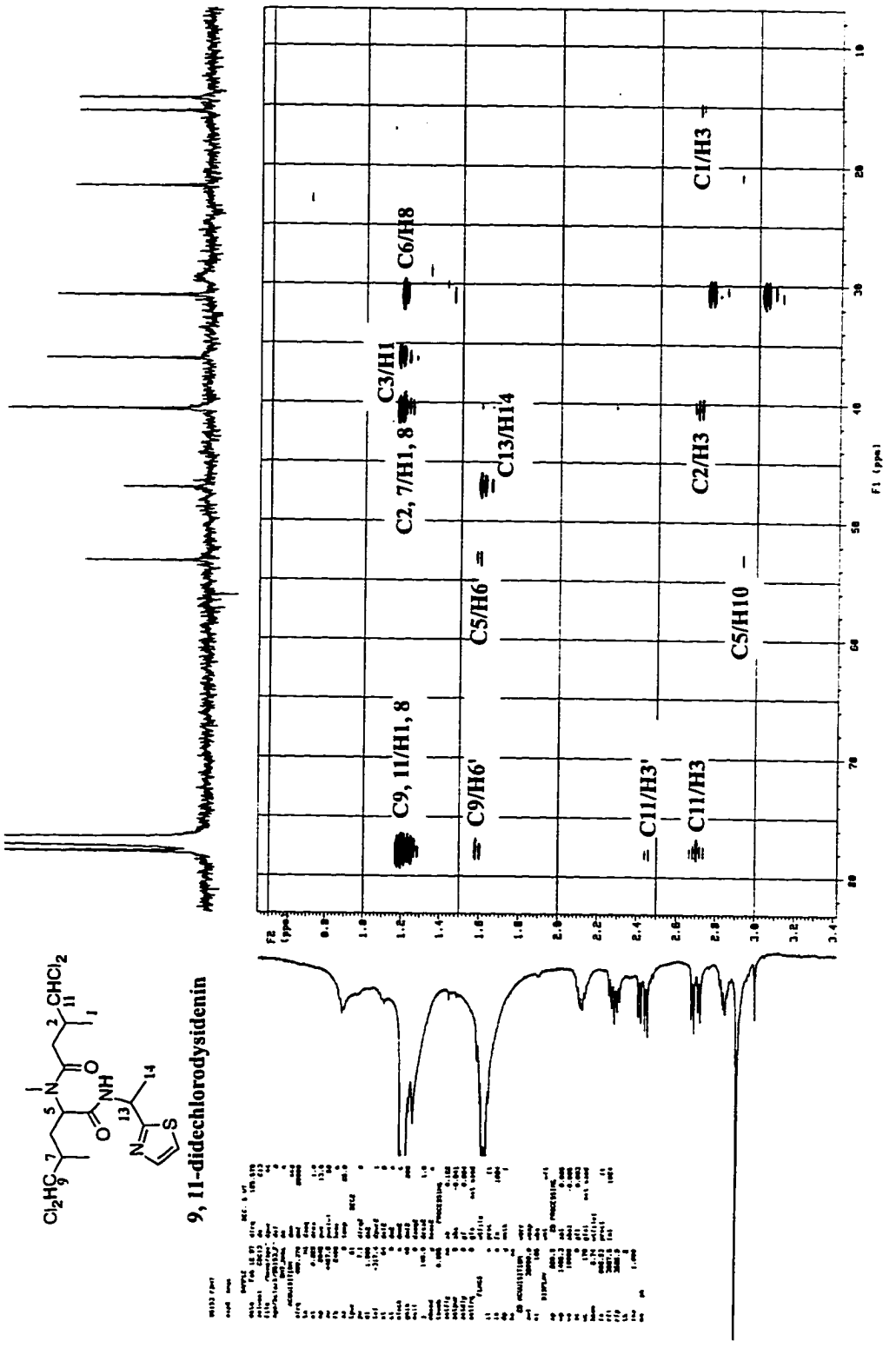


Figure 4.54. HMBC Expansion NMR Spectra ($CDCl_3$, 500 MHz).



Supplementary Material-
tetrabromodioxin

Figure 4.55. ^{1}H-NMR Spectra (CD_3OD, 500 MHz).

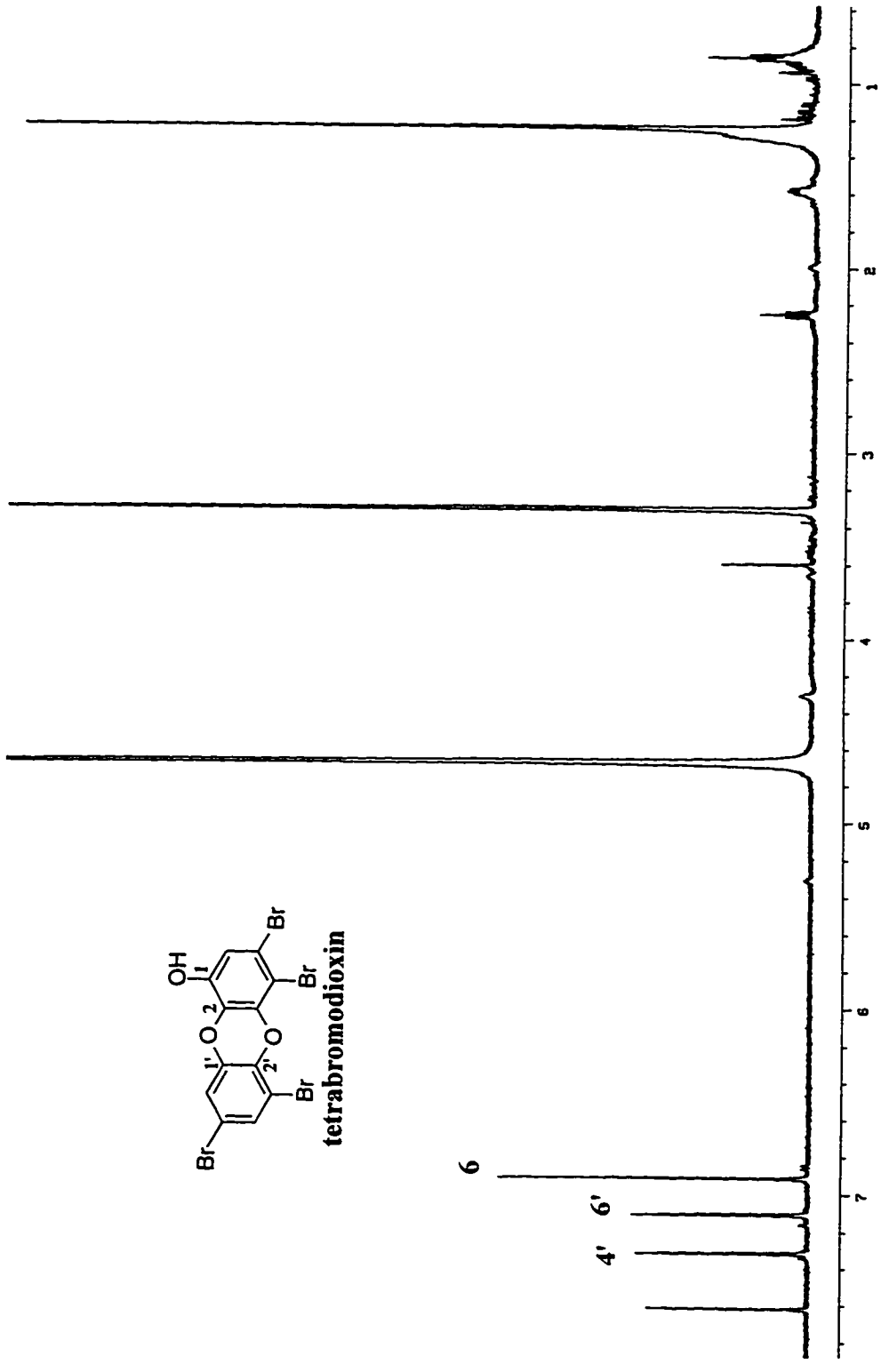


Figure 4.56. ^{13}C-NMR Spectra (CD_3OD, 125 MHz).

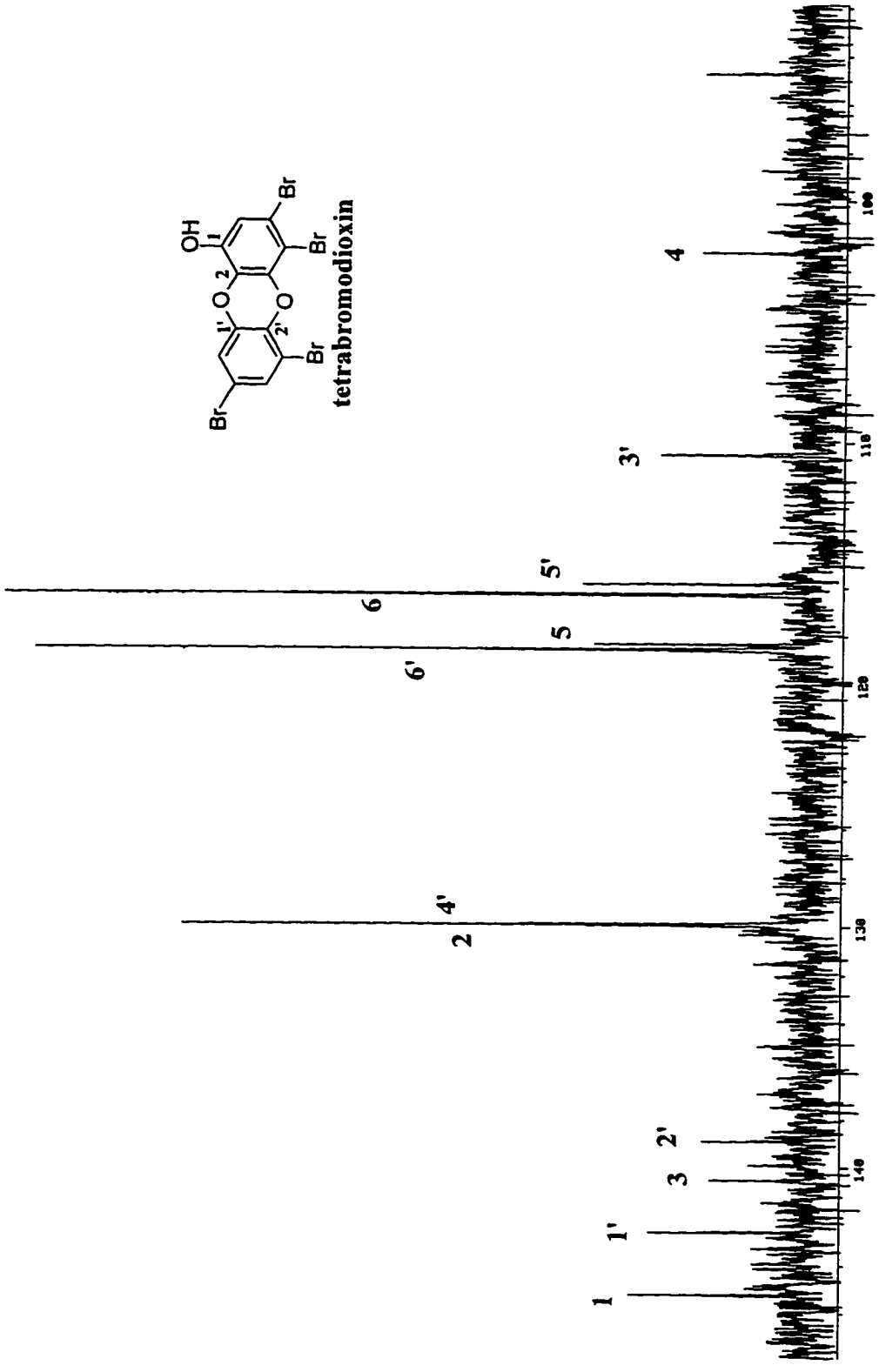


Figure 4.57. LREI Mass Spectra.

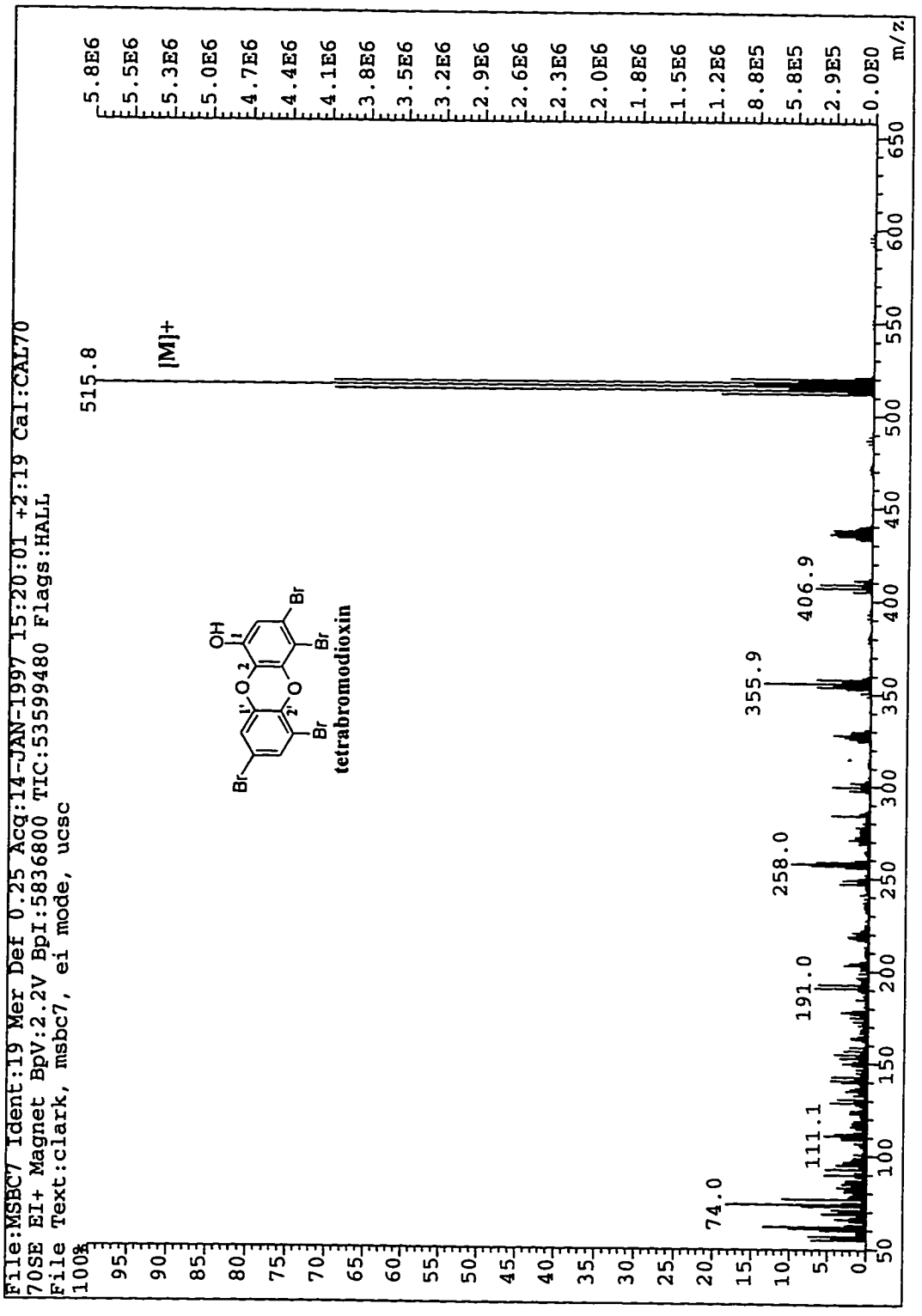


Figure 4.58. IR Spectra.

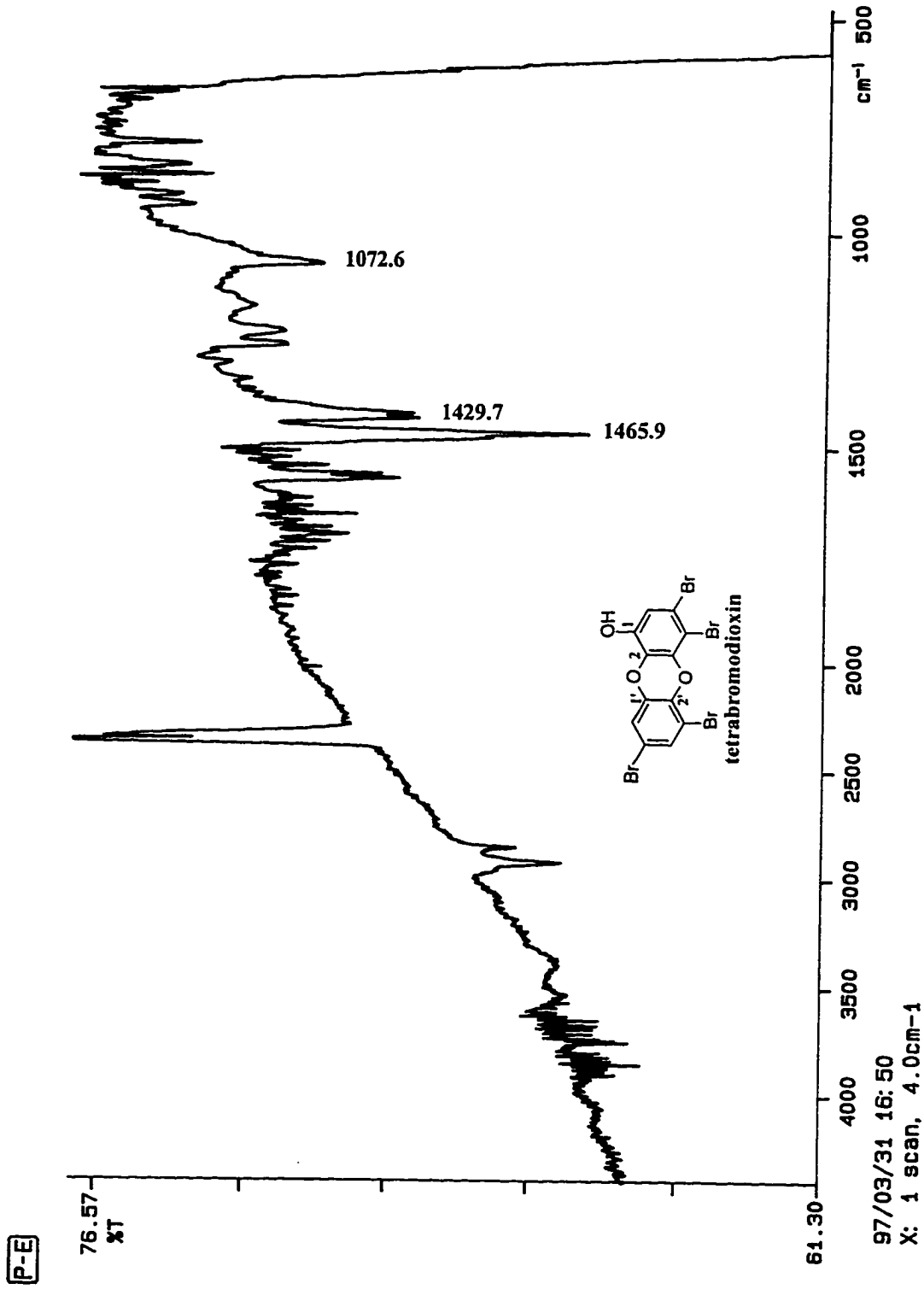


Figure 4.59. HMQC NMR Spectra (CD_3OD, 500 MHz).

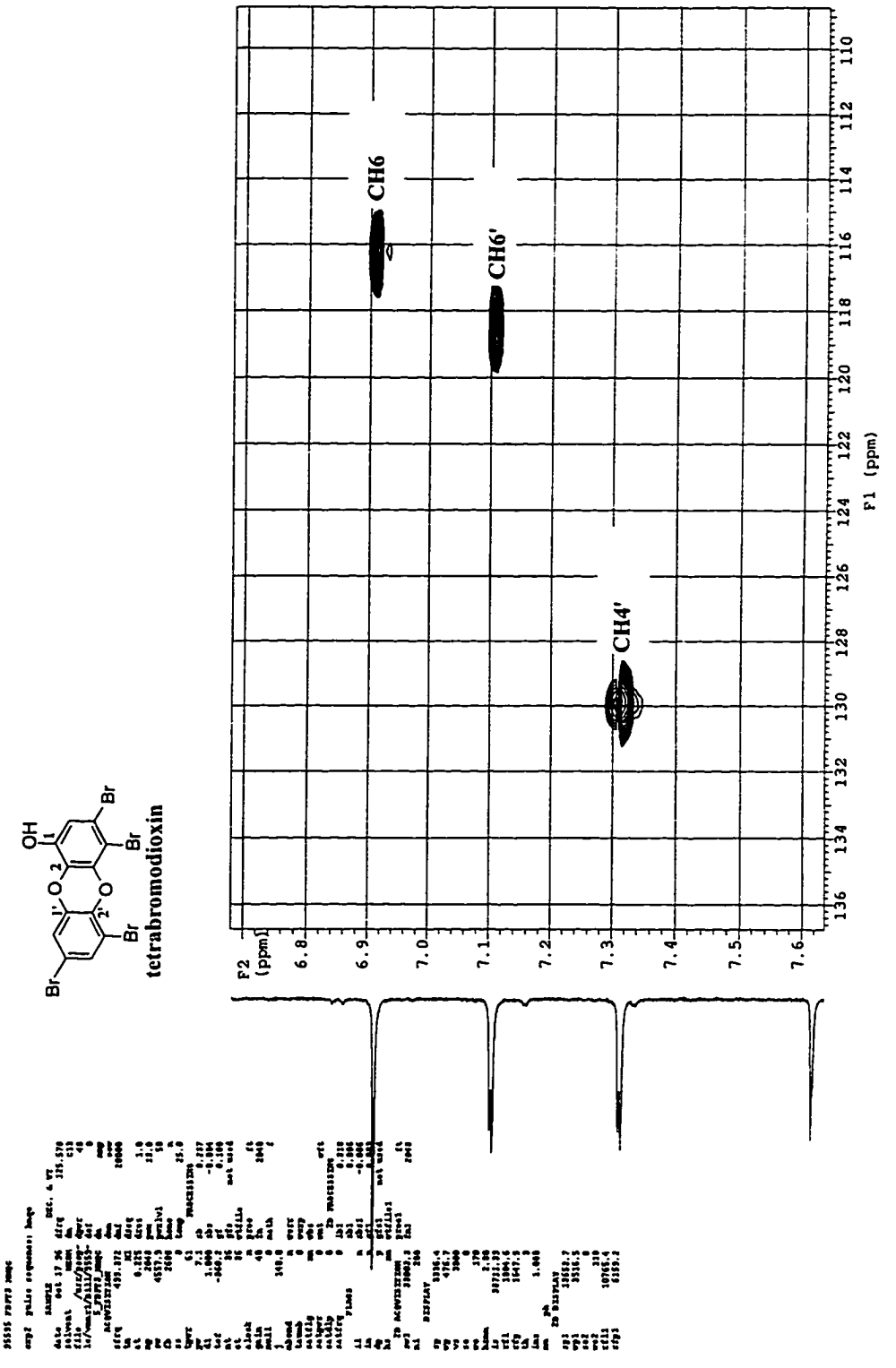


Figure 4.60. HMBC NMR Spectra (CD_3OD, 500 MHz).

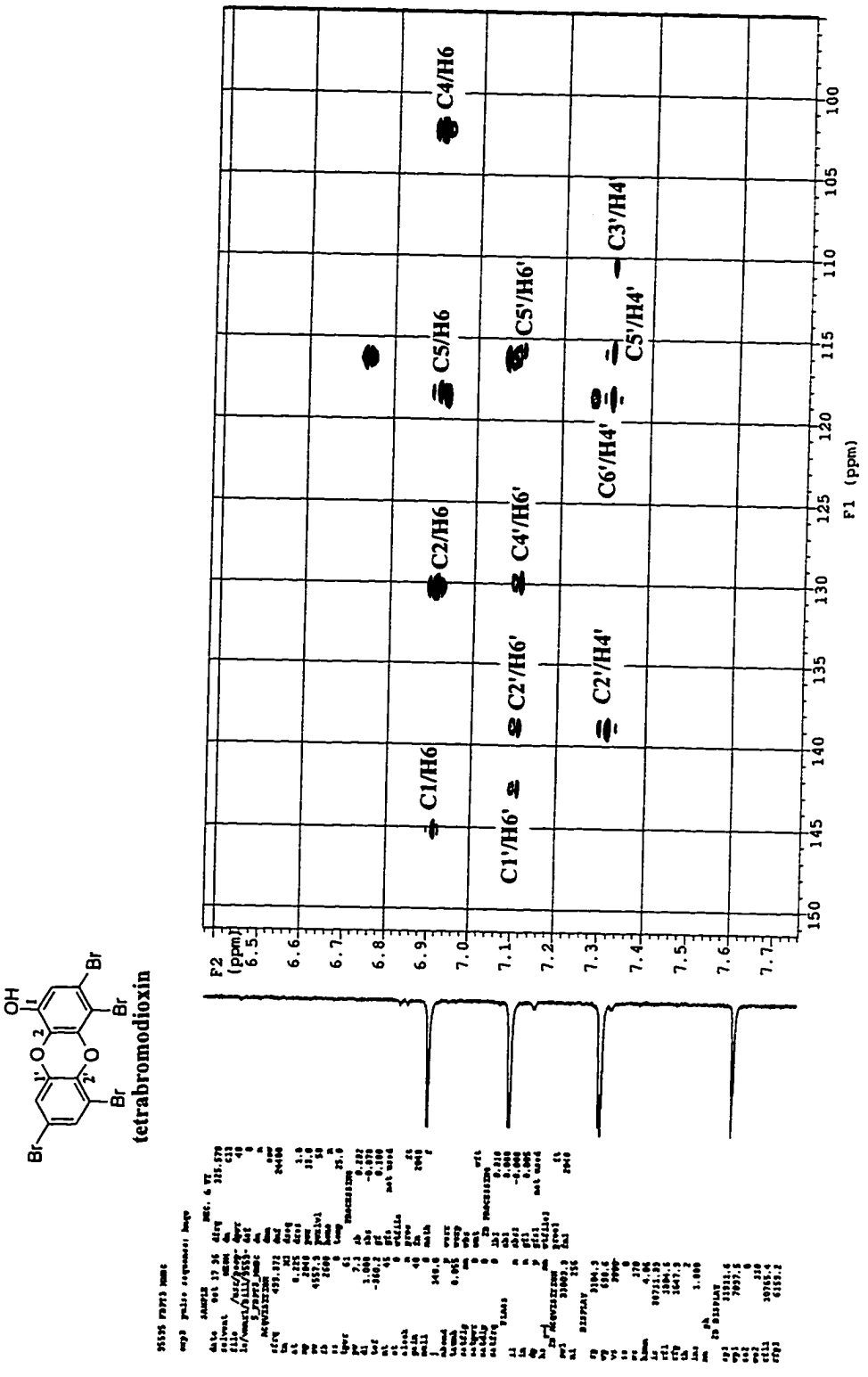


Figure 4.61. ^{1}H-NMR Spectra (CD_2Cl_2, 500 MHz).

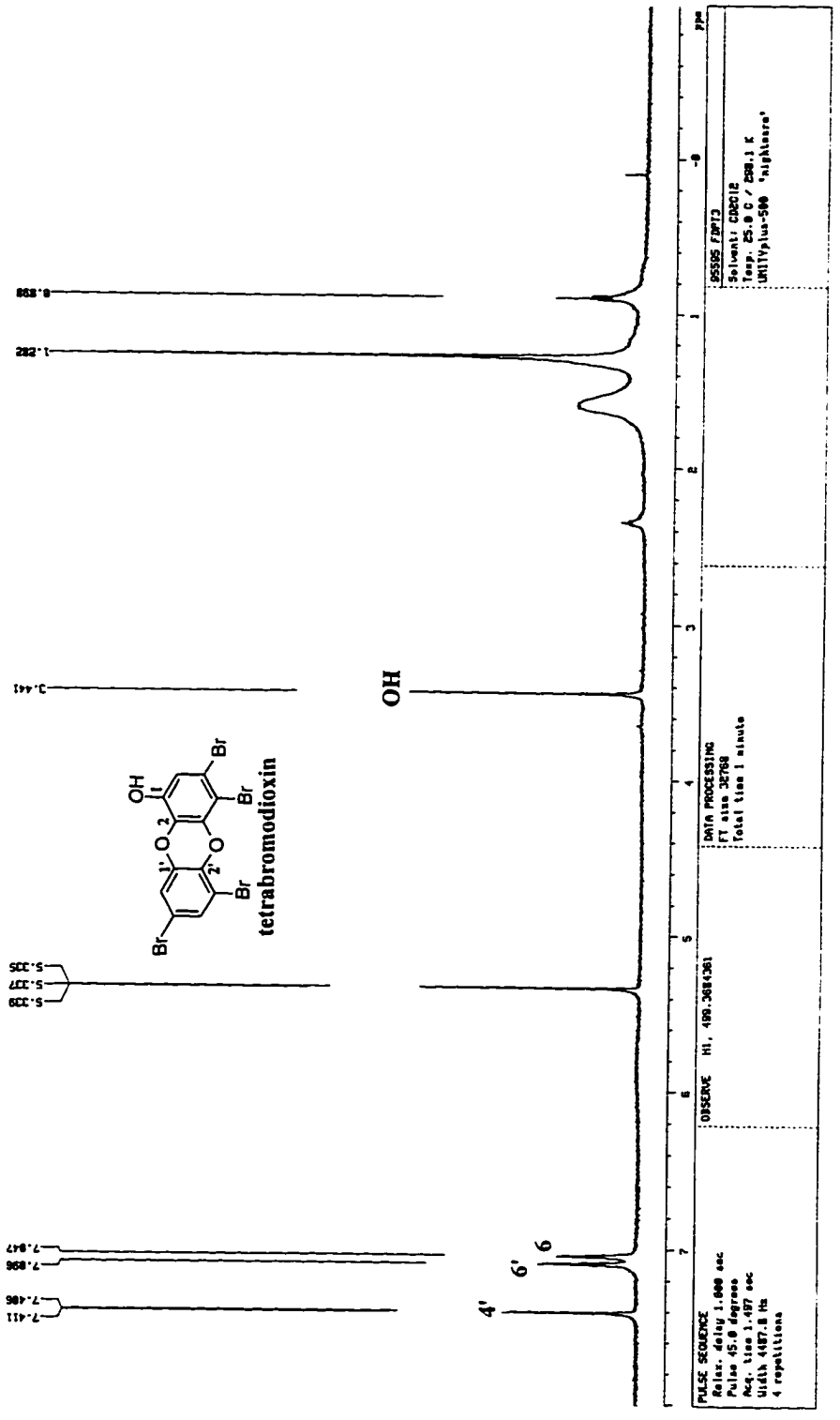


Figure 4.62. T_1 Analysis of Hydroxy Proton (CD_2Cl_2, 500 MHz).

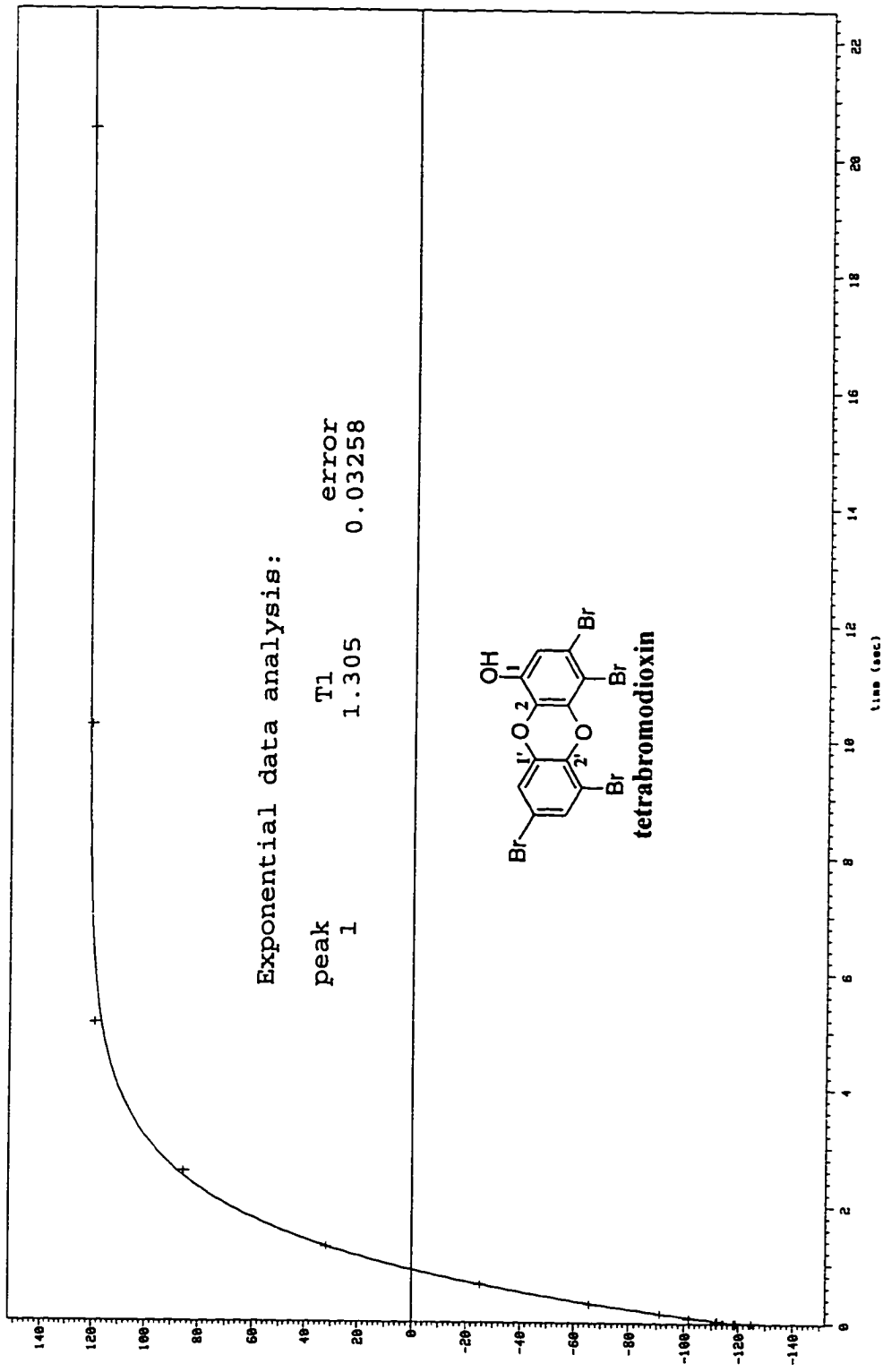


Figure 4.63. T_1 Analysis of H4' Aromatic Proton (CD_2Cl_2, 500 MHz).

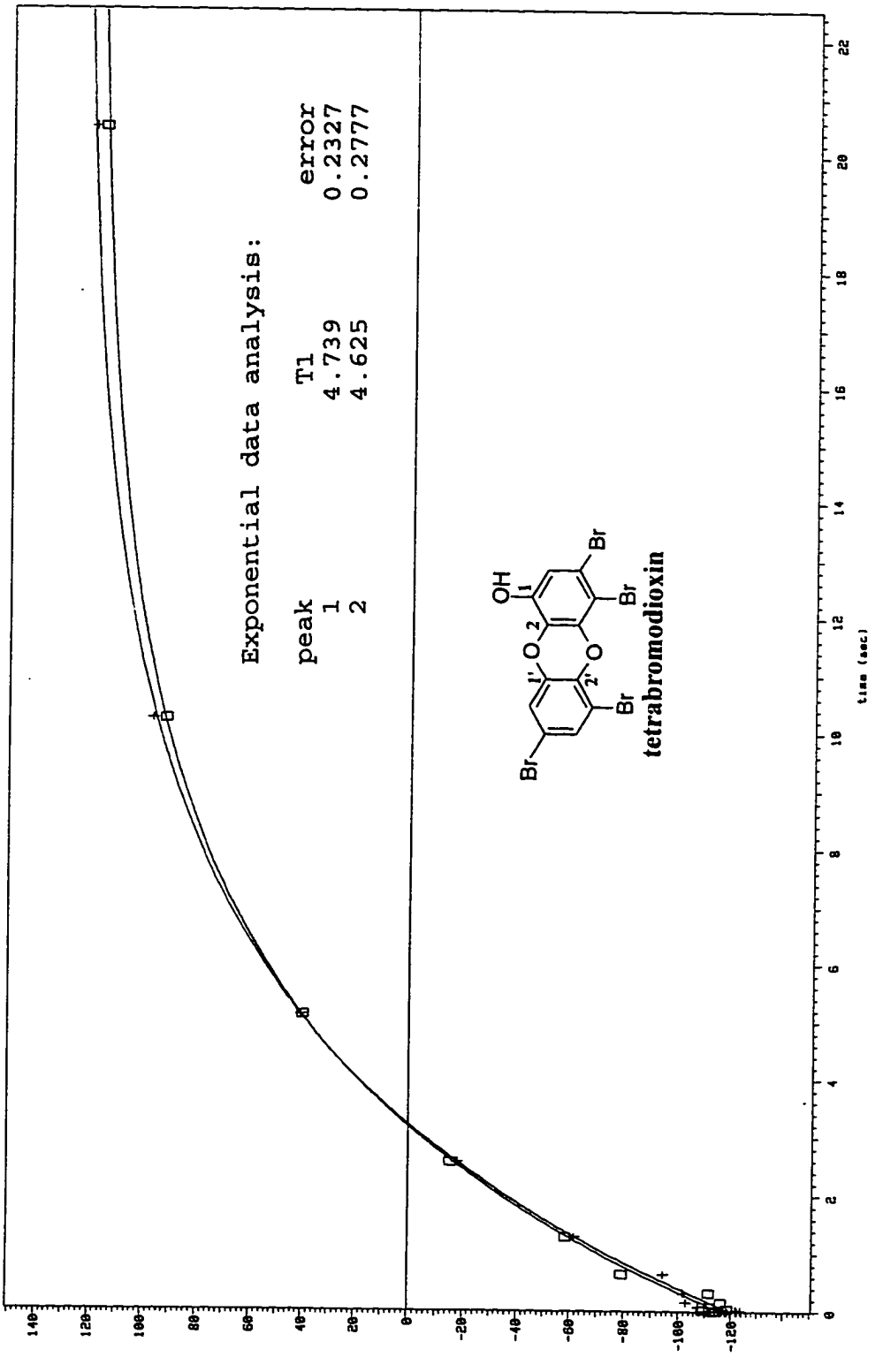


Figure 4.64. NOESY NMR Spectra (CD_2Cl_2, 500 MHz).

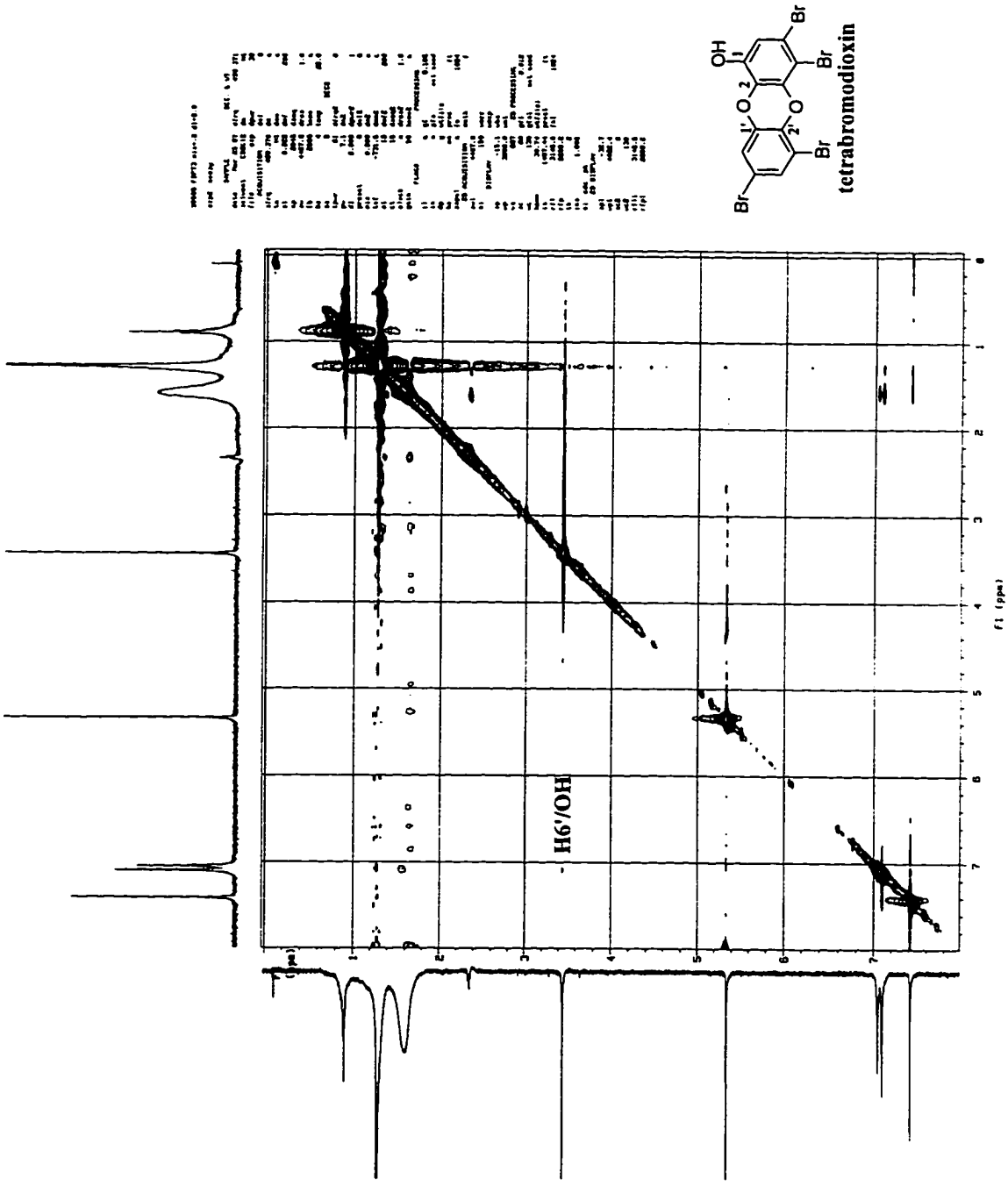
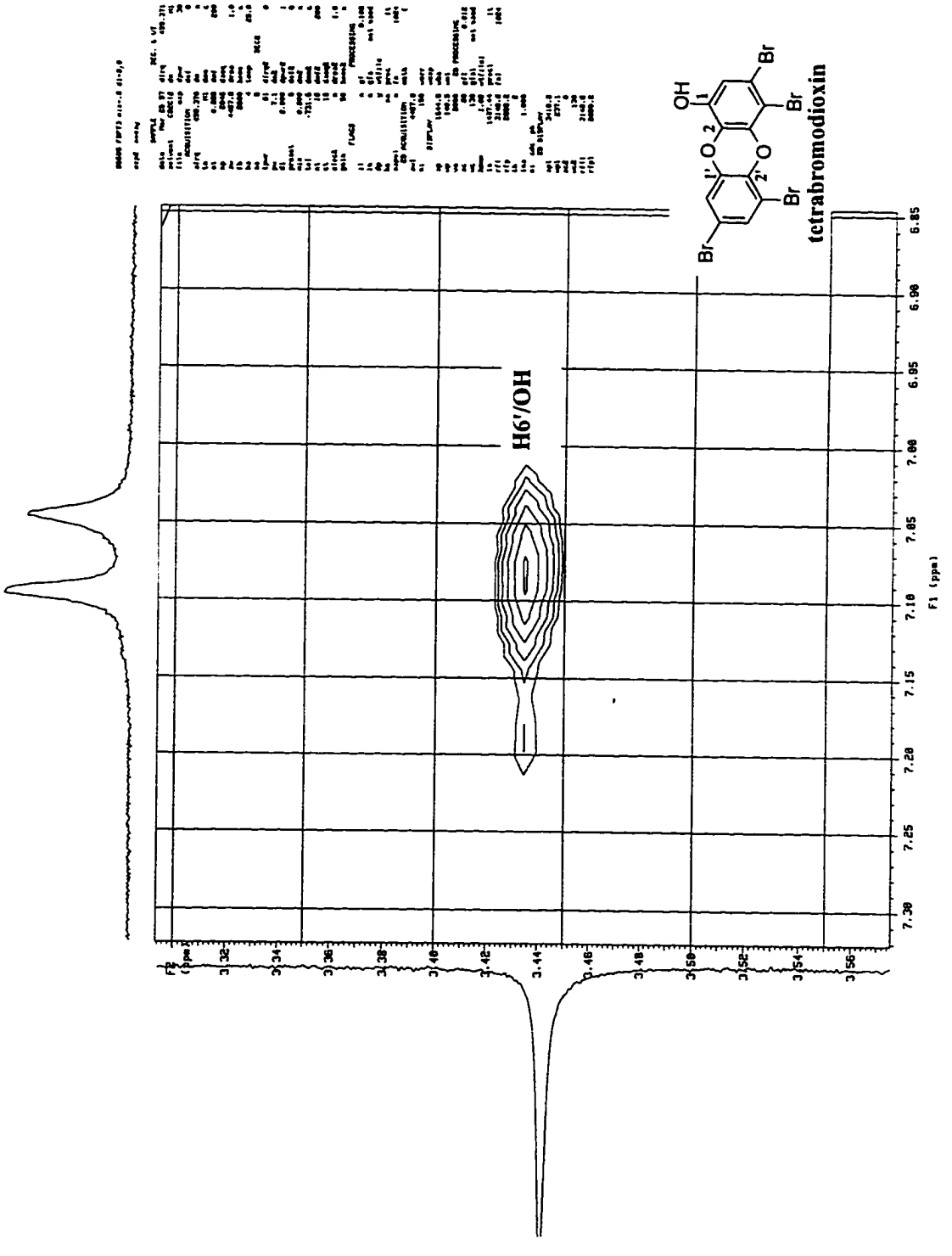


Figure 4.65. NOESY Expansion NMR Spectra (CD_2Cl_2, 500 MHz).



Supplementary Material-
tribromodioxin

Figure 4.66. ^{1}H-NMR Spectra (CD_3OD, 500 MHz).

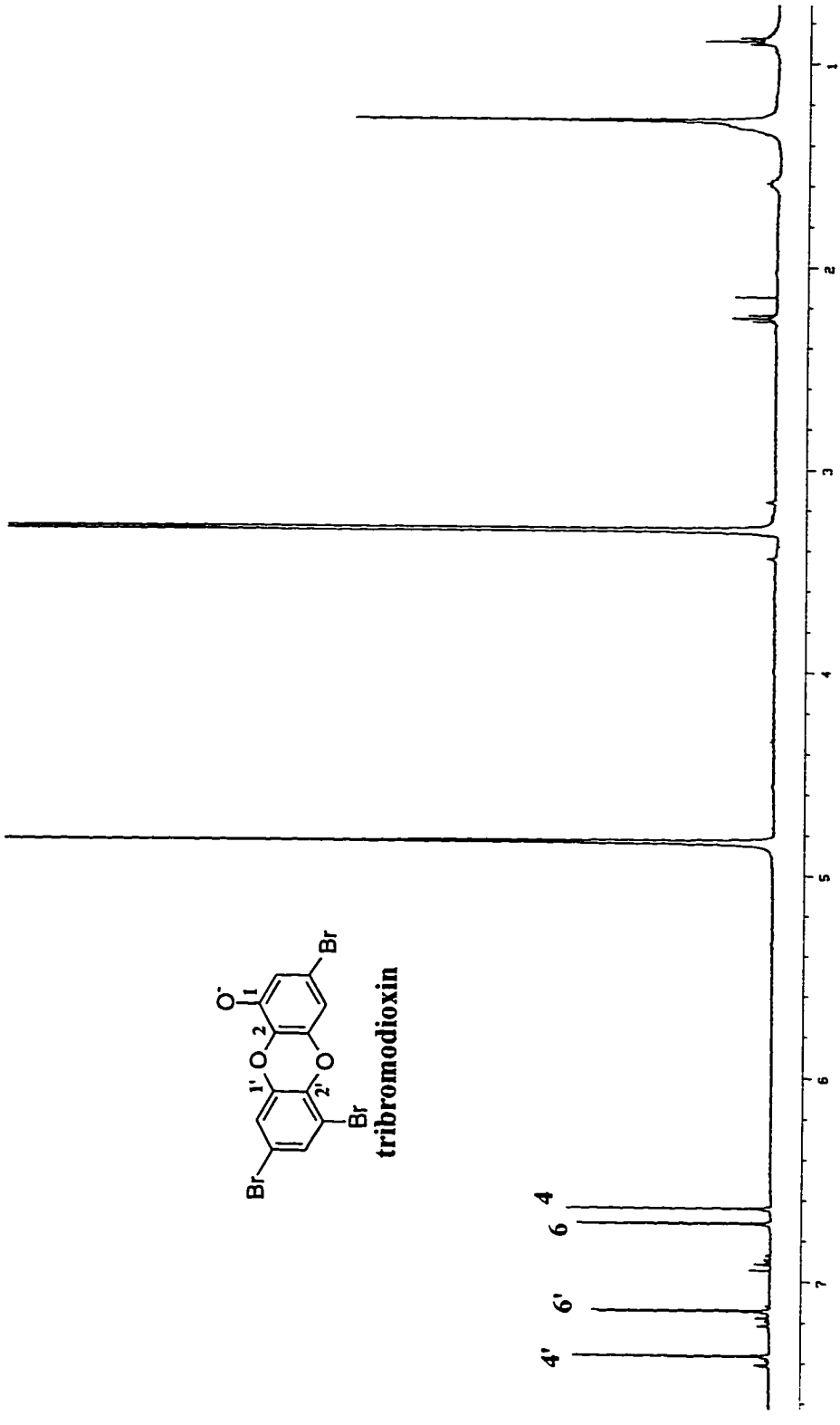


Figure 4.67. ^{13}C-NMR Spectra (CD_3OD, 125 MHz).

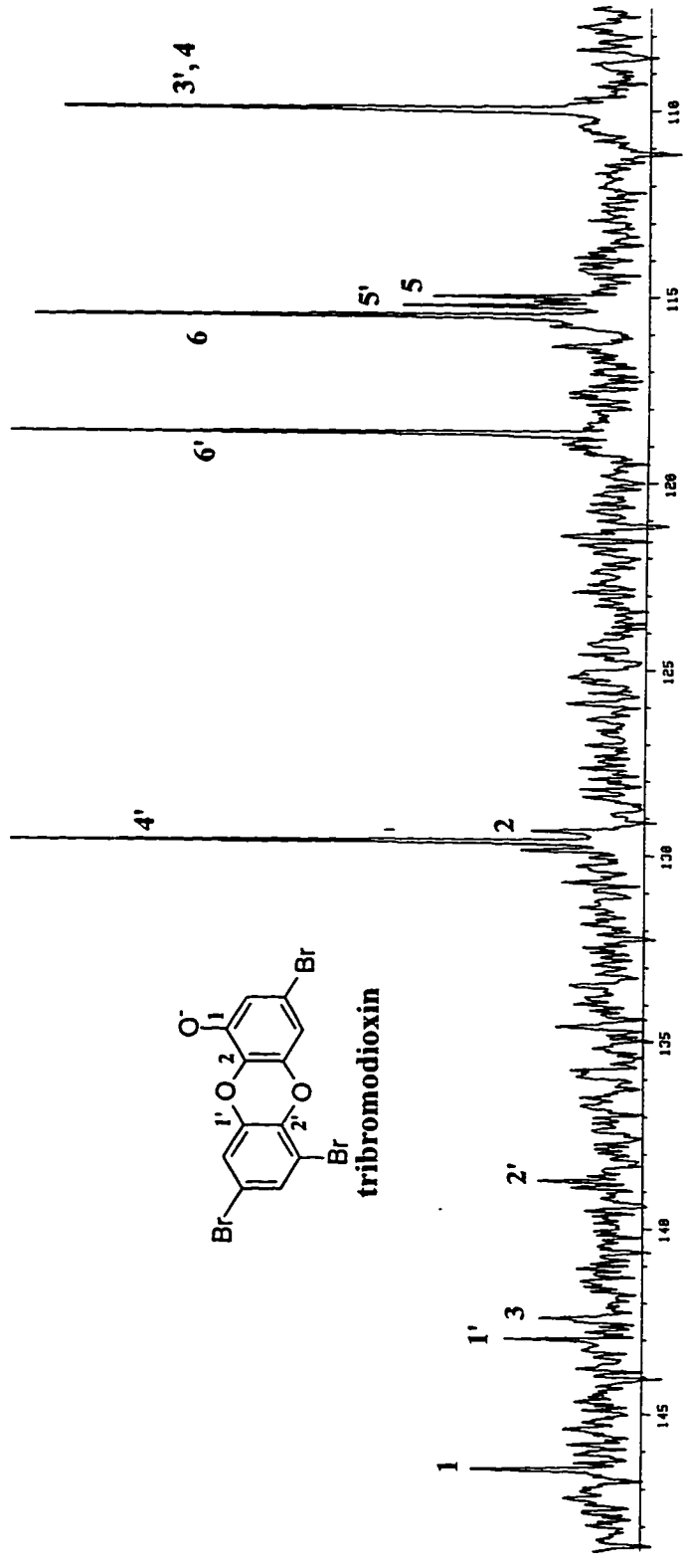


Figure 4.68. LREI Mass Spectra.

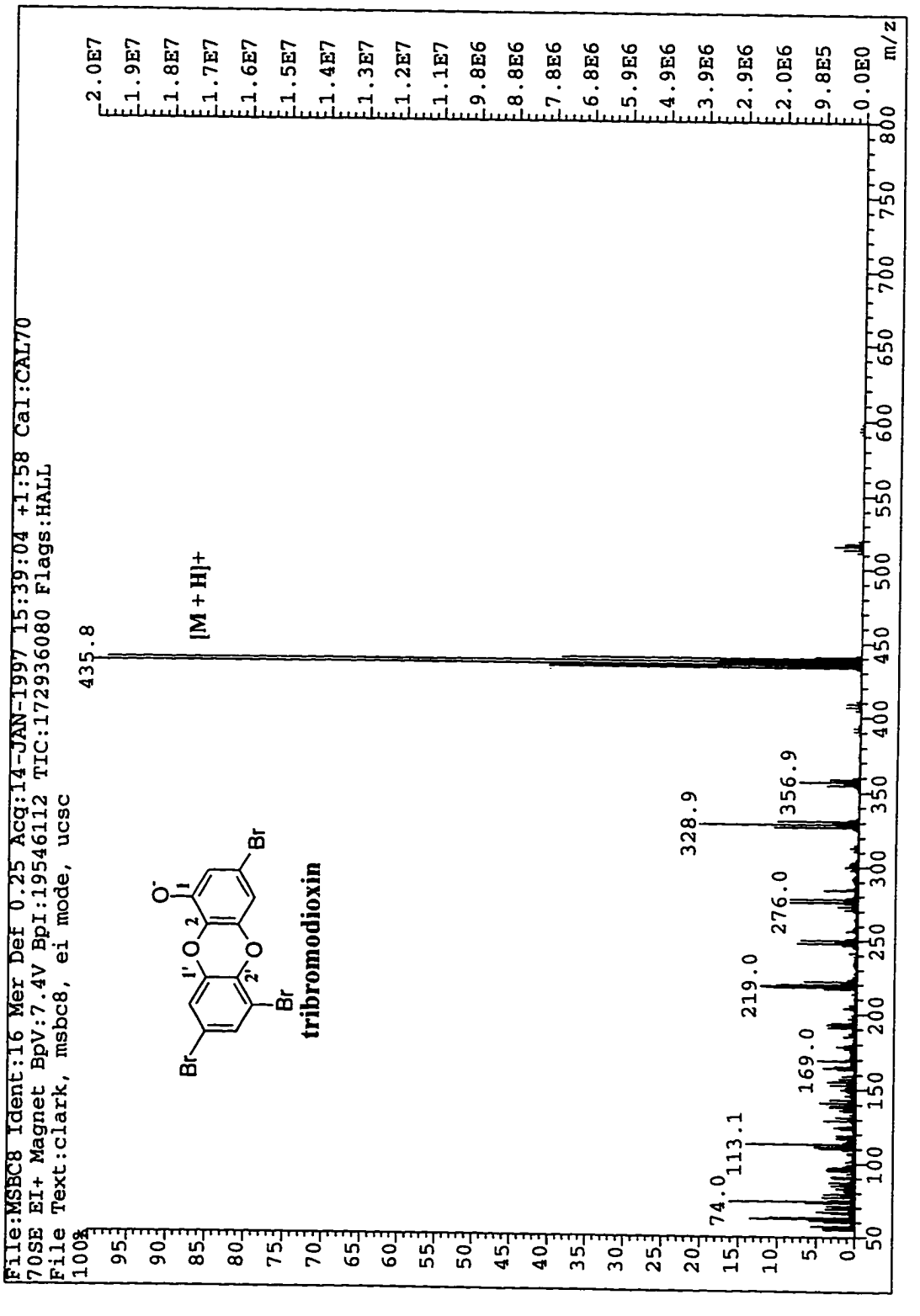


Figure 4.69. IR Spectra.

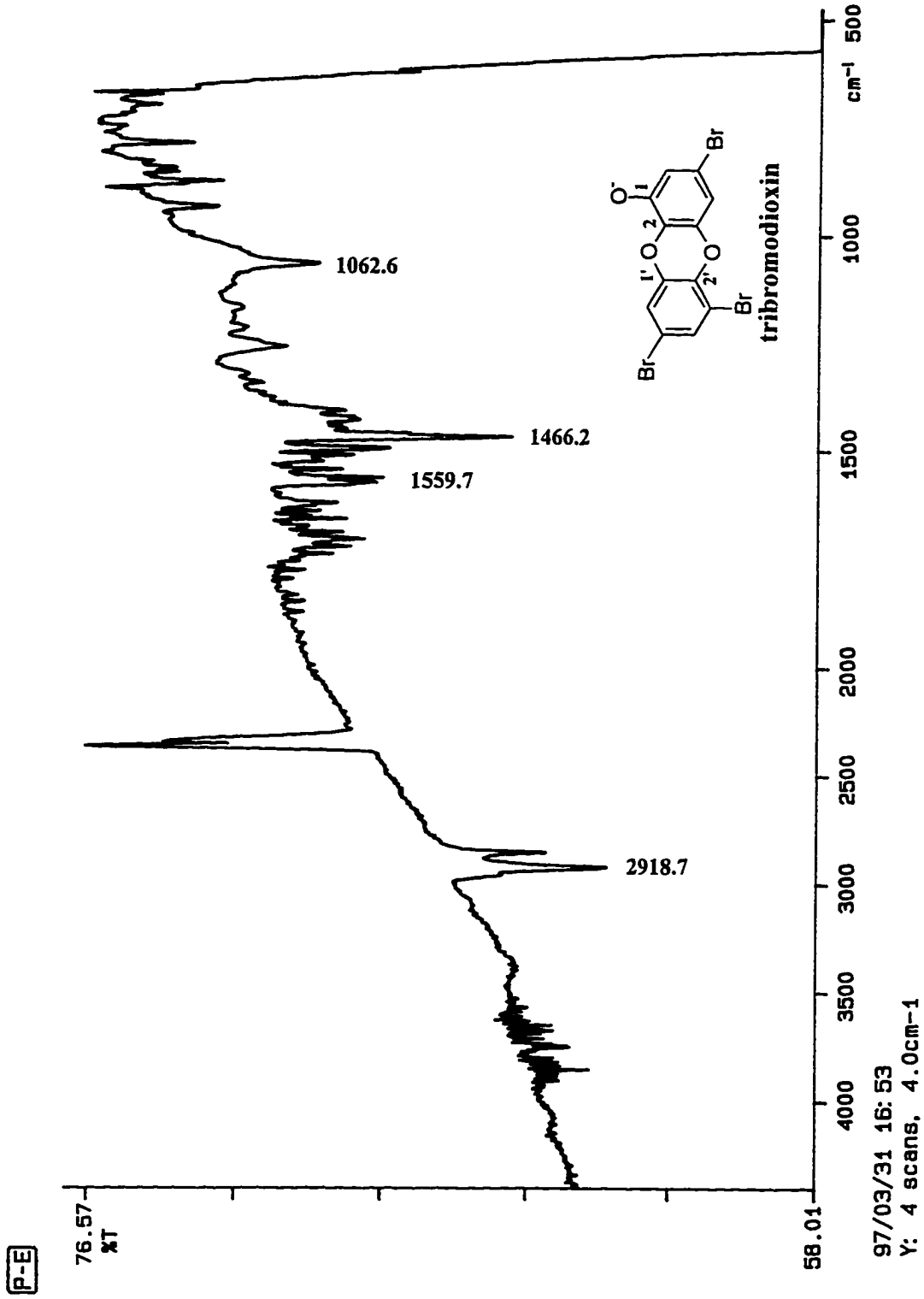


Figure 4.70. ^{1}H-NMR Spectra (CD_2Cl_2, 500 MHz).

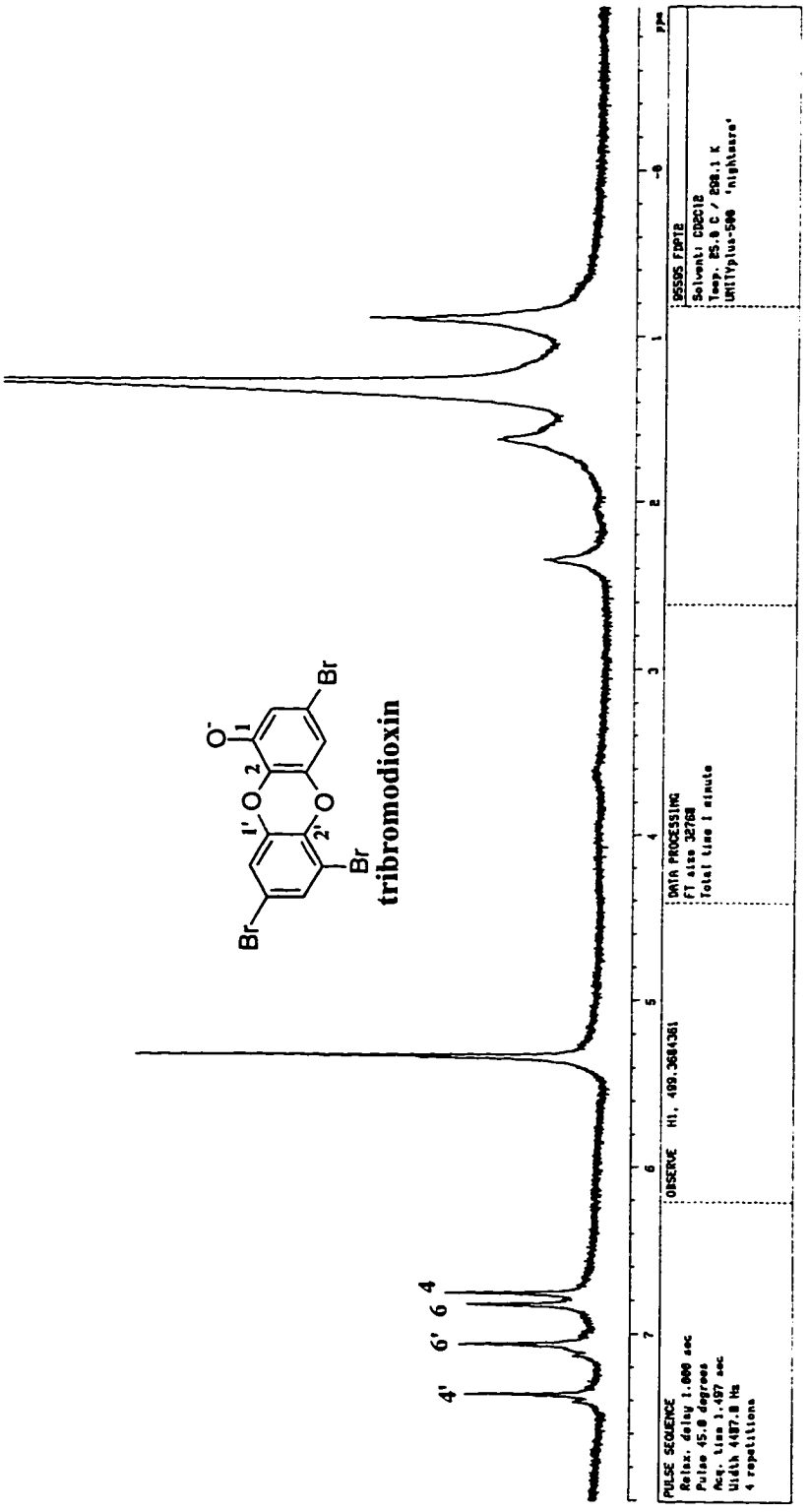


Figure 4.71. COSY NMR Spectra (CD_3OD, 500 MHz).

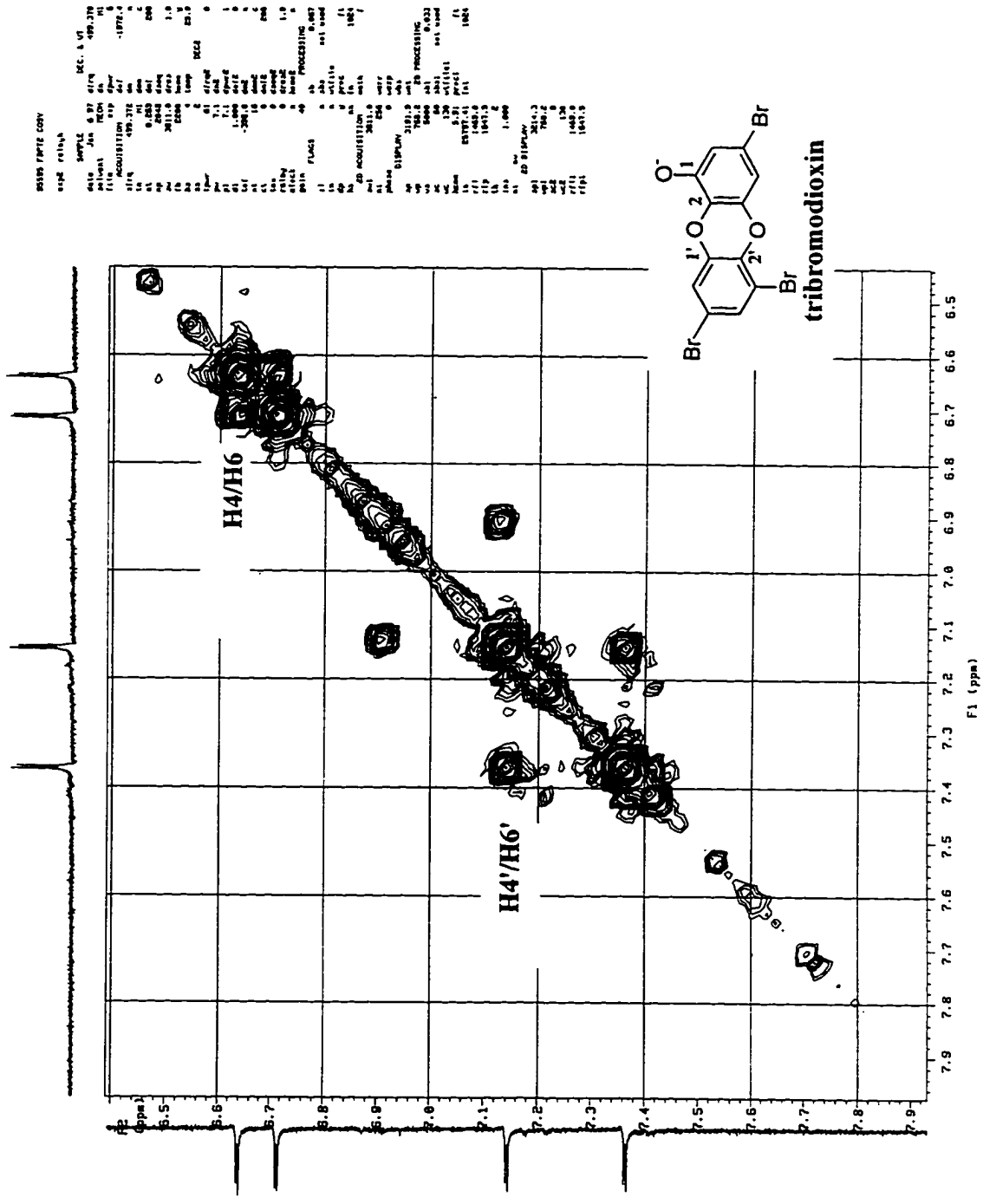


Figure 4.72. HMQC NMR Spectra (CD_3OD, 500 MHz).

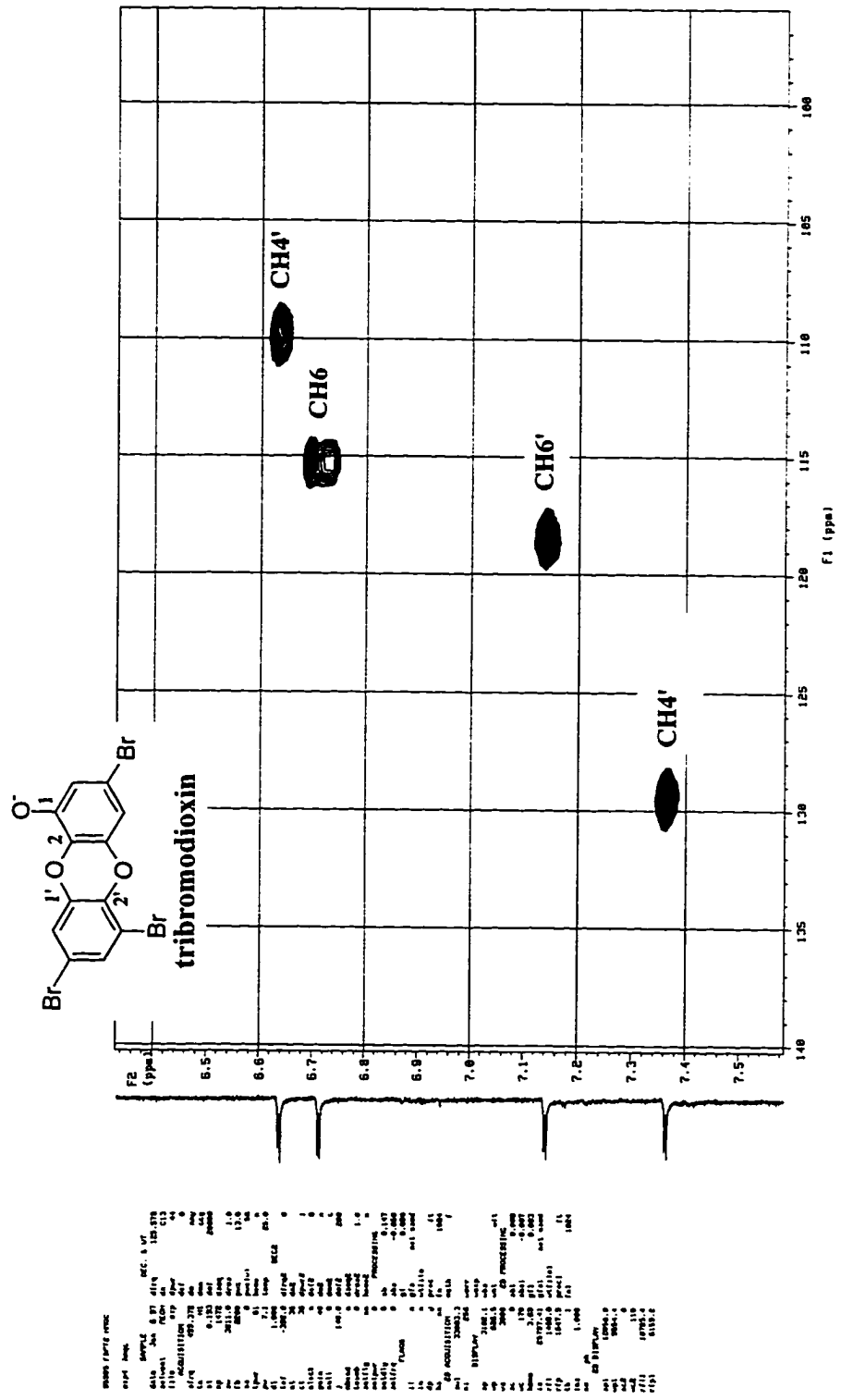
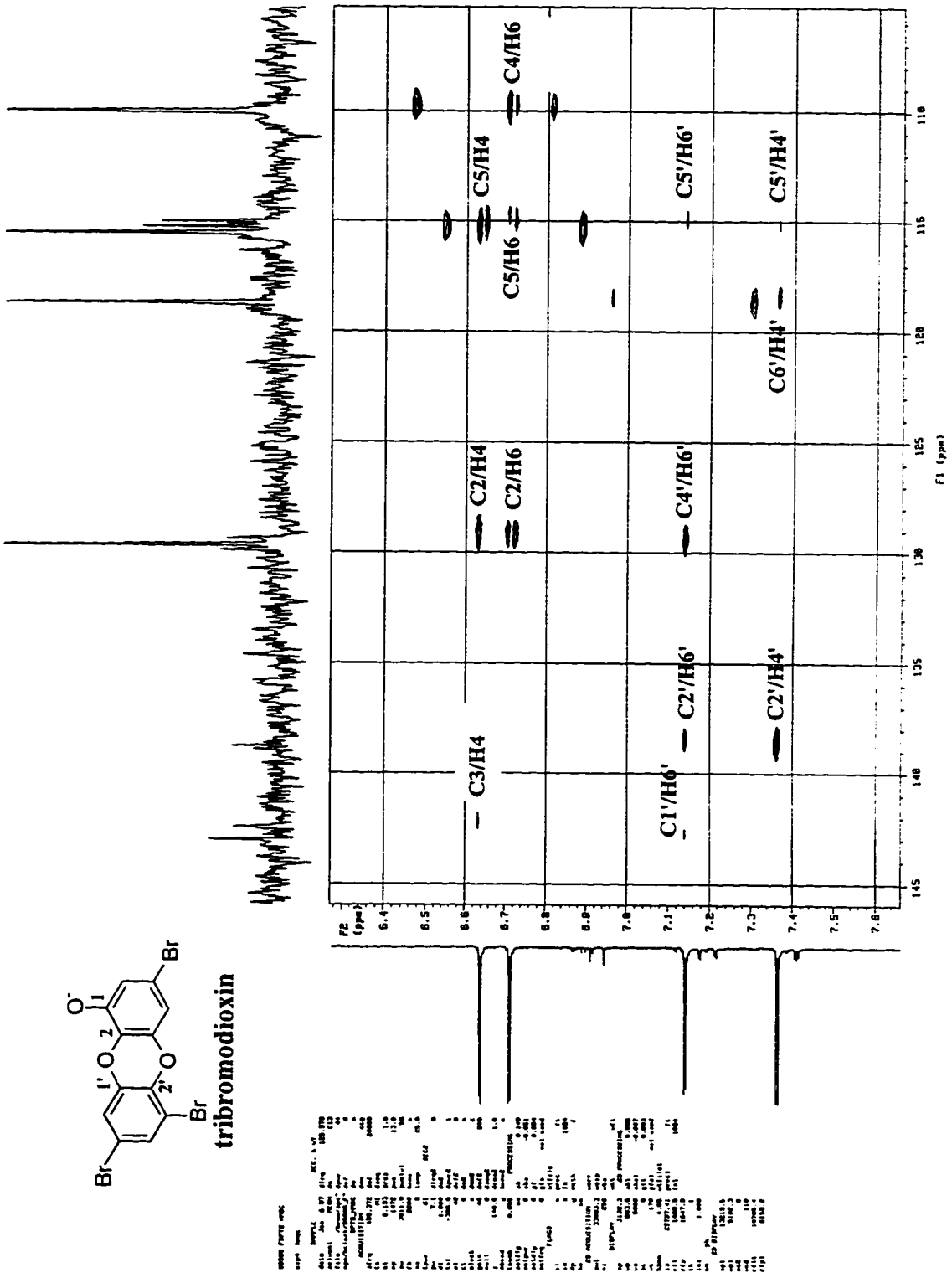


Figure 4.73. HMBC NMR Spectra (CD_3OD, 500 MHz).



Supplementary Material-
herbamide B

Figure 4.74. ^{1}H-NMR Spectra ($CDCl_3$, 300 MHz).

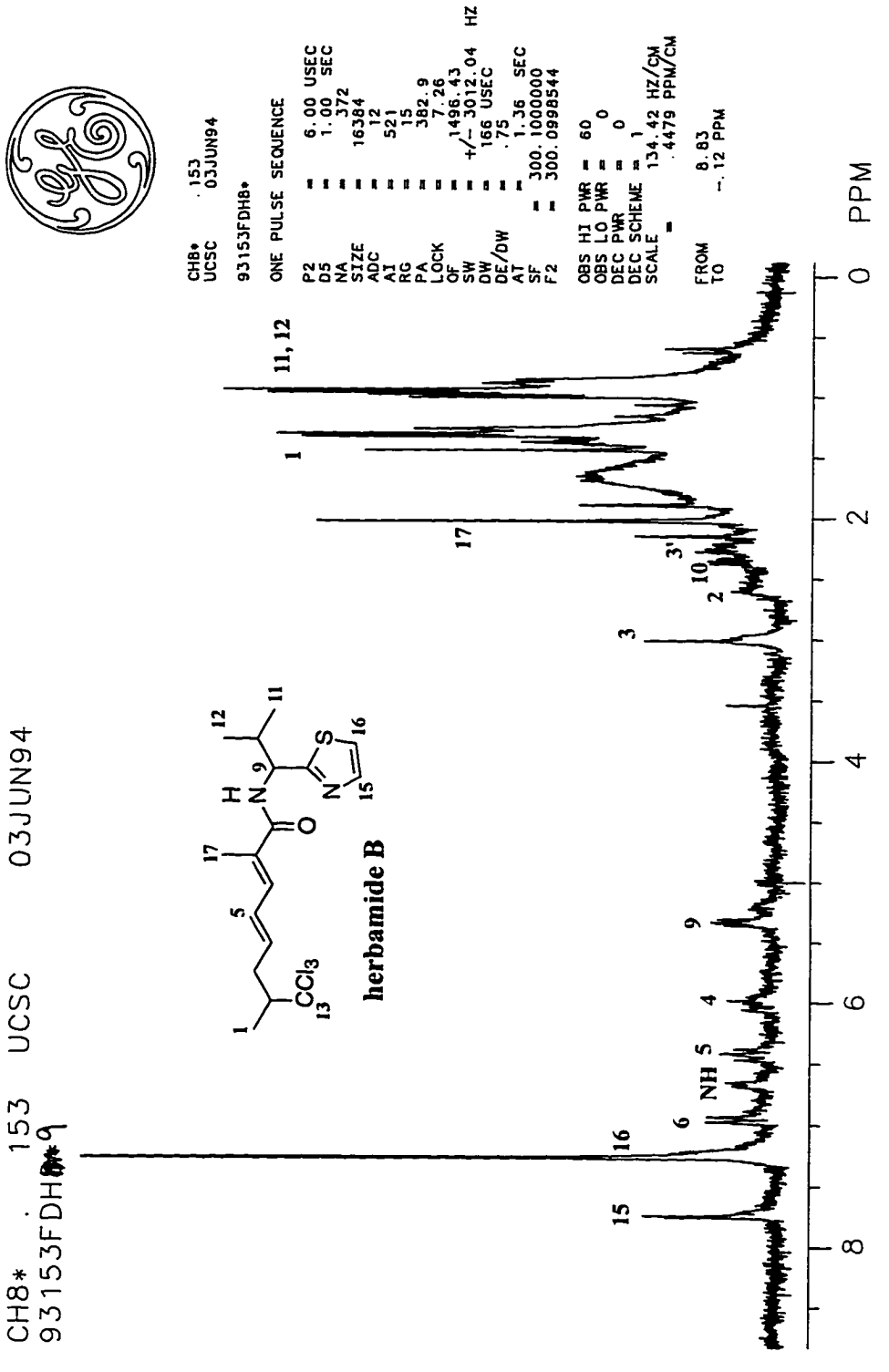


Figure 4.75. ^{13}C-NMR Spectra ($CDCl_3$, 75.5 MHz).

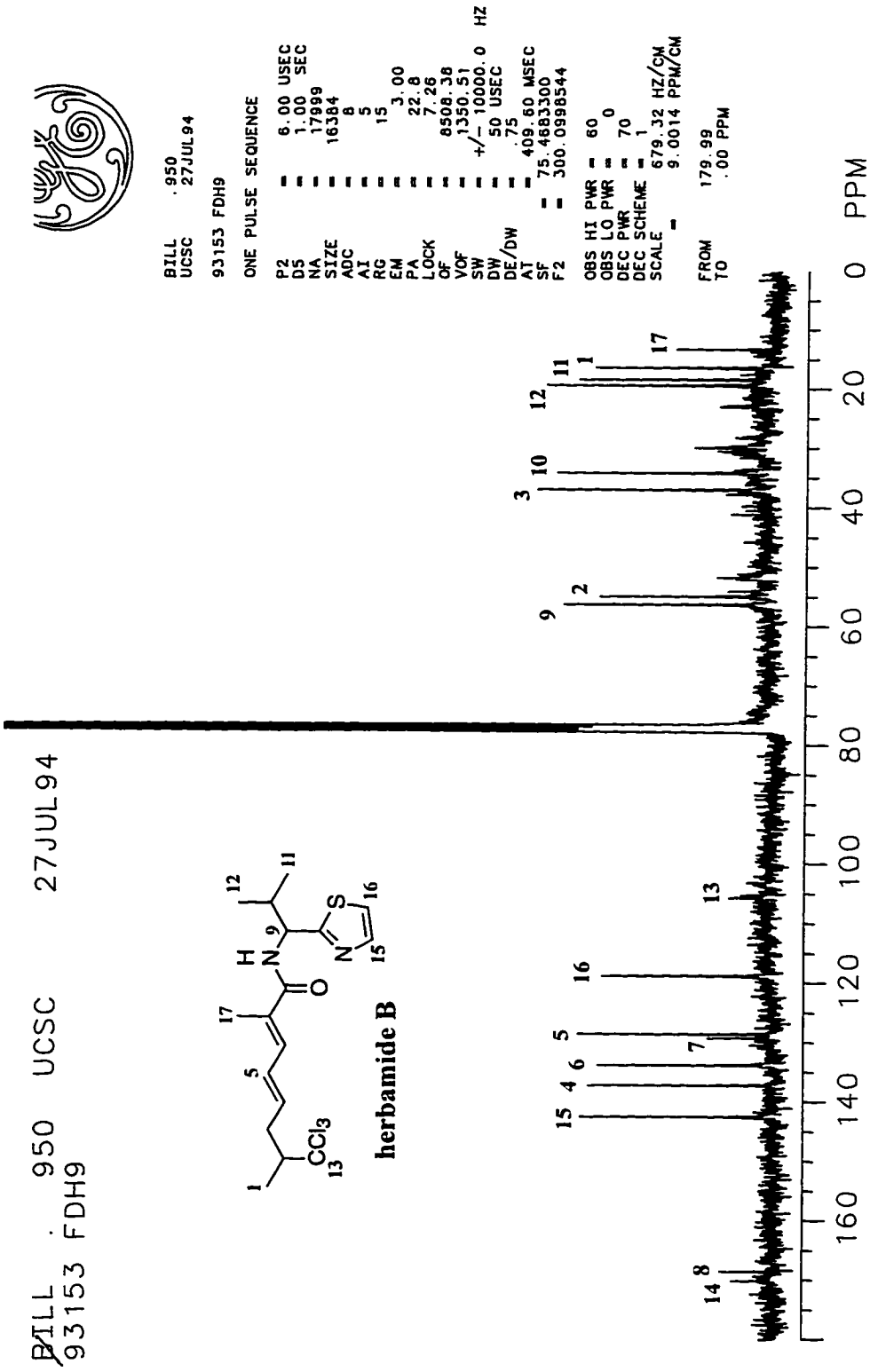


Figure 4.76. APT NMR Spectra ($CDCl_3$, 75.5 MHz).

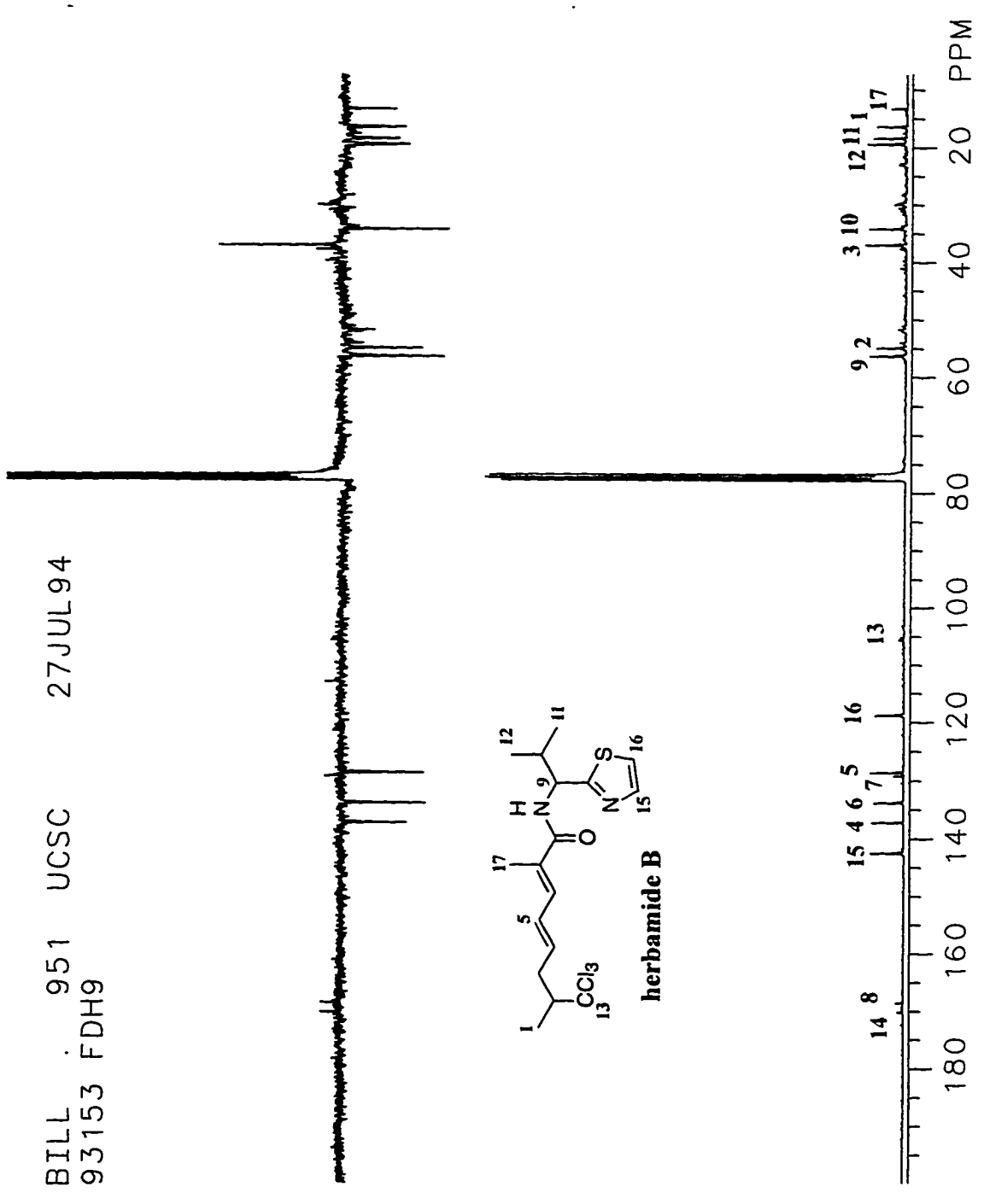


Figure 4.77. LRFAB Mass Spectra.

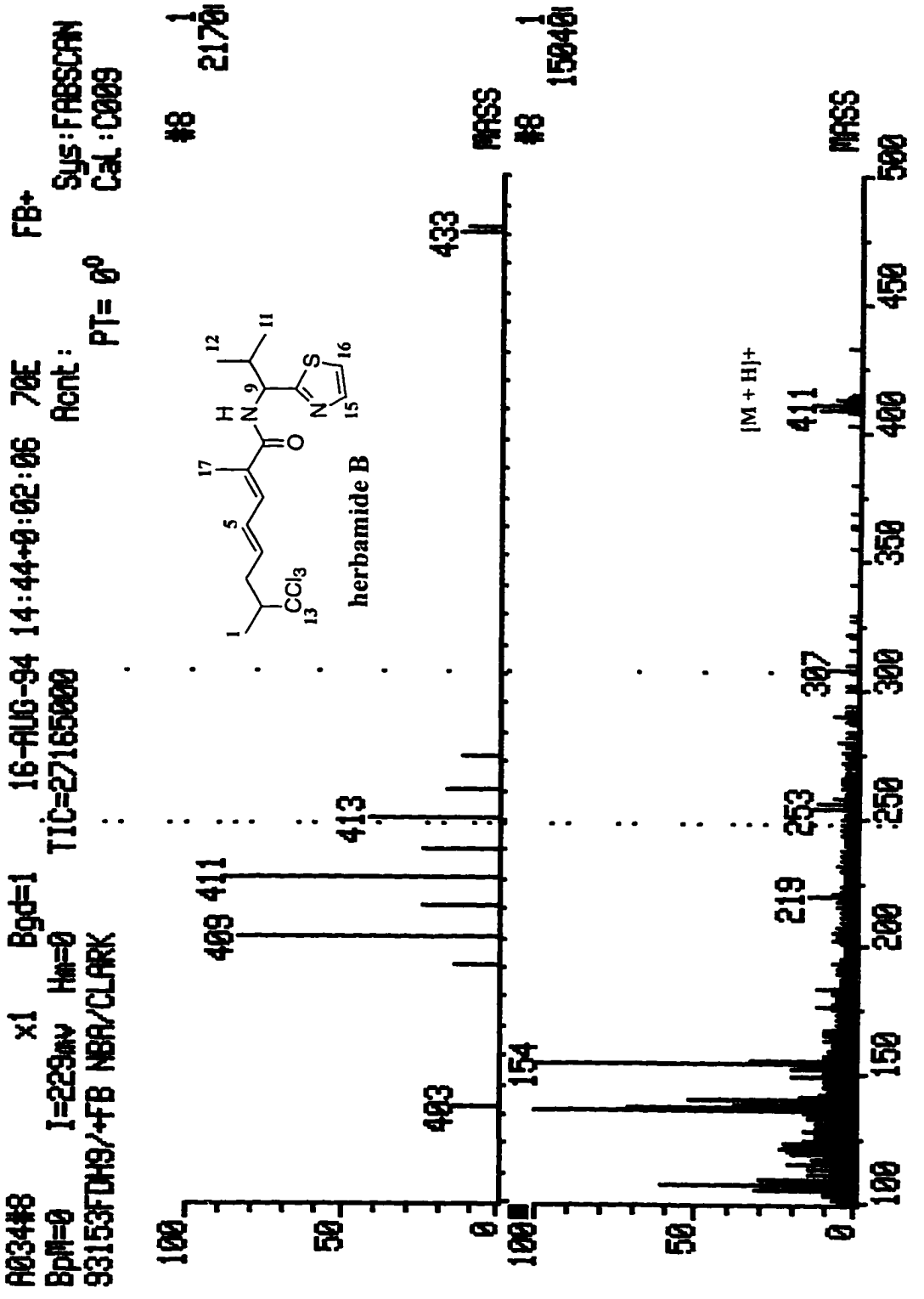
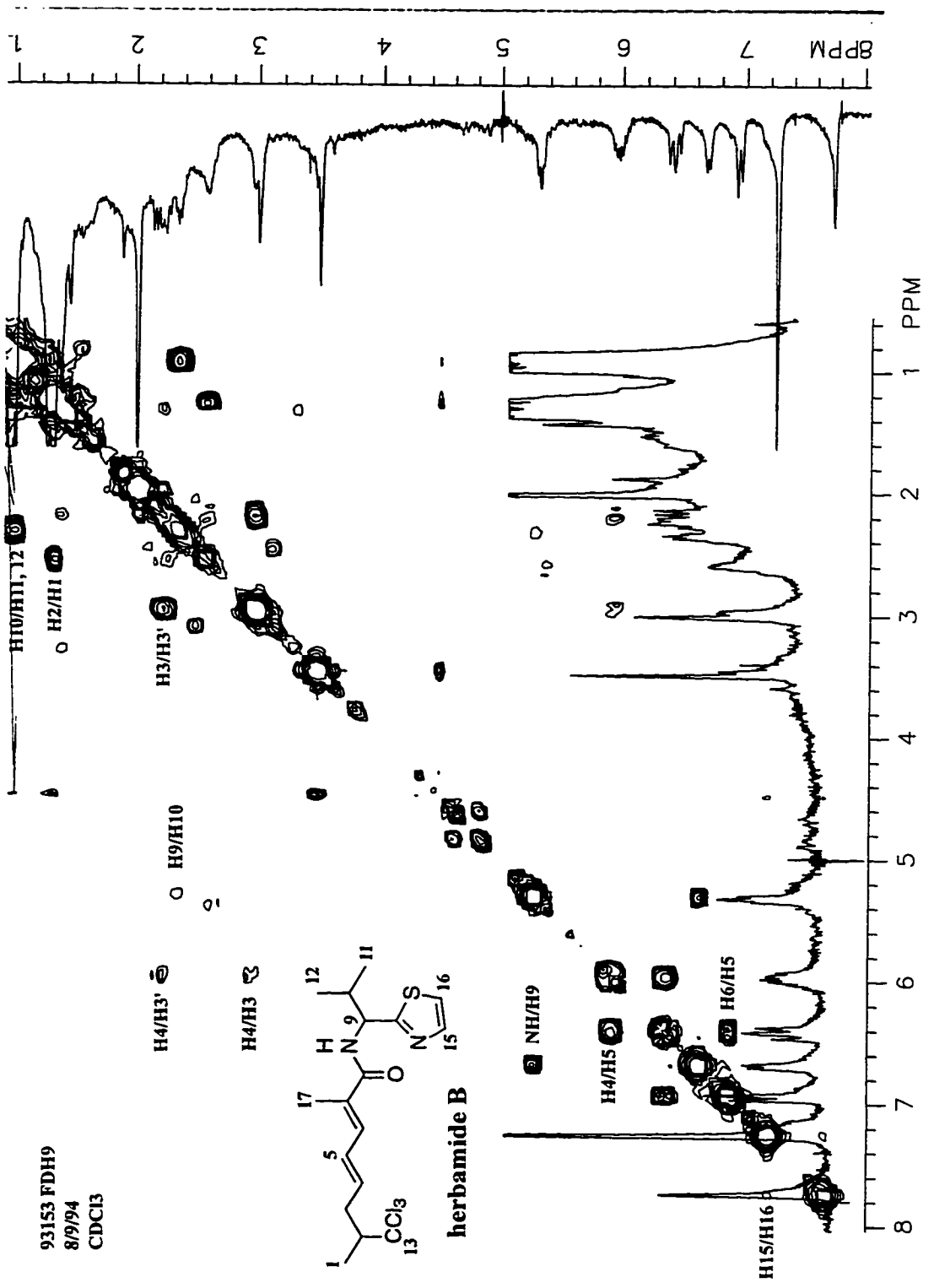


Figure 4.78. COSY NMR Spectra ($CDCl_3$, 300 MHz).



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Chapter 5-Species Differentiation in the Chemotaxonomic Analysis of Foliose Indo-Pacific Sponges of the Genus *Dysidea* (Dysideidae: Dictyoceratida)

Marine Sponges-Papua New Guinea Photo Courtesy of Jay Burreson, Ph.D.

Abstract

A comparison of the morphological features and chemical profiles of various foliose Indo-Pacific sponges of the *Dysidea* species was recently investigated. Forty-three specimens were collected in three distinct geographic locales; Northern and Southern Sulawesi, Indonesia and Milne Bay, Papua New Guinea. To assist in the characterization of the species a polybrominated diphenyl ether or polychlorinated peptide profile was identified by using electrospray ionization mass and ^{1}H-NMR spectra of the dichloromethane fractions of the specimens. Taxonomic analysis revealed five distinct *Dysidea herbacea*-like morphological types; all carrying endosymbiotic filamentous cyanobacteria (*Oscillatoria*-like) and each showing direct correlation with their chemical profiles. One morphotype exhibited a polybrominated diphenyl ether chemical profile while two others exhibited a polychlorinated peptide chemical profile. The remaining two morphotypes contained both chemical profiles which was dependent on geographic locale. The degree of bromination or chlorination of the major component directly correlated with the morphological grouping. A possible biogenesis of the brominated diphenyl ethers and chlorinated peptides is discussed.

Background

This work involves a chemotaxonomic analysis of an extensive collection of *Dysidea* sponges the UCSC marine natural products research group collected during three expeditions over a two year period (March 1994-May of 1996) from Papua New Guinea and Indonesia. The work was conducted jointly with Dr. M. Cristina Diaz, Institute of Marine Sciences, UCSC. She provided the taxonomic analyses of all the specimens and identification of the cyanobacteria present in the sponges. The results add enlightening new information to the already well-studied sponge by clarifying *Dysidea*'s difficult taxonomic nature. The results identify new species of *Dysidea* (order Dictyoceratidea, family Dysididae) and establish the fact that all the previously reported chemistry associated with *Dysidea herbacea* is in fact from more than one species. The quantitative approach of using ^{1}H-NMR coupled with electrospray ionization mass spectrometry in a chemotaxonomic analysis is the first of its kind and paves the way for future studies in this area. A manuscript derived from the work outlined in Chapter 4 has been submitted for publication in *Biochemical Systematics and Ecology*.

Introduction

The Indo-Pacific sponge *Dysidea herbacea* (Dysideidae; Dictyoceratida) associated with the filamentous cyanobacterium *Oscillatoria spongeliae* has been intensely studied for nearly 20 years. Yet, it still continues to be a rich source of chemical and taxonomic information. Early on in the study of *Dysidea herbacea* three very different types of secondary metabolites were reported; polychlorinated peptides[1], polybrominated diphenyl ethers[2], and furanosesquiterpenes[3] (Figure 5.1). It was known at that time that the sponge was heavily ladened with the endosymbiont *Oscillatoria spongeliae*, up to 40 % of sponge volume[4], and considered to be the possible producer

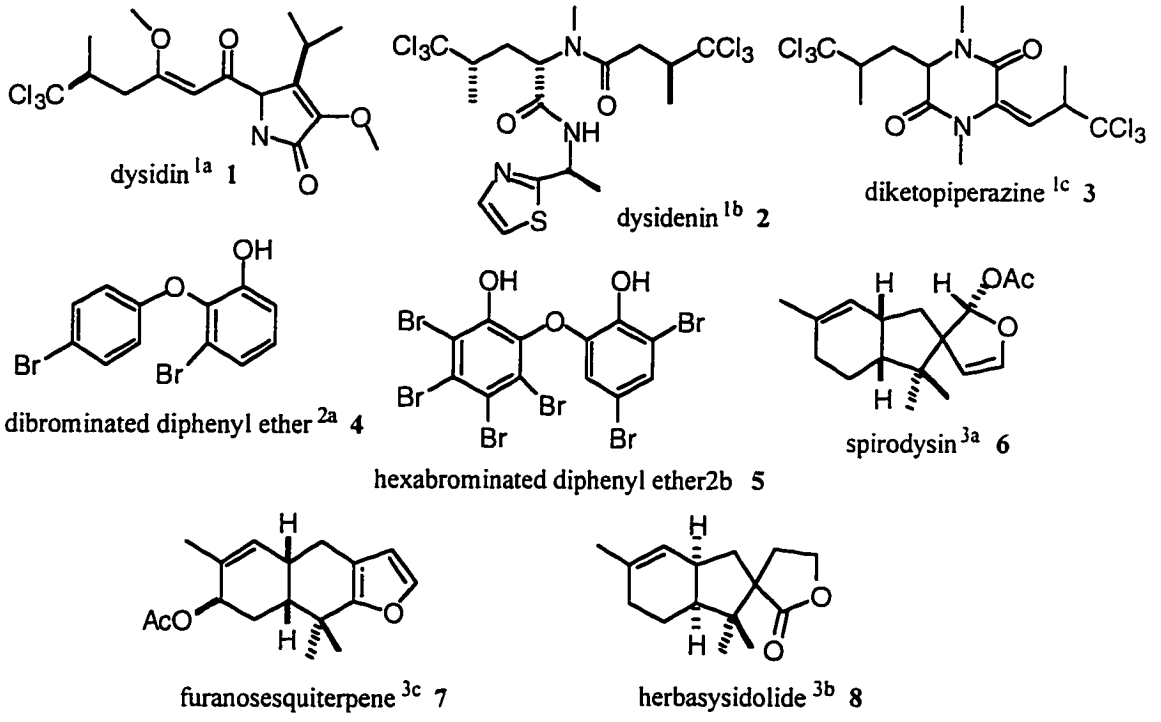


Figure 5.1. Representative Compounds of the Three Types of Metabolites Isolated from the Sponge *Dysidea herbacea*.

of these unique and unusual halogenated metabolites. This observation was proved only recently when the sponge cells were separated by flow cytometry from the symbiont *Oscillatoria* where the halogenated metabolites were concentrated.[5] The sponge cells contained furanosesquiterpenes. More recent reports show that *D. herbacea* is still a viable source of new compounds[6] (Figure 5.2) and of continued interest due to its sponge/symbiont association[7].

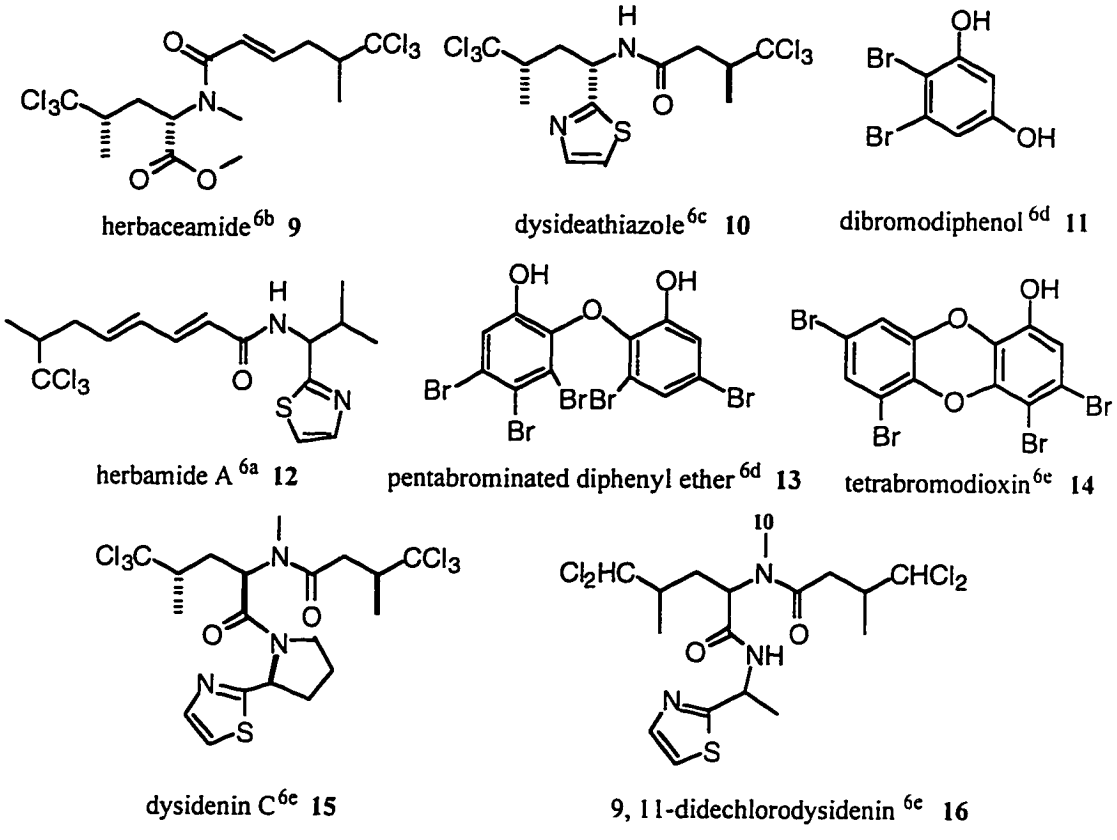


Figure 5.2. Halogenated Compounds Recently Isolated from *Dysidea herbacea*.

The sponge *D. herbacea* has been a part of previous chemotaxonomic study. One involved the analysis of the sterol composition of 55 species of Demospongiae.[8] A review looked at the presence of terpenes and brominated metabolites of the Porifera and

specifically the sesquiterpenes, brominated diphenyl ethers, and the chlorinated peptides of this species[9]. In the review, Bergquist and Wells had concluded that the presence of microorganisms and cyanobacteria in sponges would make any chemotaxonomic conclusions difficult and that the origins of the metabolites isolated from the sponge should be known. Despite the recent findings that link the production of distinct secondary metabolites (brominated diphenyl ethers vs. polychlorinated peptides) to the cyanobacterial symbiont found in *Dysidea herbacea*[5], there are numerous references of *D. herbacea* collections in which the two distinct chemotypes never overlap.[10] Interestingly, these reports, as well as our own study, continue to support the observation that neither the polychlorinated peptides or the polybrominated diphenyl ethers are isolated from the same sponge host indicating the possibility, at minimum, of two chemotypes of cyanobacteria which may be species-specific within the sponge.[5b] An uncertain issue left considering in all these studies is the conspecificity of all the specimens chemically characterized which have all been assigned as *D. herbacea*.

It is widely recognized that species of *Dysidea* are rather difficult to distinguish due to their identical or very similar internal structure and the rather plastic external morphological features of certain species.[12] The genus *Dysidea* Johnston, 1842 was erected to include "Dysideidae in which all fibers are filled with detritus".[13] *Dysidea herbacea* was originally described by Keller[14] as *Spongelia herbacea* and was redescribed by Berquist[12b] to include: lamello-digitate sponges with conules 0.4 mm high with a tendency to be aligned in vertical rows, a skeleton of an open network of fibers between 50-153 μm in diameter profusely cored by sand and foreign material and a well defined cortex of detritus. *D. chlorea* De laubenfels[15] closely associated to *D. herbacea*, presents a predominantly digitate habit with a finer fiber reticulation than *D. herbacea*.[12b] These

characters, however, have been found highly variable among certain *Dysidea* species such as *D. fragilis*.[12a] Thus it is possible that *D. herbacea* and *D. chlorea* are conspecific.

In order to further understand the nature of the morphological and chemical variability reported for *Dysidea herbacea*, as well as the issue of conspecificity, the distinct but non-overlapping chemistry, and possible morphological heterogeneity, we conducted simultaneous chemical and morphological characterizations of forty-three specimens collected in the Indo-Pacific. All studied specimens ranged from fan-shaped to digitate sponges and conformed very closely to the descriptions of *D. herbacea* Keller[14] and Berquist[12b].

Materials and Methods

Samples were collected by the Crews research group on three separate field expeditions. The first in the Fall of 1994 to Northern Sulawesi, Indonesia, the second in the Spring of 1995 to Milne Bay, Papua New Guinea and the third in the Fall of 1995 to Southern Sulawesi, Indonesia. Separate colonies were collected by SCUBA and carefully kept separate. Underwater photographs and an external morphological characterization (growth form, surface, oscule shape, color, exhudate, consistency, size and distribution) were carried out in the field.

To study skeletal composition and organization, a subsample of each specimen collected was fixed in 10% formaldehyde for 1-3 days, and then transferred to ethanol (70%). In the laboratory thick sections were made tangential and perpendicularly to the sponge surface and permanently mounted on a slide using Permount media. The sections were studied with a light microscope under 100-400x magnification.

Museum collections (National Museum of Natural History, Washington D.C) of *Dysidea herbacea*: NMNH # 23127 *Phyllospongia complex* De Laubenfels[15], Palau, reassigned to *Dysidea* by Bergquist,[12b]; NMNH # 24005 and *Dysidea chlorea*: NMNH #23705, Palau; NMNH #23999, Palau, obtained by Dr. M. C. Diaz were studied.

To detect the presence and type of photosynthetic symbiont a second smaller sponge subsample (<0.5 cm^3), including external and internal tissues, was cut into smaller pieces (0.1 cm^3), and fixed in 1 mL of Karnovsky solution (0.73% formaldehyde and 0.91% glutaraldehyde in 0.1M cacodylate buffer made up with filtered sea water). These samples were kept refrigerated at 4°C and in the dark. A small portion of this tissue sample was later sectioned and observed with a fluorescence microscope (Leitz Diaplan, fluorescent incident light excitation) using a blue filter (I2/3). Under these conditions

cyanobacterial pigments emit a bright yellow to orange light. Using this method at magnifications of 100-400X the presence and overall morphology of the cyanobacterial symbiont can be recognized. By these means cyanobacteria, *Oscillatoria spongeliae*-like, filaments with individual cells that are wider than they are long[16] were detected associated to the internal tissues of the samples studied.

Sponge samples for chemical analysis were preserved in the field by soaking the specimen in (50:50; ethanol:sea water) for 24 hours. The pour-off was discarded and the sponge specimens transported to the UCSC labs. Upon arrival at UCSC the sponges were stored at 10°C in methanol. The methanol extract was processed according to our standard procedure (see Appendix A).[17] The dichloromethane solvent partition fractions of the specimens were analyzed by electrospray ionization mass and ^{1}H-NMR spectroscopy. Proton NMR spectra were obtained in $CDCl_3$ or CD_3OD on a Varian 500 spectrometer operating at 500 MHz. Electrospray ionization mass spectra (ESIMS) were obtained on a VG Quattro in both positive (ESI^+) and negative (ESI^-) mode. Chemical profiles were developed based on these data and the specimens grouped according to their profiles.

Results

The specimens were all identified in the field as being *Dysidea herbacea*-like based on external morphological characteristics (growth form, surface, oscule shape and distribution) particular to the sponge[12b,13] (Table 5.1). They were all found to be heavily associated with cyanobacterial symbionts indicated initially by characteristic darker purplish coloring of the ectosome and later proved by epifluorescence (Figure 5.3). After initial identification, the specimens were subjected to a more detailed morphological and chemical characterization and grouped according to their morphological and chemical profiles for comparative analysis.

The dichloromethane solvent partition fractions of 43 specimens were subjected to analysis by electrospray ionization mass and ^{1}H-NMR spectroscopy in order to develop chemical profiles that would assist in the characterization of the specimens. Electrospray ionization mass spectrometry (ESIMS) is considered to be the "softest" ionization technique known.[18] This minimizes or eliminates fragmentation and results in the visualization of only protonated molecular ions ($[M + H]^+$, positive mode) in the spectra.[19] ESIMS is quite sensitive making it the technique of choice to unequivocally assign a particular chemotype to a specimen[20] and has been used in the dereplication of natural products extracts[21]. Fast atom bombardment-mass spectroscopy (FABMS) and GC-mass spectroscopy have previously been used in chemotaxonomic analyses.[22] Recently, ESIMS was used in the secondary metabolite profiling and taxonomy of crude fungal extracts.[23] However, to our knowledge this is the first report where the technique of ESIMS is used in the chemotaxonomic study of a marine organism. Other methods, such as thin layer chromatography, have been used in the development of chemical profiles in species differentiation with some success[11], but due to the nature of the

Table 5.1. Specimens of *Dysidea* Analyzed by ESIMS and ^{1}H-NMR.

Entry No.	Collection No.*	Site†	Depth‡	Color§	Morphotype¶
1	95006	MB	20-40	yl-rd-gy	I
2	95007	MB	20-40	yl-rd-gy	I
3	95080	MB	20-40	yl-rd-gy	I
4	95081	MB	20-40	yl-rd-gy	I
5	95083	MB	20-40	yl-rd-gy	I
6	95084	MB	20-40	yl-rd-gy	I
7	95085	MB	20-40	yl-rd-gy	I
8	95098	MB	20-40	yl-rd-gy	I
9	95154	MB	20-40	yl-rd-gy	I
10	95661	TB	20-30	yl-rd-gy	I
11	94601	SI	20-40	gr-gy	II
12	94603	SI	20-40	gy-rd	II
13	94604	SI	20-40	gy-rd	II
14	94605	SI	20-40	gy-rd	II
15	94608	SI	20-40	gy-rd	II
16	94626	SI	20-40	gr-tn	II
17	94631	SI	20-40	gr-tn	II
18	94632	SI	20-40	gr-tn	II
19	95594	TB	6-10	gr-tn	II
20	95595	TB	6-10	yl	II
21	95596	TB	6-10	gr-tn	II
22	94555	SI	20-40	gy-rd	III
23	94602	SI	20-40	gy-rd	III
24	94607	SI	20-40	gy-rd	III
25	95612	TB	20-30	gy-rd	III
26	95616	TB	20-30	gy-rd	III
27	94557	SI	20-40	gr-gy	IV
28	94558	SI	20-40	gr-gy	IV
29	94635	SI	20-40	gr-gy	IV
30	95078	MB	20-40	gr-gy	IV
31	95082	MB	20-40	gr-gy	IV
32	95095	MB	20-40	gr-gy	IV
33	95152	MB	20-40	gr-gy	IV
34	95643	TB	20-30	gr-gy	IV
35	95153	MB	20-40	gy-rd	V
36	95588	TB	20-30	gy-rd	V
37	95589	TB	20-30	gy-rd	V
38	95590	TB	20-30	gy-rd	V
39	95591	TB	20-30	gy-rd	V
40	95592	TB	20-30	gy-rd	V
41	95610	TB	20-30	gy-rd	V
42	95614	TB	20-30	gy-rd	V
43	95664	TB	20-30	gy-rd	V

* The first two digits indicate the year and the last three digits indicate the sample number.

† Site: SI = Sangihe Islands-Northern Sulawesi, Indonesia; TB = Tomini Bay-Southern Sulawesi, Indonesia; MB = Milne Bay, Papua New Guinea.

‡ Depth is in feet.

§ Color: gr = green, gy = grey, tn = tan, rd = red, yl = yellow.

¶ Morphological Type: I-*Dysidea sp. A*; II-*Dysidea sp. B*; III-*D. herbacea* 1; IV- *D. herbacea* 2; V-*D.chlorea*.

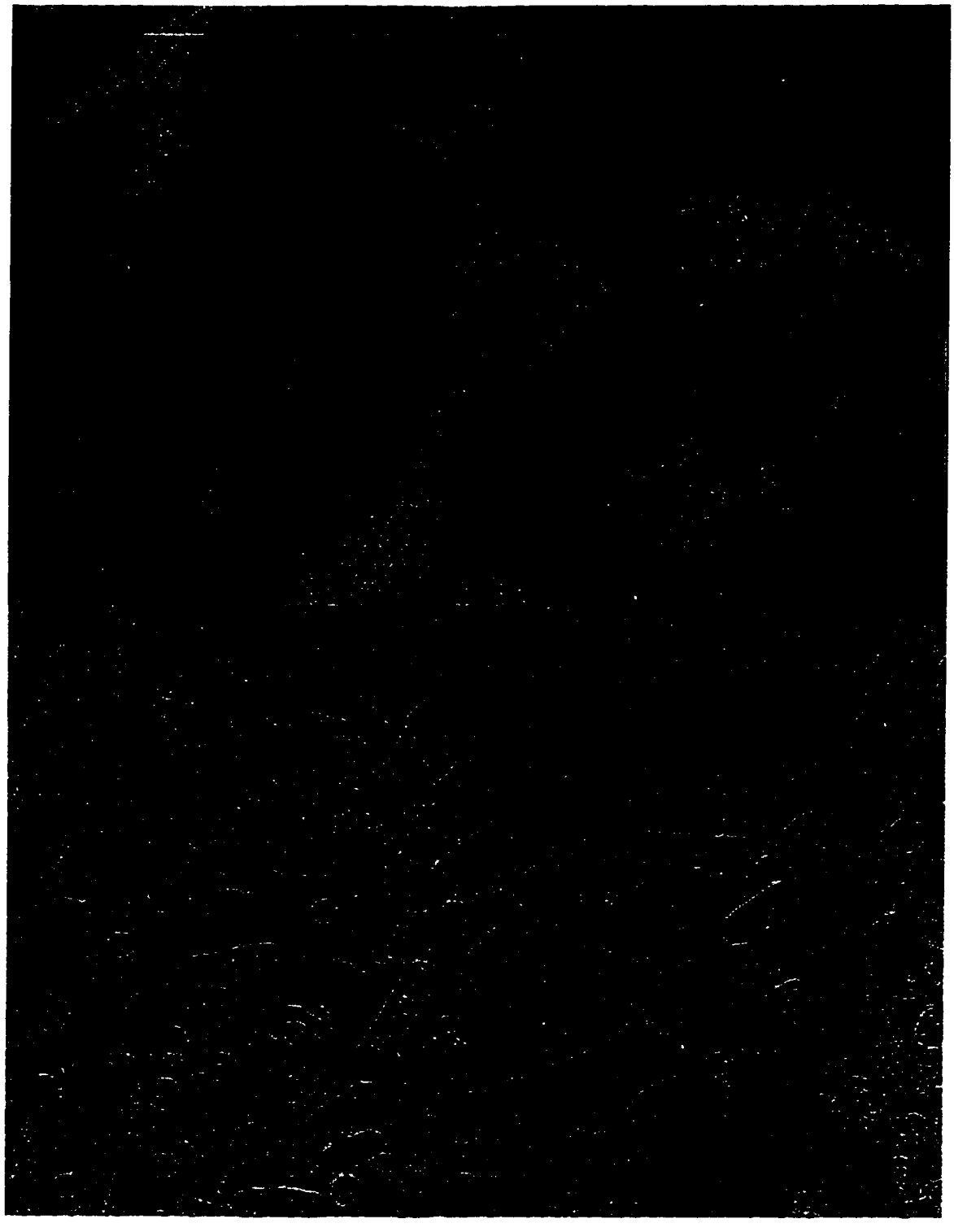


Figure 5.3. *Oscillatoria spongeliae*-like Cells Visualized under Fluorescent (A) and Normal Light (B).

metabolites in the current study (high halogen count), ESIMS was deemed more appropriate.

Polybrominated and polychlorinated compounds exhibit distinctly different isotope distribution patterns due to their natural abundance in nature (Figure 5.4).[24] In addition, the brominated diphenyl ethers ionized only in negative mode (ESI^-) and the chlorinated peptides only in positive mode (ESI+) further simplifying the characterization (Figures 5.5, 5.6).[25] This technique coupled with ^1H-NMR spectroscopy proved to be an excellent, quantitative approach for quickly identifying which halogenated metabolites were present in the sponge; chlorinated peptides or brominated diphenyl ethers. The identity of specific metabolites was determined by comparison of molecular ion peaks (ESIMS) to masses of known compounds in mass database (see Tables 4.1 and 4.2 in Chapter 4).

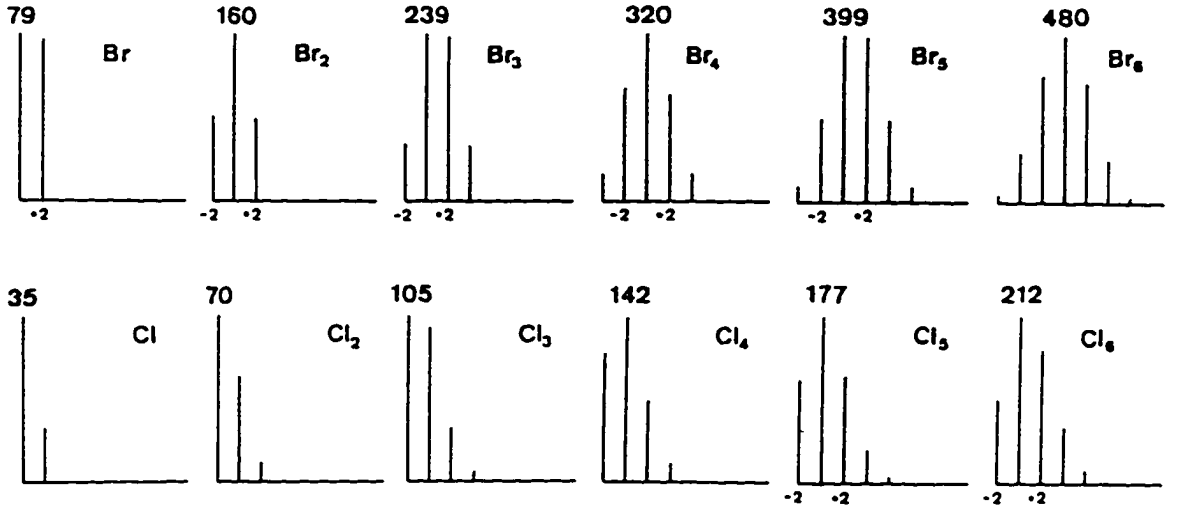


Figure 5.4. Isotope Distribution Patterns for Chlorine and Bromine.

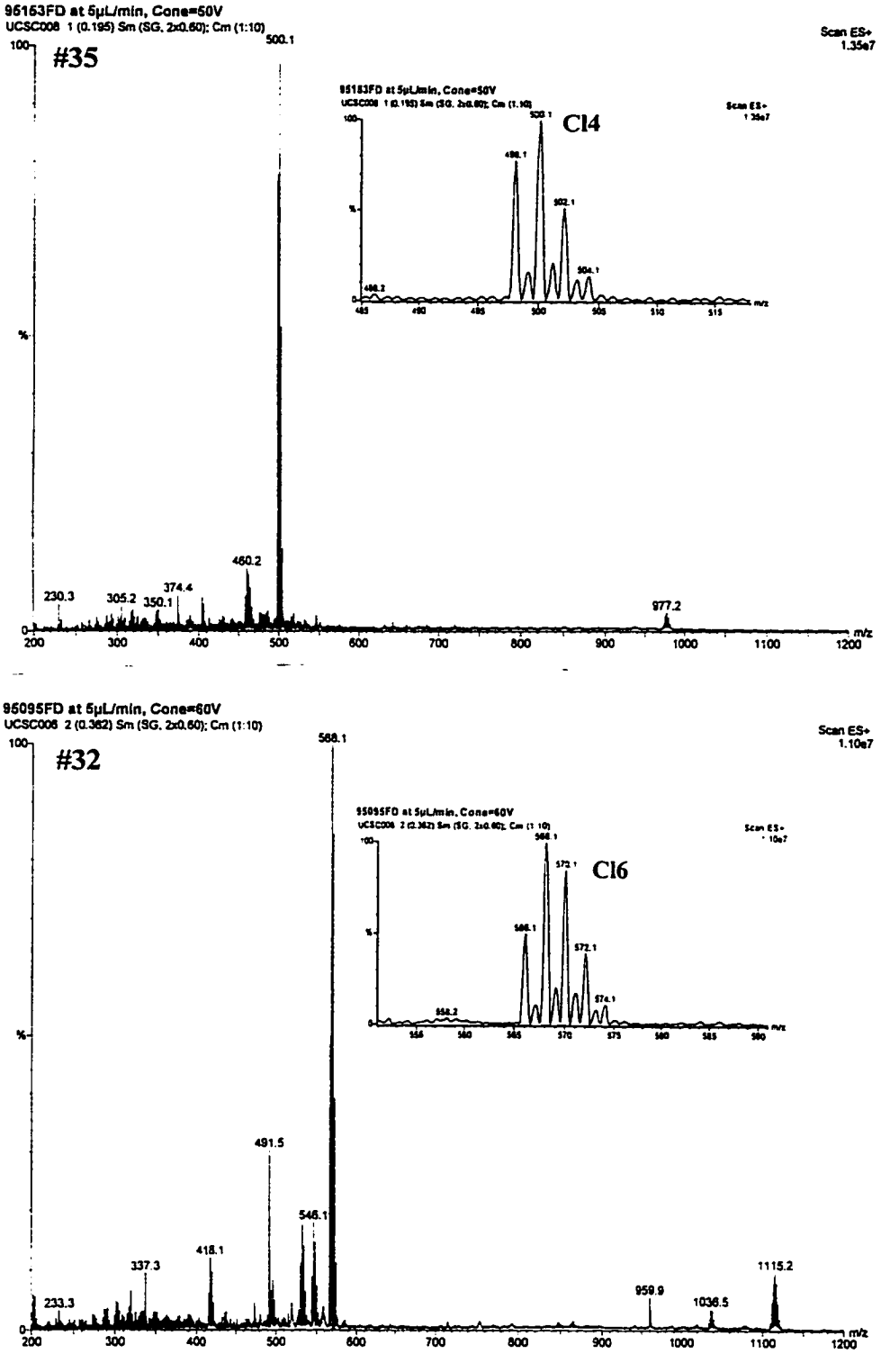


Figure 5.5. Sample ESI$^+$ Mass Spectra for Chlorinated Peptides.

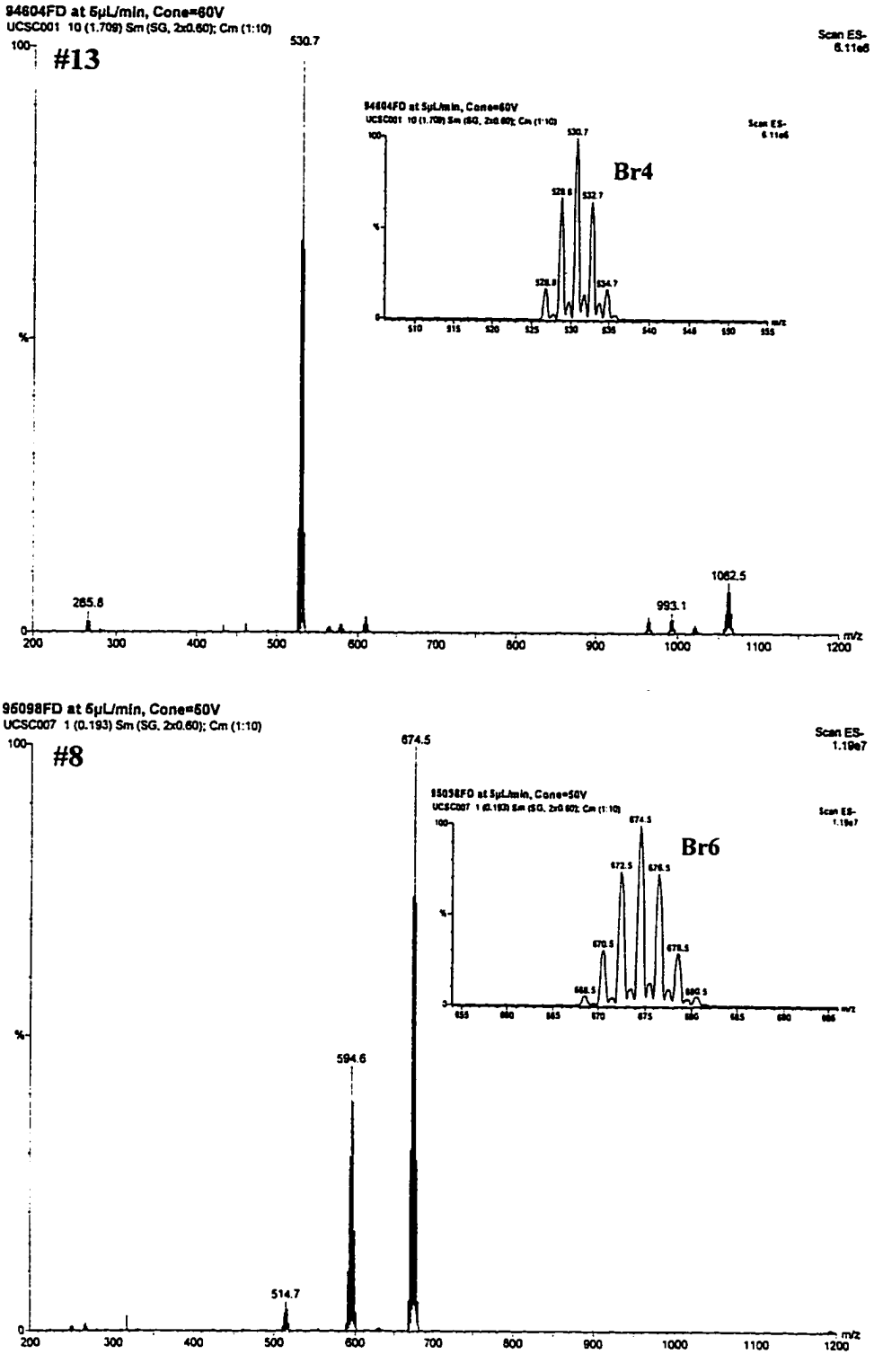


Figure 5.6. Sample ESI$^-$ Mass Spectra for Brominated Diphenyl Ethers.

Proton NMR spectra were analyzed for key chemical shifts corresponding to functionalities known to be associated with the chlorinated peptides and brominated diphenyl ethers.[1b,2c] In the bromophenols all of the protons are clustered in the δ 6.00-8.00 region and nowhere else except for the occasional methoxy peak at ≈ δ 4.00. The chlorinated peptides, on the other hand, exhibit quite different resonances due to the type of functionality present. The majority of the chlorinated peptides contain a thiazole ring which features resonances for its two protons at ≈ δ 7.30 and δ 7.80 with the majority of the its remaining resonances residing upfield. There are also characteristic shifts for the *N*-methyls usually found at ≈ δ 3.00. Therefore, spectra showing a clustering of several downfield peaks with no others indicates brominated diphenyl ethers and spectra with just a few downfield peaks, with the majority of the peaks residing upfield, indicates the presence of chlorinated peptides. Additionally, it has been reported that no terpenes are isolated when brominated diphenyl ethers are present in the sponge[3a,c,5b] further differentiating the two types of ^{1}H-NMR spectra in the δ 1.00-4.00 region.

Five morphotypes were obvious among the *Dysidea herbacea*-like sponges studied here. The morphotypes include two undescribed species of *Dysidea*, two different forms of *D. herbacea*, and *D. chlorea*. The comparative analyses between the museum types of *D. herbacea* and *D. chlorea* studied showed their extreme morphological similarity leaving only their rather variable growth habit (lamello-digitate in *D. herbacea* and only digitate in *D. chlorea*), which sometimes was found to overlap, as the differentiating character. Although Bergquist[12c] indicates that only *Dysidea herbacea* possesses cyanobacterial symbionts, we found that all the specimens here belonging to either *D. herbacea*, *D. chlorea*, or to distinct *Dysidea* spp. possessed *Oscillatoria*-like cyanobacteria as symbionts. A characterization with chemical and morphological profiles of the five morphotypes is presented below.

Morphotype I - Dysidea sp. A

Geography. Nine specimens in this group were collected in Milne Bay, Papua New Guinea (10°15'S, 150°30'E between 20-40 ft) and one specimen, 95661, was collected in Southern Sulawesi, Indonesia (Tomini Bay; 0°30'N-0°30'S, 121°30'-125°E between 20-30 ft) (Table 5.1).

Chemistry. The ESIM and ^{1}H-NMR spectra of this group were characterized by two brominated diphenyl ethers **17**, **18** as the major compounds (Figure 5.9, Table 5.2). The ESI$^-$ mass spectra of morphotype I indicated the presence of only brominated diphenyl ethers which were hexa and pentabrominated as evident by the isotope distribution patterns and peak intensities in the mass spec (*m/z* 669.5, 591.6) (Table 5.3, Supp. Mat. Type I). The ^{1}H-NMR spectra showed characteristic resonances in the downfield region of the spectra at δ 6.40-7.40 indicating bromophenol type compounds (Figure 5.7, Supp. Mat. Type I).

General description. The sponge is a flat, thin fan-shape (1-2 mm in thickness, up to 10-20 cm in length), usually lacking any projection or deformation from the main slender lamella(ae) (Figure 5.8). Specimens are found erect or laying down on the substrate and lack a stalk. The color alive is yellowish-gray externally and reddish-gray internally. The sponge is compressible, but firm in consistency. The surface appears smooth, but under magnification densely arranged microconules, less than 0.1 mm in height and 0.1- 0.2 mm apart, become evident. Small round oscules, 1-2 mm in diameter are regularly distributed on the top side of the fan with radial canals converging onto them. A dense and tight reticulation of heavily cored fibers (40-80 μm in diameter, mostly by sand) formed mostly oval meshes (240-480 μm in diameter). No clear sand cortex was observed.

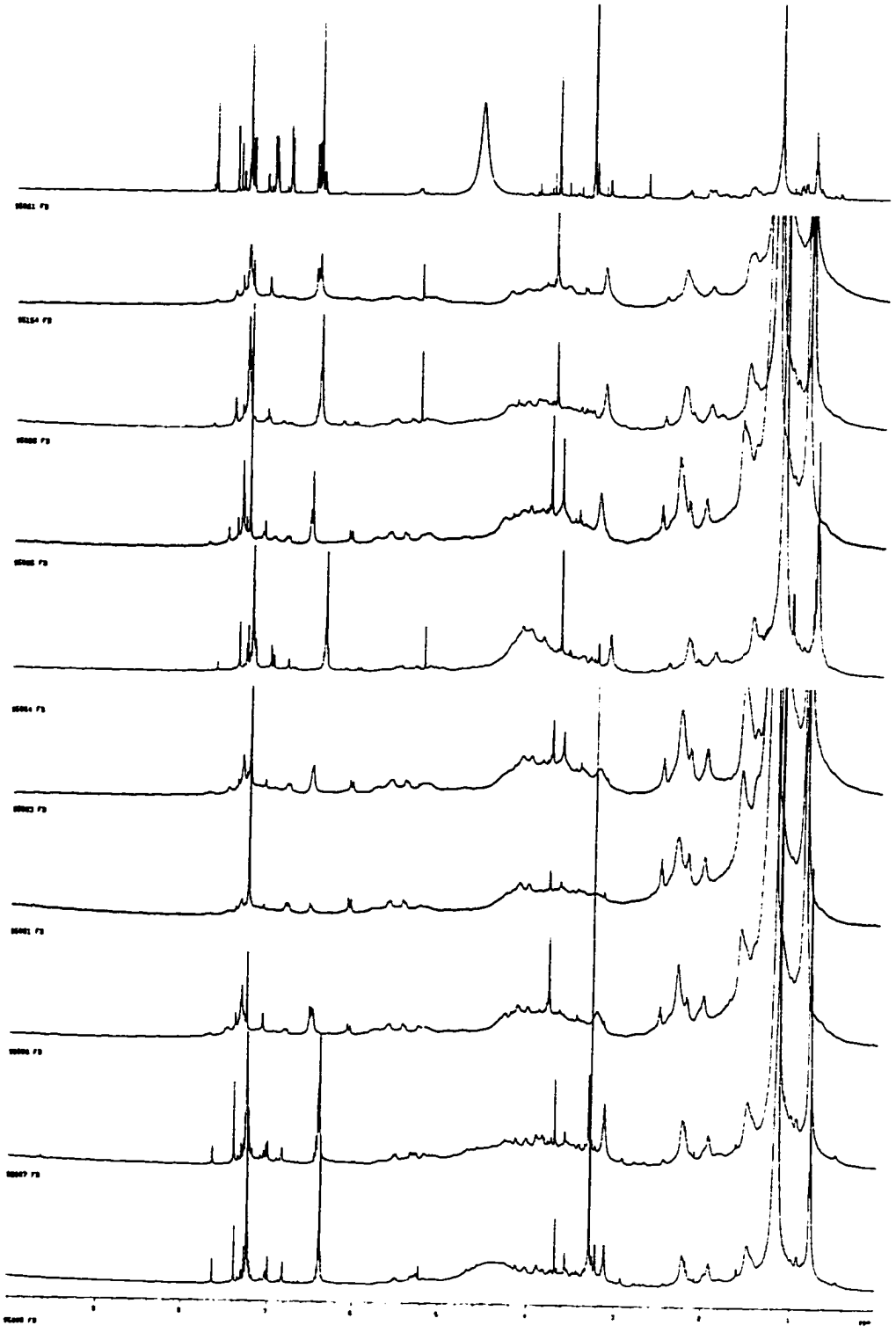


Figure 5.7. ^{1}H-NMR Spectra of Morphotype I.

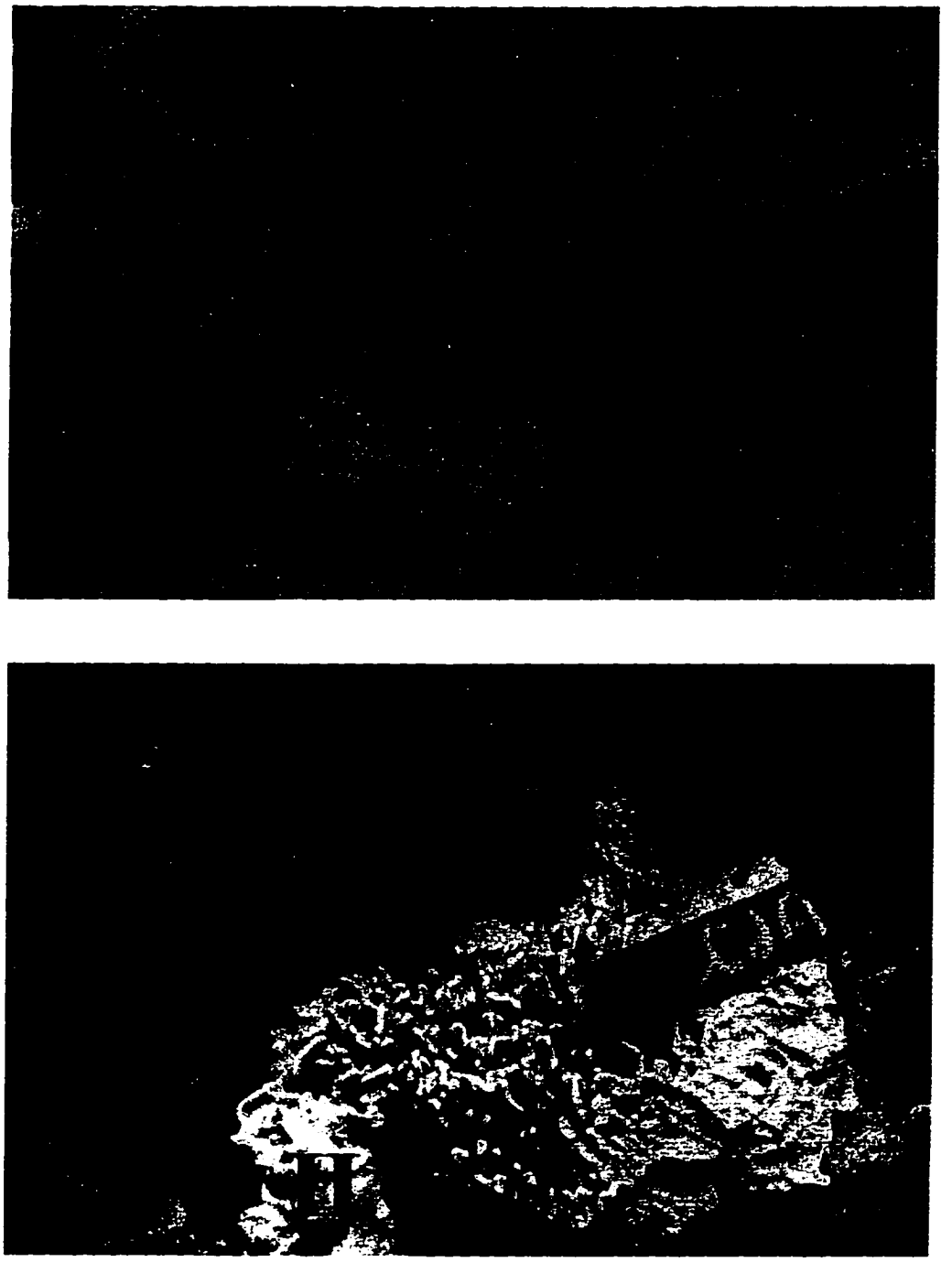


Figure 5.8. Underwater Photographs of Morphotypes I (95080) and II (94603).

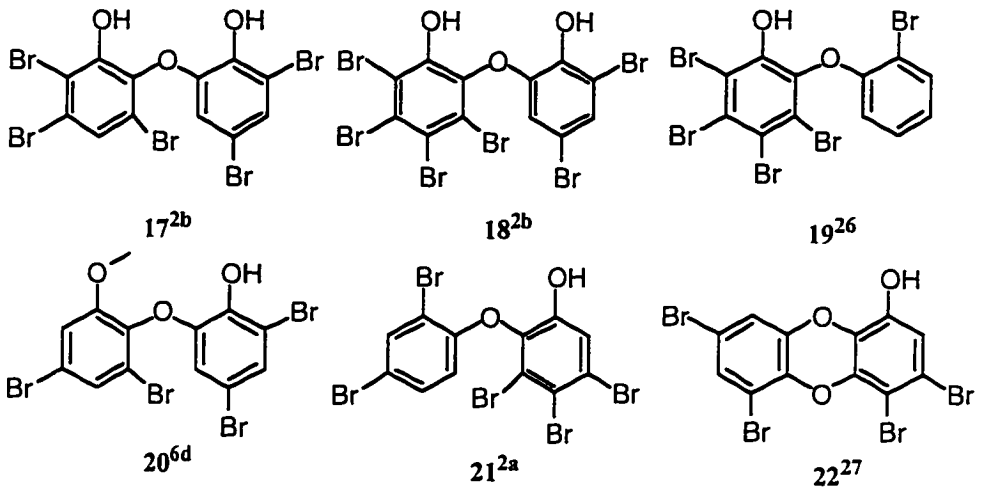


Figure 5.9. Major Brominated Diphenyl Ethers Identified in Morphotypes I, II, III.

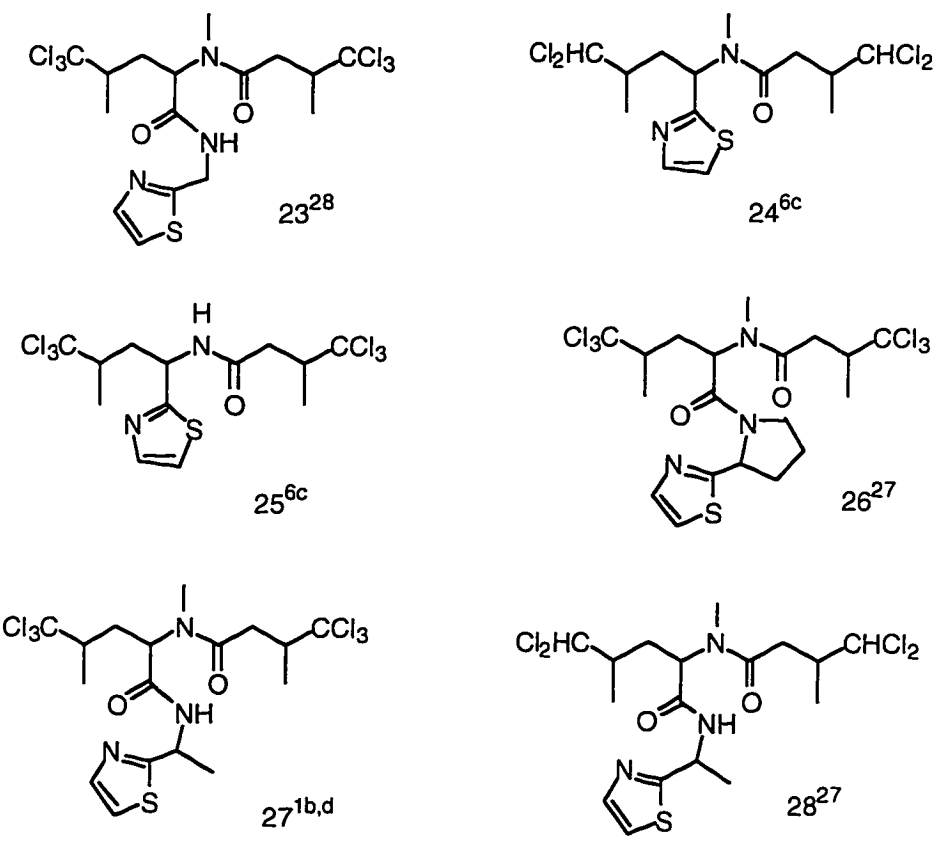


Figure 5.10. Major Chlorinated Peptides identified in Morphotypes II, III, IV, V.

Morphotype II - Dysidea sp. B

Geography. Eight specimens were collected in Northern Sulawesi, Indonesia (Sangihe Islands; 1°40'-2°50'N, 121°10'-30'E between 20-40 ft) and three specimens in Southern Sulawesi, Indonesia (Tomini Bay; 0°30'N-0°30'S, 121°30'-125°E between 6-10 ft) (Table 5.1).

Chemistry. The ESIM and ^{1}H-NMR spectra of this group of specimens fit both the brominated diphenyl ether and chlorinated peptide chemical profiles depending on the geographic locale the specimens were collected. The Northern Sulawesi collection was characterized by three different brominated diphenyl ethers as the major components **19** (94601), **20** (94604) and **21** (94603, 605, 608, 626, 632) versus **17** and **18** identified in morphotype I (Figure 5.9, Table 5.2). Collection numbers 95594 and 95596 from Southern Sulawesi were characterized by the chlorinated peptide demethyldysidenin **23** while 95595 was characterized by the brominated dioxin **22** (Figures 5.9, 5.10). The ESI$^-$ mass spectra of the Northern Sulawesi collection and 95595 exhibited characteristic isotope distribution patterns associated with tetra and pentabrominated compounds (*m/z* 575.5, 527.7, 511.8) while the ESI$^+$ mass spectra for the Southern Sulawesi specimens 95594 and 95596 exhibited the characteristic isotope distribution pattern for a compound containing six chlorine atoms (*m/z* 528.9) (Table 5.3, Supp. Mat. Type II). The ^{1}H-NMR spectra for those specimens containing brominated diphenyl ethers showed characteristic downfield resonances in the δ 6.40-7.80 region (Figure 5.11, Supp. Mat. Type II). The ^{1}H-NMR spectra for specimens 95594 and 95596 were exemplified by typical resonances in the δ 1.00-4.00 region which included a peak at δ 3.00 for a *N*-methyl and resonances characteristic of a thiazole ring at δ 7.30 and δ 7.80 (Figure 5.11, Supp. Mat. Type II).

General description. The sponge has short lamello-digitate projections (1.0-4.0 mm thick and 1.0-5.0 cm in length) growing out of a base that creeps on the substrate (Figure 5.8). Occasionally, the projections diverge at right angles from each other creating a honey-combed appearance when looked at from the top. The color alive is gray-greenish to yellowish externally and reddish-green internally. The sponge has a smooth-looking surface with densely arranged microconules, less than 0.1 mm in height and 0.1-0.2 mm apart. Microconules can be found arranged in rows or regularly distributed on the sponge surface. Occasionally, conules fuse and form ridged projections 0.5 mm high and 2.0-3.0 mm long. Small round oscules are distributed on the top of the lamella or digitate projections (1.0-2.0 mm in diameter), with canals that can be distinguished running up towards the oscules along the projection's length. Both sides of the bodies present similar surfaces. The sponge is compressible, but firm in consistency exhibiting a dense and tight reticulation of heavily cored fibers with primary and secondary fibers distinguished (primaries 50-160 μm in diameter; secondaries 16-30 μm in diameter). The reticle forms oval to loosely shaped meshes 80-160 μm in diameter close to the surface and 200-400 μm in diameter towards the interior of the projections. Towards the center of the lamella the incorporation of foreign material in the fibers decreases. In cross sections the surface seems loaded mostly with sand. It should be noted that past collections of this morphotype extend its distribution to Papua New Guinea and the Solomon Islands and were represented by the bromophenol chemical profile.[29]

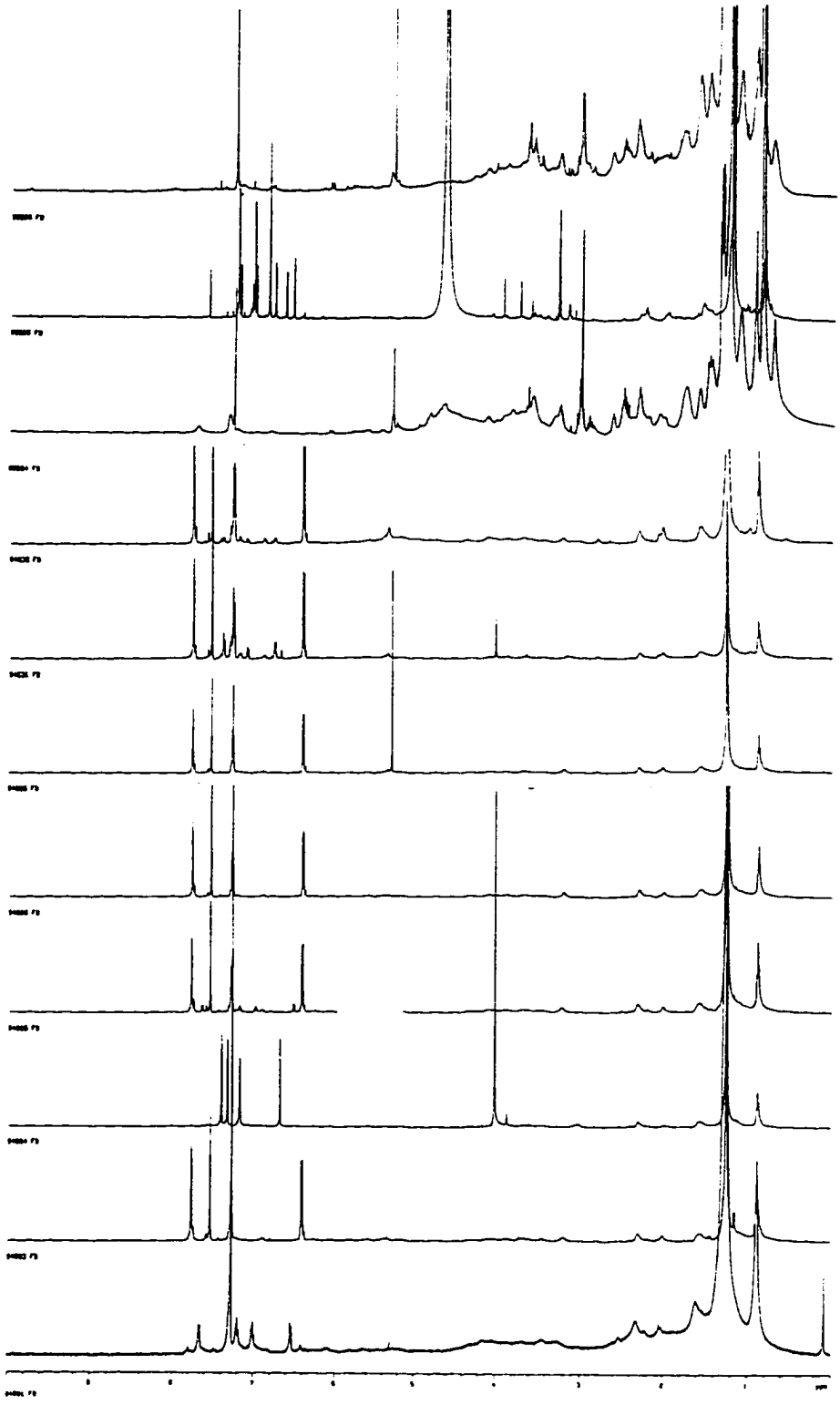


Figure 5.11. ^{1}H-NMR Spectra of Morphotype II.

Morphotype III - Dysidea herbacea 1

Geography. Three specimens were collected in Northern Sulawesi, Indonesia (Sangihe Islands; 1°40'-2°50'N, 121°10'-30'E between 20-40 ft) and two specimens in Southern Sulawesi, Indonesia (Tomini Bay; 0° 30'N-0° 30'S, 121°30'-125°E between 20-30 ft) (Table 5.1).

Chemistry. The ESIM and ^{1}H-NMR spectra of this group of specimens also indicated the presence of brominated diphenyl ethers and chlorinated peptides depending on geographic locale and were characterized by bromophenols **17** (94555) and **21** (94602 and 94607) as well as 9,10-didechloro-N-methyldysideathiazole **24** (95612, 95616) (Figures 5.9, 5.10, Table 5.2). The ESI$^-$ mass spectra of 94555, 602, and 607 exhibited the isotope distribution pattern of pentabrominated diphenyl ethers (*m/z* 591.5, 575.5) while the ESI$^+$ mass spectra of 95612 and 95616 indicated a tetrachlorinated compound (*m/z* 424.0) (Table 5.3, Supp. Mat. Type III). The ^{1}H-NMR spectra of bromophenols **17** and **21** exhibited typical chemical shift resonances in the δ 6.40-7.80 range (Figure 5.12, Supp. Mat. Type III). The ^{1}H-NMR spectra of 9,10-didechloro-N-methyldysidea-thiazole **24** showed characteristic resonances of protons in the thiazole ring at δ 7.30 and δ 7.80 along with chemical shifts in the δ 1.00-4.00 region (Figure 5.12, Supp. Mat. Type III).

General description. The sponge presents slender erect or creeping fans (1.0-2.0 mm in thickness, up to 20 cm in length) that never branch, but occasionally are observed with small, variously shaped projections that depart from the main lamella (Figure 5.13). The rim of the fan is rather regular and smooth. The color alive is reddish-gray externally and internally. Each side of the fan possesses microconules (0.5-1.0 mm in height and width) that present distinct surfaces. On the top side of the fan the microconules are arranged on top of ridges formed by fibers that run parallel to each other and tangential to

the sponge surface. Connecting fibers between these ridges give the top surface of the sponge a highly reticulated appearance. These ridges are mostly absent from the lower side of the fan and the microconules are either arranged in rows or evenly spaced. Oscules are present on the top side of the fan (1.0-2.0 mm in diameter) and are regularly distributed. The sponge is compressible and soft in consistency. A loose, open network of fibers (40-80 μm in diameter) is found forming rather rectangular meshes (300-1000 μm in diameter) on the top side of the fan, while on the bottom side the meshes are more irregular in shape. No clear sand cortex is observed.

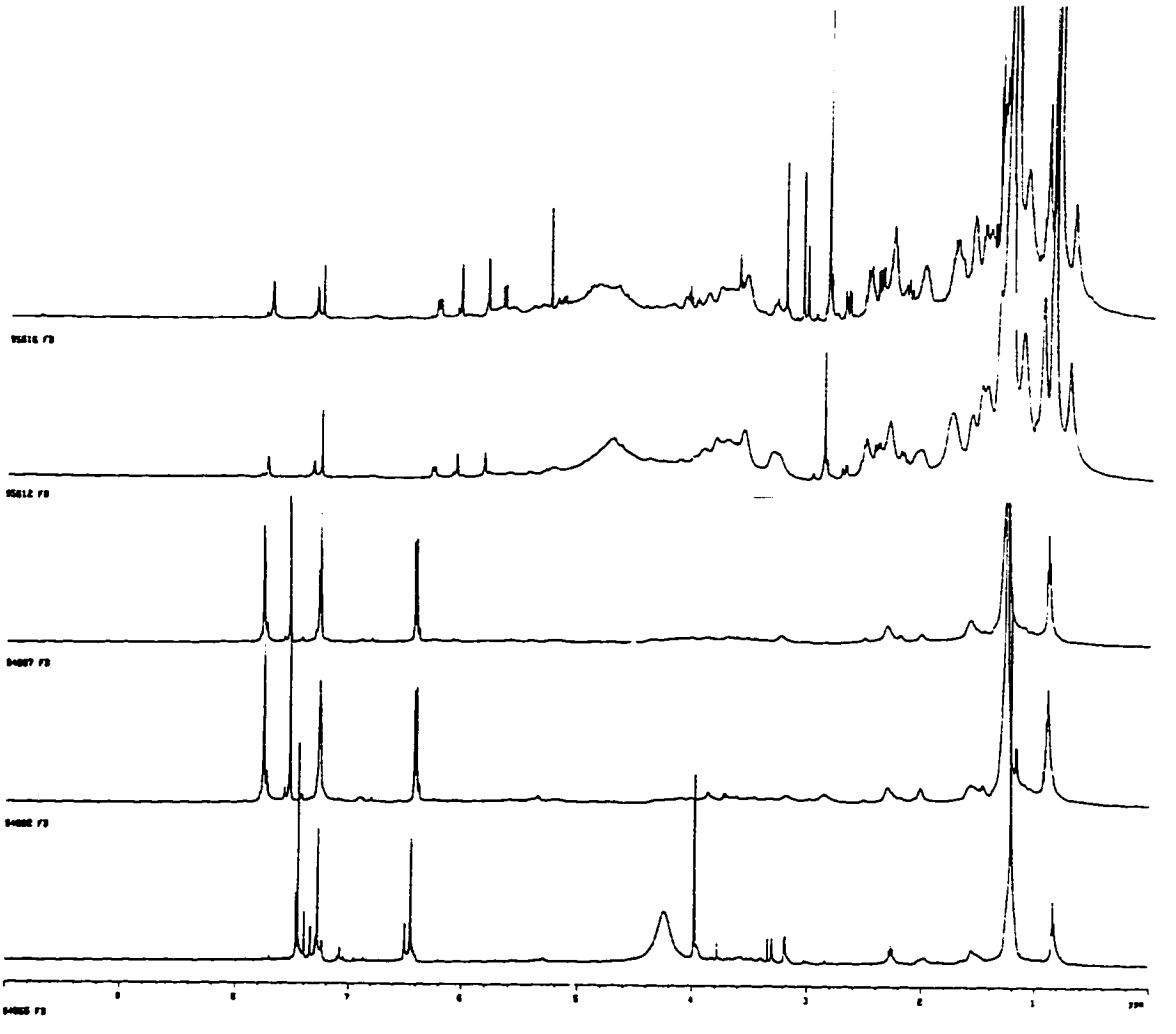


Figure 5.12. ^{1}H-NMR Spectra of Morphotype III.

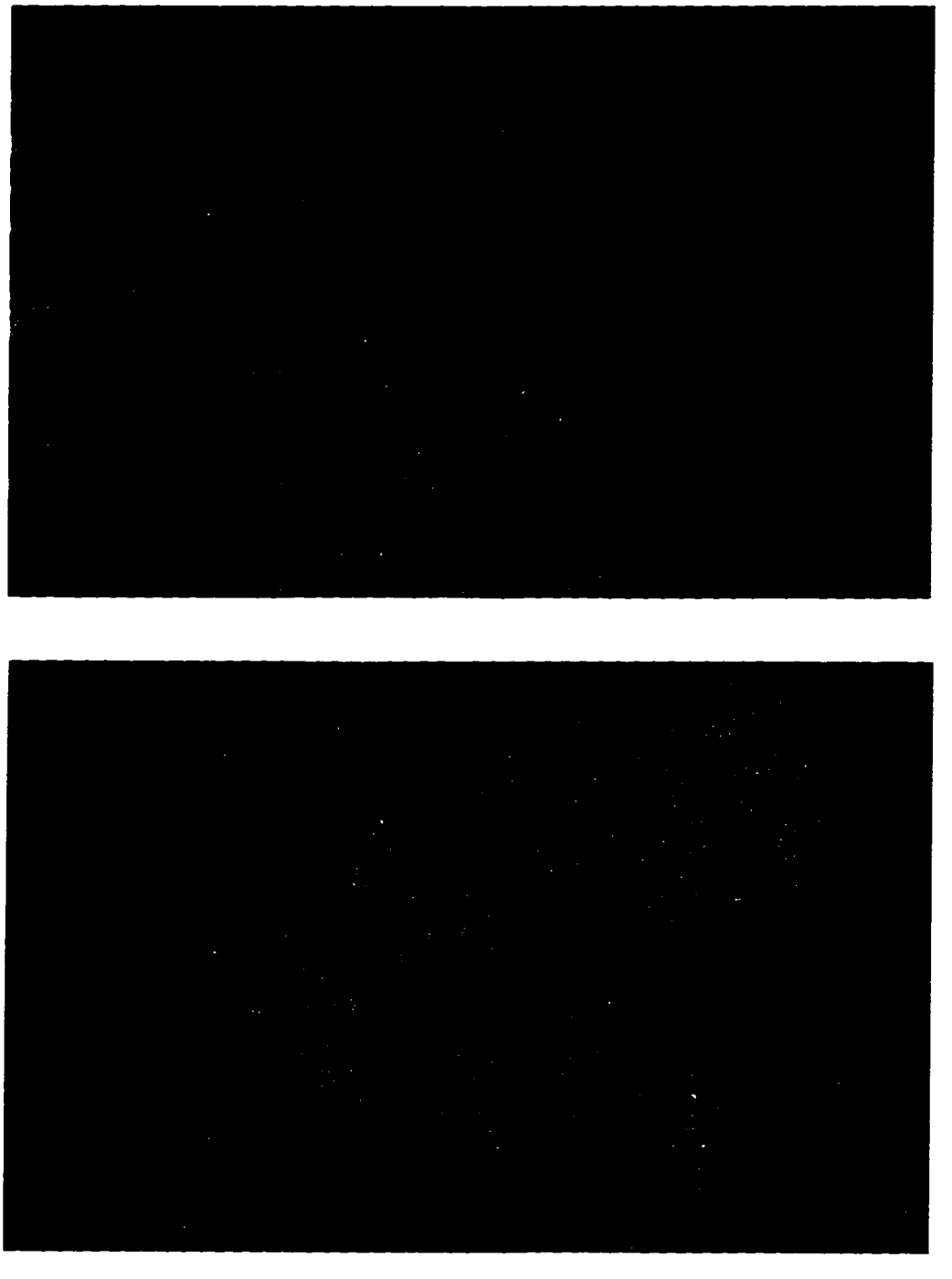


Figure 5.13. Underwater Photographs of Morphotypes III (94555) and IV (94557).

Morphotype IV - Dysidea herbacea 2

Geography. Eight specimens of this morphotype were collected from all three geographic locales. Three specimens were collected in Northern Sulawesi, Indonesia (Sangihe Islands; 1°40'-2°50'N, 121°10'-30'E in 20-40 ft); four specimens in Milne Bay, Papua New Guinea (10°15'S, 150°30'E in 20-40 ft) and one specimen in Southern Sulawesi, Indonesia (Tomini Bay; 0°30'N-0°30'S, 121°30'-125°E in 20-30 ft) (Table 5.1).

Chemistry. The ESIM and ^{1}H-NMR spectra of all the specimens identified polychlorinated peptides as the major metabolites with the exception of 95643 which appeared to contain only terpenes. The Northern Sulawesi collection was characterized by the chlorinated peptides 13-demethyldysidenin **23** (94557, 94558) and dysideathiazole **25** (94635) and the Milne Bay collection by dysidenin C **26** (95078, 95082) and dysidenin **27** (95095, 95152) (Figure 5.10, Table 5.2). The only sample from Southern Sulawesi, 95643, appeared to contain terpenes. The ESI^+ mass spectra for all samples, except 95643, identified the major components as containing six chlorine atoms (*m/z* 457.9, 528.9, 543.1 and 569.1) by analysis of the isotope distribution patterns (Table 5.3, Supp. Mat. Type IV). Analysis of the ESI^+ mass spectra of 95643 indicated the lack of chlorinated molecular ion peaks. Instead, there were peaks distinguished in the low molecular weight range (*m/z* 200-325), as the major components, possibly attributed to terpenes or non-halogenated lipids (Table 5.3, Supp. Mat. Type IV). The ^{1}H-NMR spectra of chlorinated peptides **23**, **25**, **26** and **27** exhibited characteristic resonances for the protons in the thiazole ring at δ 7.30 and δ 7.80 along with chemical shifts in the δ 1.00-4.00 region particular to each compound (Figure 5.14, Supp. Mat. Type IV). Confirmation of the presence of terpenes came from an APT NMR spectra ($CDCl_3$, 500 MHz) with diagnostic methyl shifts at ~ δ 16.0 and 19.0 and a ^{1}H-NMR spectra

exemplified by numerous peaks in the vinylic region of δ 5.00-6.00 as well as numerous peaks in the δ 1.00-4.00 region (Figures 5.14, 5.71, 5.72 in Supp. Mat. Type IV).

General description. The sponge is thin, fan-shaped and found mostly creeping on the substrate with the occasional erect leafy branches projecting from the main body (1.0-3.0 mm in thickness) (Figure 5.13). The color alive is greenish-gray externally and dark-green internally.The rim of the fan is rather irregular with sharp microconules (0.5-1.0 mm in height and width) distributed along rows giving the surface a very characteristic ribbed appearance. Both sides of the body present very similar surfaces with oscules (<0.5 mm in diameter) irregularly distributed on the body's top side. The sponge is compressible and very soft. A very loose open network of fibers (60-100 μm in diameter) is found forming rectangular to oval meshes (300-1400 μm in diameter).

Table 5.2. Summary of Constituents Isolated from Morphotypes I-V.

	Dysidea sp. A I	*Dysidea* sp. B II	*D. herbacea* 1 III	*D. herbacea* 2 IV	*D. chlorea* V
bromophenols	17, 18	19, 20, 21	17, 21	-	-
bromodioxin	-	22	-	-	-
chloropeptides	-	23	24	23, 25, 26, 27	24, 28
terpenes	-	-	-	yes	-

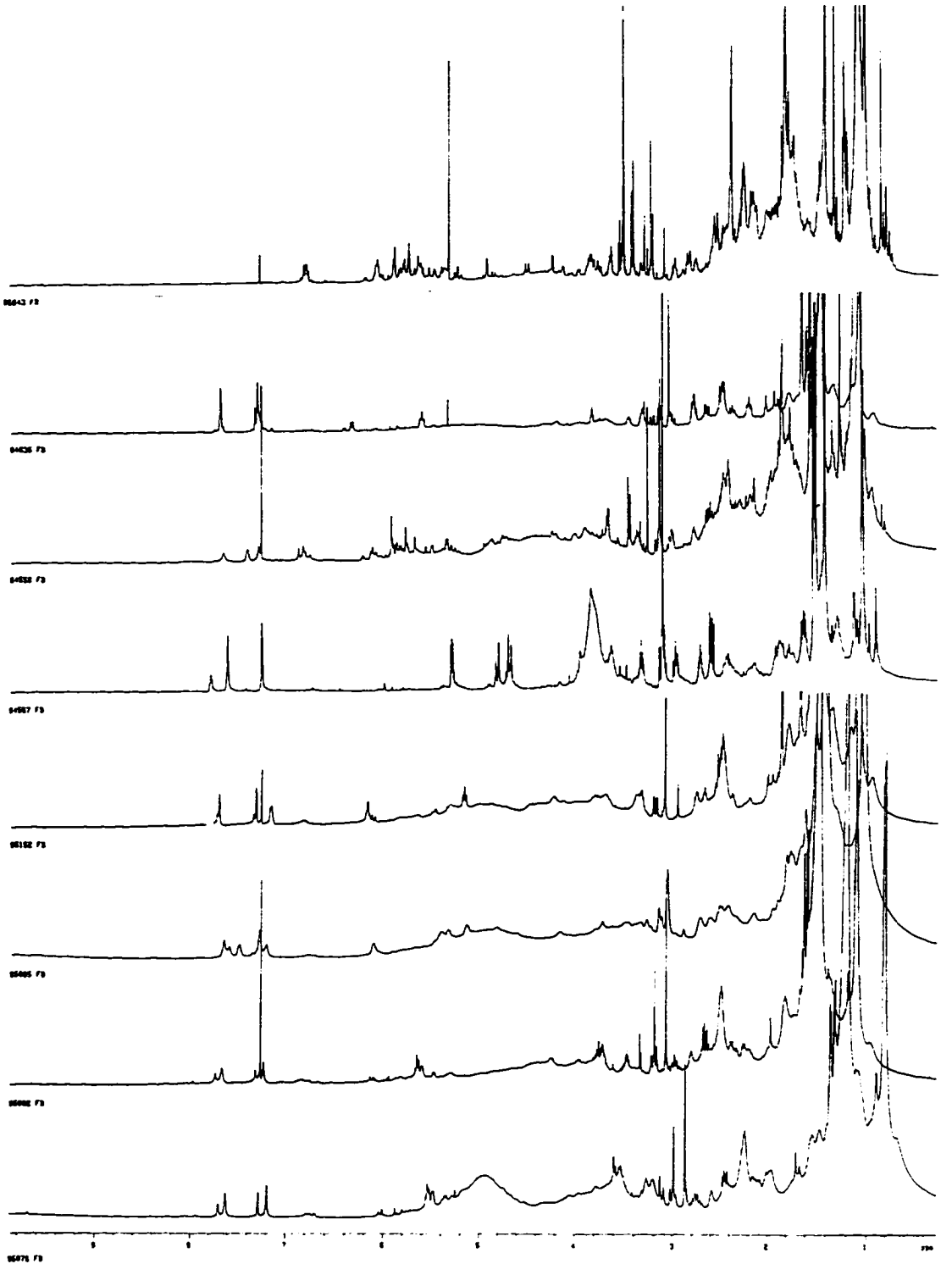


Figure 5.14. ^{1}H-NMR Spectra of Morphotype IV.

Morphotype V - Dysidea chlorea

Geography. Eight of the nine specimens identified as this morphotype were collected in Southern Sulawesi, Indonesia (Tomini Bay; 0°30'N-0°30'S, 121°30'-125°E in 20-30 ft). The remaining specimen was collected in Milne Bay, Papua New Guinea (10°15'S, 150°30'E in 20-40 ft) (Table 5.1).

Chemistry. The ESIM and ^{1}H-NMR spectra of these samples indicated the presence of two types of polychlorinated peptides. The Southern Sulawesi collection was distinguished by 9,10-didechloro-N-methyldysideathiazole **24** while 95153 was characterized by 9, 11-didechlorodysidenin **28** (Figure 5.10, Table 5.2). The ESI$^+$ mass spectra attested to the presence of tetrachlorinated compounds, as the major components, in all the samples as apparent by the isotope distribution patterns of the molecular ion peaks (*m/z* 404.0, 498.1) (Table 5.3, Supp. Mat. Type V). The ^{1}H-NMR spectra of 9,10-didechloro-N-methyldysideathiazole **24** (95588, 95589, 95590, 95591, 95592, 95610, 95614 and 95664) showed typical resonances for the thiazole ring at δ 7.30 and δ 7.80 as well as characteristic chemical shifts in the δ 1.00-4.00 region for this particular compound (Figure 5.15, Supp. Mat. Type V). The ^{1}H-NMR spectra of 9, 11-didechlorodysidenin **28** contained resonances in the δ 1.00-4.00 and δ 7.00-8.00 region characteristic of this compound (Figure 5.15, Supp. Mat. Type V).

General description. The sponge is a creeping to erect fan (1.0-3.0 mm thick) with abundant digitate projections irregularly branching off the main body (Figure 5.16). The shape, abundance, and length of these projections was quite variable between the specimens studied. The surface of this sponge is covered by microconules, 0.5-1.0 mm high and 2.0-3.0 mm apart, more or less regularly distributed. The color alive is grayish to dark red externally and dark red internally. The oscules (1.0-2.0 mm in diameter) are regularly distributed on the sponge body. The sponge is compressible and soft, but rather

flimsy in consistency. A peculiarity of all the specimens of this species is that the alcohol turned dark-red once the specimens were transferred from the formalin fixative to the alcohol. An extremely loose open network of fibers (40-160 μm in diameter) forms a reticle with poor mesh definition.

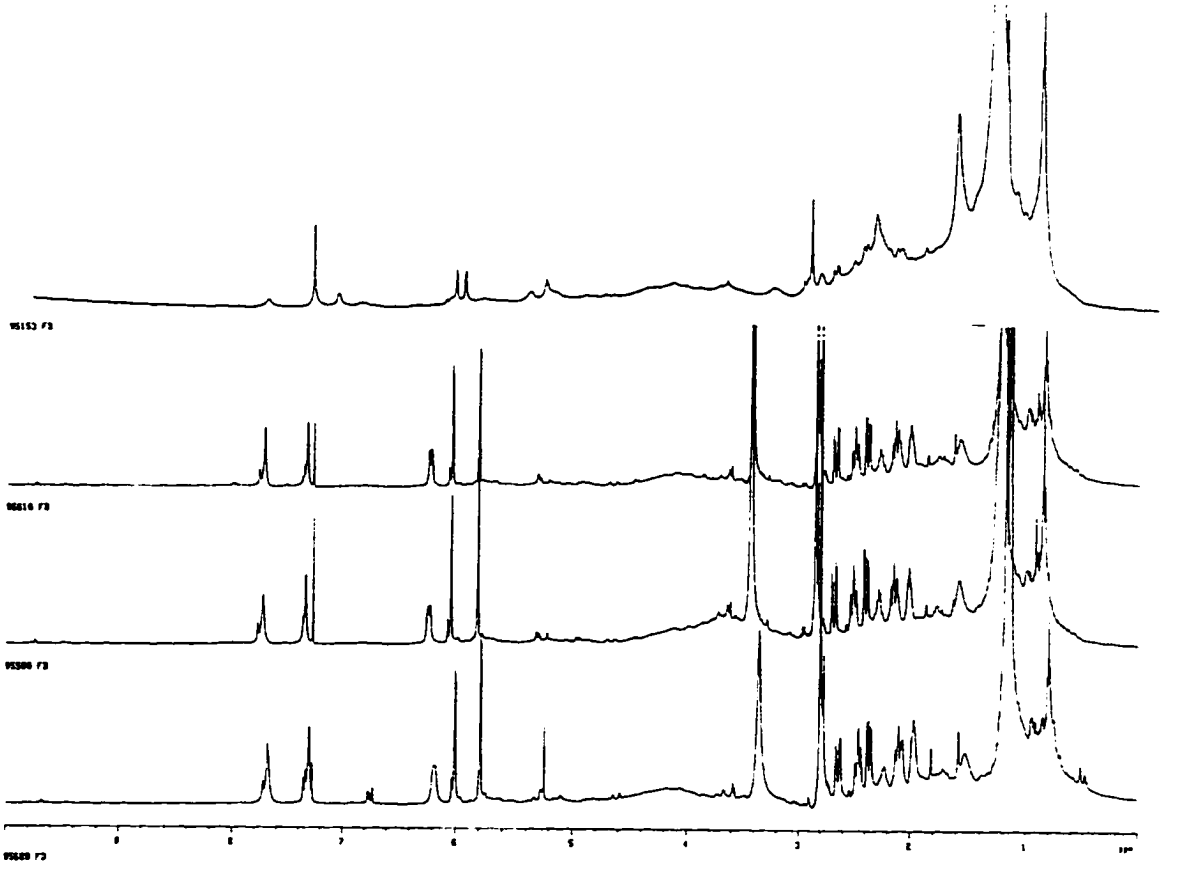


Figure 5.15. ^{1}H-NMR Spectra of Morphotype V.

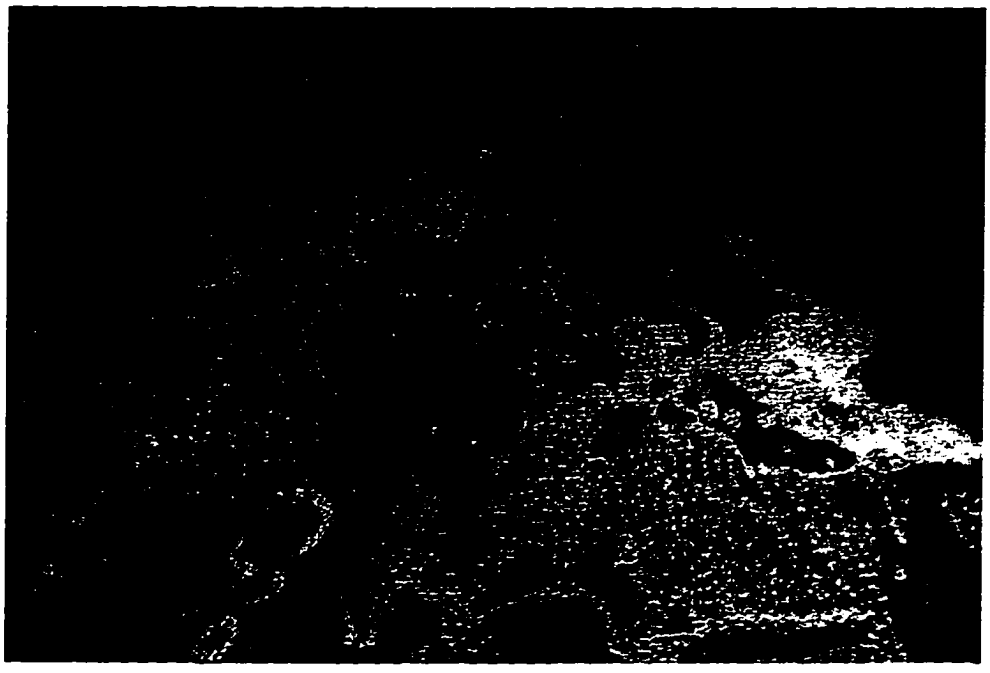


Figure 5.16. Underwater Photograph of Morphotype V (95592).

Discussion

The use of ESIMS coupled with ^{1}H-NMR spectra proved to be an excellent, quantitative tool in the chemotaxonomic study of *Dysidea*. Even though this method is not applicable to field study, it offers intriguing possibilities for the chemotaxonomic study of other genera of marine organisms that may be difficult to analyze using the present methods. One major advantage is that only a small amount of sponge is required for the analysis. Electrospray ionization mass and ^{1}H-NMR spectrometry are both very sensitive techniques allowing unequivocal identification of the metabolites present, provided there is a working knowledge of the chemistry of the sponge.

In the present study these techniques were able to determine the metabolites present in the sponge and thereby classify them into either a brominated diphenyl ether or polychlorinated peptide chemical profile which was directly correlated with the morphotype identified (Table 5.4). Specimens of morphotype I (no. 1-10) were found to contain bromophenols while morphotypes IV (no. 27-34) and V (no. 35-43) contained chlorinated peptides. Morphotypes II and III exhibited either the brominated diphenyl ether (no. 11-18, 22-24) or chlorinated peptide chemical profile (no. 19, 21, 25, 26) depending on the geographic locale of collection (SI vs. TB respectively) (Table 5.4). One exception to this trend was specimen 95595 which exhibited the brominated diphenyl ether chemical profile while 95594 and 95596, from the same geographic locale, contained chlorinated peptides (Table 5.4). It is interesting to note that the color of 95595 was distinctly different from 95594 and 95596 and that these three were collected in only 6-10 ft of water whereas all other specimens were collected in a minimum depth of 20 ft (Table 5.1). Additionally, brominated dioxins were isolated for the first time from this same specimen further differentiating it from 95594 and 95596 as well as the other

Table 5.3. Chemical Profiles Developed by ESI Mass Spectroscopy.*

No.	Coll. No.	Type	Halogen count	m/z (exact mass)	Peak intensities (%)	Ratio/Halo.
1	95006	I	Br2, Br4, Br5, Br6	265.9, 513.7, 591.6, 669.5	10, 10, 70, 100	1:1:7:10
2	95007	I	Br2, Br4, Br5, Br6	265.9, 513.7, 591.6, 669.5	10, 10, 70, 100	1:1:7:10
3	95080	I	Br2, Br4, Br5, Br6	265.9, 513.7, 591.6, 669.5	9, 9, 58, 100	1:1:6:10
4	95081	I	Br2, Br4, Br5, Br6	265.9, 513.7, 591.6, 669.5	9, 9, 58, 100	1:1:6:10
5	95083	I	Br2, Br4, Br5, Br6	265.9, 513.7, 591.6, 669.5	9, 9, 58, 100	1:1:6:10
6	95084	I	Br2, Br4, Br5, Br6	265.9, 513.7, 591.6, 669.5	10, 11, 72, 100	1:1:7:10
7	95085	I	Br2, Br4, Br5, Br6	265.9, 513.7, 591.6, 669.5	9, 9, 58, 100	1:1:6:10
8	95098	I	Br4, Br5, Br6	513.7, 591.6, 669.5	5, 45, 100	.5:5:10
9	95154	I	Br2, Br4, Br5, Br6	265.9, 513.7, 591.6, 669.5	9, 9, 58, 100	1:1:6:10
10	95661	I	Br3, Br4, Br5, Br6	435.8, 513.7, 591.6, 669.5	5, 18, 48, 100	.5:2:5:10
11	94601	II	Br5, Br6, Br5	575.6, 653.5, 689.5	100, 30, 10	10:3:1
12	94603	II	Br5	575.5	100	10
13	94604	II	Br4	527.7	100	10
14	94605	II	Br5	575.5	100	10
15	94608	II	Br5	575.5	100	10
16	94626	II	Br5	575.5	100	10
17	94631	II	Br5	575.5	100	10
18	94632	II	Br5	575.5	100	10
19	95594	II	Cl6, Cl6, Cl6 + Na	515.1, 528.9, 551.9	28, 48, 100	1:5
20	95595	II	Br3, Br4, Br5	433.8, 511.8, 591.6	5, 100, 10	.5:10:1
21	95596	II	Cl6, Cl6, Cl6 + Na	515.1, 528.9, 551.9	12, 12, 100	1:9
22	94555	III	Br2, Br5, Br5	265.9, 591.5, 605.6	8, 100, 5	1:10:.5
23	94602	III	Br5	575.5	100	10
24	94607	III	Br5	575.5	100	10
25	95612	III	Cl3, Cl4, Cl4 + Na	368.2, 404.0, 424.0	40, 45, 100	1:4
26	95616	III	Cl3, Cl4, Cl4 + Na	368.2, 404.0, 424.0	40, 45, 100	1:4
27	94557	IV	Cl4, Cl6, Cl6 + Na	516.9, 528.9, 550.8	16, 30, 100	1:8
28	94558	IV	Cl4, Cl6, Cl6 + Na	516.9, 528.9, 550.8	10, 100, 10	1:11
29	94635	IV	Cl6, Cl6	457.9, 471.9	100, 44	10:4
30	95078	IV	Cl4, Cl6, Cl6 + Na	557.2, 569.1, 591.1	20, 20, 100	1:6
31	95082	IV	Cl4, Cl6, Cl6 + Na	557.2, 569.1, 591.1	20, 20, 100	1:6
32	95095	IV	Cl6, Cl6, Cl6 + Na	529.1, 543.1, 565.1	18, 18, 100	1:7
33	95152	IV	Cl6	542.8	100	10
34	95643	IV	terpenes	233.3, 255.2, 303.2	48, 100, 90	-
35	95153	V	Cl4 + Na	498.1	100	10
36	95588	V	Cl3, Cl4, Cl4 + Na	368.1, 404.0, 424.0	60, 95, 100	1:3
37	95589	V	Cl3, Cl4, Cl4 + Na	368.1, 404.0, 424.0	60, 95, 100	1:3
38	95590	V	Cl3, Cl4, Cl4 + Na	368.1, 404.0, 424.0	60, 95, 100	1:3
39	95591	V	Cl3, Cl4, Cl4 + Na	368.1, 404.0, 424.0	60, 95, 100	1:3
40	95592	V	Cl3, Cl4, Cl4 + Na	368.1, 404.0, 424.0	60, 95, 100	1:3
41	95610	V	Cl3, Cl4, Cl4 + Na	368.1, 404.0, 424.0	60, 95, 100	1:3
42	95614	V	Cl3, Cl4, Cl4 + Na	368.1, 404.0, 424.0	60, 95, 100	1:3
43	95664	V	Cl3, Cl4, Cl4 + Na	368.1, 404.0, 424.0	60, 95, 100	1:3

*Each mass number represents molecular ion [M ± H] peaks for individual compounds identified in the specimens and corresponds directly to the halogen count and peak intensities shown in the same order of sequence.

Table 5.4. Secondary Metabolites Identified by ESIMS and ^{1}H-NMR.

No.	Collection No.	Morphotype	Site†	Chlorinated peptides	Brominated phenols	Bromodioxin	Terpenes
1	95006	I	MB		◆		
2	95007	I	MB		◆		
3	95080	I	MB		◆		
4	95081	I	MB		◆		
5	95083	I	MB		◆		
6	95084	I	MB		◆		
7	95085	I	MB		◆		
8	95098	I	MB		◆		
9	95154	I	MB		◆		
10	95661	I	TB		◆		
11	94601	II	SI		◆		
12	94603	II	SI		◆		
13	94604	II	SI		◆		
14	94605	II	SI		◆		
15	94608	II	SI		◆		
16	94626	II	SI		◆		
17	94631	II	SI		◆		
18	94632	II	SI		◆		
19	95594	II	TB	◆			
20	95595	II	TB			◆	
21	95596	II	TB	◆			
22	94555	III	SI		◆		
23	94602	III	SI		◆		
24	94607	III	SI		◆		
25	95612	III	TB	◆			
26	95616	III	TB	◆			
27	94557	IV	SI	◆			
28	94558	IV	SI	◆			
29	94635	IV	SI	◆			
30	95078	IV	MB	◆			
31	95082	IV	MB	◆			
32	95095	IV	MB	◆			
33	95152	IV	MB	◆			
34	95643	IV	TB				◆
35	95153	V	MB	◆			
36	95588	V	TB	◆			
37	95589	V	TB	◆			
38	95590	V	TB	◆			
39	95591	V	TB	◆			
40	95592	V	TB	◆			
41	95610	V	TB	◆			
42	95614	V	TB	◆			
43	95664	V	TB	◆			

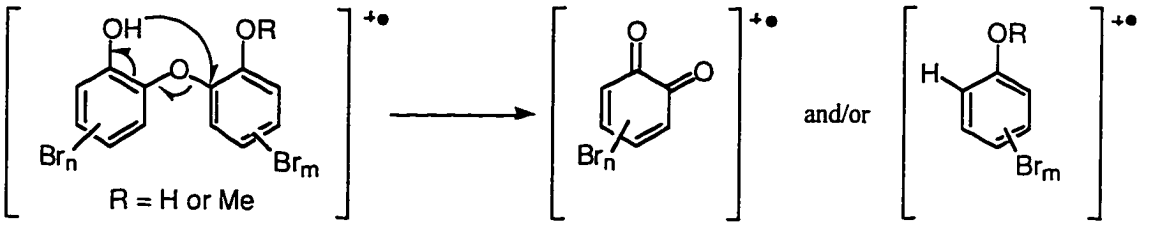
† Site: SI = Sangihe Islands-Northern Sulawesi, Indonesia; TB = Tomini Bay-Southern Sulawesi, Indonesia; MB = Milne Bay, Papua New Guinea.

specimens which contained the usual bromophenolic compounds (Figure 5.9). Another outlier was 95643 which contained terpenes and no evidence of halogenated compounds even though cyanobacteria were present in the specimen (Table 5.4).

A pattern in the degree of bromination and chlorination of the metabolites was also evident upon evaluation of the morphological and chemical data of the ESIM spectra (Table 5.3). Morphotypes I, II, and III containing brominated diphenyl ethers, differed in the number of bromine substituents present on the major component of each type as can be seen by the halogen ratios in Table 5.3. Morphotype I (no. 1-10) contained a hexabrominated diphenyl ether as the major component while morphotypes II and III (no. 11-18, 22-24) contained a pentabrominated diphenyl ether as the major component. Morphotypes II, III, IV, and V exhibited differences in degree of chlorination as well. A tetrachlorinated peptide was the major component of morphotypes III (no. 25, 26) and V (no. 35-43) while the major component of morphotypes II (no. 19,20) and IV (no. 27-34) were hexachlorinated constituents (Table 5.3). This pattern correlated directly with the morphotype (Table 5.4).

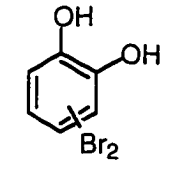
Further evaluation of the ESIM spectra also revealed some insights into the biogenesis of the chlorinated peptides and bromophenols (Table 5.3; Supp. Mat. Types I-V). In morphotypes I, II, III which gave compounds **17**, **18**, **20** and **22** (Figure 5.9, Table 5.2) the presence of a dibromo-hydroxyphenol precursor at *m/z* 266 ($C_6H_4Br_2O_2$) was identified in the ESIM spectra of many of the specimens at low threshold levels (<10% peak intensity) (Figures 5.19, 5.21, 5.25, 5.46, 5.50 in Supp. Mat. Types I-III). In morphotypes II and III which gave compounds **19** and **21** a dibromophenol precursor was identified at *m/z* 250 ($C_6H_5Br_2O$) (Figure 5.40 in Supp. Mat. Type II). These peaks can be ruled out as a result of fragmentation for the following reasons. First, the mass *m/z* 266 does not match any of the possible fragmentation ions one would normally observe

in an ortho-hydroxy ether rearrangement (Scheme 5.1).[6d,30] Secondly, the *m/z* 266 peak has been previously identified[6d] and most importantly, only one peak in the low molecular weight range is observed; not a pair as would be expected from the fragmentation of penta- and hexabrominated diphenyl ethers (Figures 5.19, 5.21, 5.25, 5.46, 5.40, 5.50 Supp. Mat. Types I-III). In our study, these observations suggest that

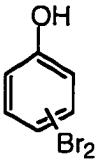


Scheme 5.1. Mass Spectral Fragments Observed from Brominated Diphenyl Ethers.

these precursors give rise to a tetrabromodiphenyl ether with further halogenation to give penta- and hexabrominated diphenyl ethers after the joining of two precursor molecules. Either subsequent methylation of the phenol or dehalogenation of bromine results in all the bromophenols identified (Figure 5.9). As well, these precursor molecules can explain all of the possible bromophenols that have been isolated (see Table 1.4 in Chapter 1).



$C_6H_4Br_2O_2$
m/z 266



$C_6H_5Br_2O$
m/z 250

Analysis of the ESIM spectra of morphotypes II-V containing chlorinated peptides revealed a different story in regards to biogenesis of these metabolites (Figures 5.44, 5.48, 5.55, 5.57, 5.59, 5.61, 5.63, 5.66, 5.68, 5.74, 5.76 in Supp. Mat. Types II-V). No chlorinated amino acid precursor molecules were evident in the mass spectra (e.g. trichlorovaline at *m/z* 219 and trichloroleucine at *m/z* 233). Additionally, none have ever been reported in the literature. In our study, this suggests that the precursor molecules of the chlorinated peptides are amino acids with chlorination occurring after the peptide is formed. Mass spectral ion peaks at *m/z* 221, 255, 415 were visible in the ESIM spectra, but these were attributed to some of the normal fragmentation ions observed in the analysis of chlorinated peptides[1b,6c] and were non-isolable. We also observed the same peak with *m/z* 415 in the electrospray mass spectra of pure dysidenin **23** and dysidenin C **26**. Consequently, this is one example where minimal fragmentation is observed during electrospray ionization.

From the five *Dysidea herbacea*-like morphotypes studied here, two major groups can be distinguished. The first comprises morphotypes I and II (*Dysidea* sp.A and *Dysidea* sp.B) which are quite distinct from the *D.chlorea* and *D. herbacea* types analyzed. Morphotypes I and II included lamellate (I) or digitate (II) sponges with an apparently smooth body surface covered by minute microconules, only visible under the microscope, and a compact, tight *Dysidea* fiber reticulation. These two species were quite distinct morphologically and in their habits and probably conform two undescribed species.

The other three morphotypes (III-V) present surface and skeleton characteristics that associate them to the *D. chlorea* and *D. herbacea* types. These morphotypes included lamellate or digitate sponges with larger conules on the surface and an open-loose *Dysidea* fiber reticulation. Morphotypes III and IV comprised predominantly lamellate to

foliose forms while morphotype V comprised a branching-digitate form with widely spaced conules and a looser fiber reticulation. Morphotypes III and IV are considered conspecific representatives of *Dysidea herbacea*. Comparisons of the specimens in this study with the museum types of *D. herbacea* denoted the same morphological characteristics, but with rather variable growth forms. We maintained them separate to search for any possible correlations between their morphological differences and secondary metabolite profile.

Morphotype V was assigned to *Dysidea chlorea* considering mainly its branching, irregular shapes, distinct surface and reticle. However, the possibility of the conspecificity of *D. herbacea* and *D. chlorea* can not be discarded at this point and remains an issue to be resolved in a further systematic evaluation of these species. Therefore, it is feasible that morphotypes III-V conform to one species complex possessing a wide range of habits. Full taxonomic descriptions of the two new species of *Dysidea* and a reevaluation of *D. herbacea* and *chlorea* are currently in progress.

In this particular case study, the presence of *Oscillatoria spongeliae*-like cyanobacteria in the sponge specimens was confirmed. Current data suggests that this organism is responsible for the production of the brominated diphenyl ethers and polychlorinated peptides found in *Dysidea herbacea*.[5a,b] The observation that the degree of halogenation of the major halogenated metabolite is associated with a specific morphotype and the fact that only one chemical profile (either chlorinated peptide or bromophenol) is identified in a particular geographic locale suggests there is a morphological/evolutionary trend associated with a particular chemotype of cyanobacteria. It is believed that prokaryotic cyanobacteria evolved in symbiotic relationship with ancestral sponges during the Pre-Cambrian period and it is feasible that one or more cyanobacterial strain could have established itself with a sponge host in a

certain geographic locale.[31] Also, it has been suggested very recently that the application of morphological trends of evolution can be applied in the phylogenetic interpretation of chemotaxonomic data.[32] The authors specifically looked at the production of triterpene glycosides from sea cucumbers as the basis for their theory. In light of this information and examination of the data in our study, it seems that a particular chemotype or strain of cyanobacteria has evolved with a particular species of *Dysidea* governed by its geographic location.

In conclusion, a total of five morphological types were identified, each consistently distinct from the others with respect to their surface (conule size and distribution), reticle dimensions, habit and chemical profiles. We have shown that the halogenated metabolites found in all the specimens assisted in this characterization based on chemical profiles developed from electrospray ionization mass and ^{1}H-NMR spectra of the extracts. It would be of great value to continue to explore the chemotaxonomy of *Dysidea* sponges from other geographic locales in the South Pacific. Additionally, further characterization of *Oscillatoria* (DNA sequencing) is necessary to confirm the presence of the suggested chemotypes of cyanobacteria. Finally, we have demonstrated that through the use of electrospray ionization mass and ^{1}H-NMR spectroscopy one can obtain secondary metabolite profiling of a particular sponge and that these techniques can be applied in the chemotaxonomic and biogenetic analyses of other marine organisms in general.

Supplementary Material

Morphotype I

Figure 5.17. ^{1}H-NMR Spectra-95006 (CD_3OD).

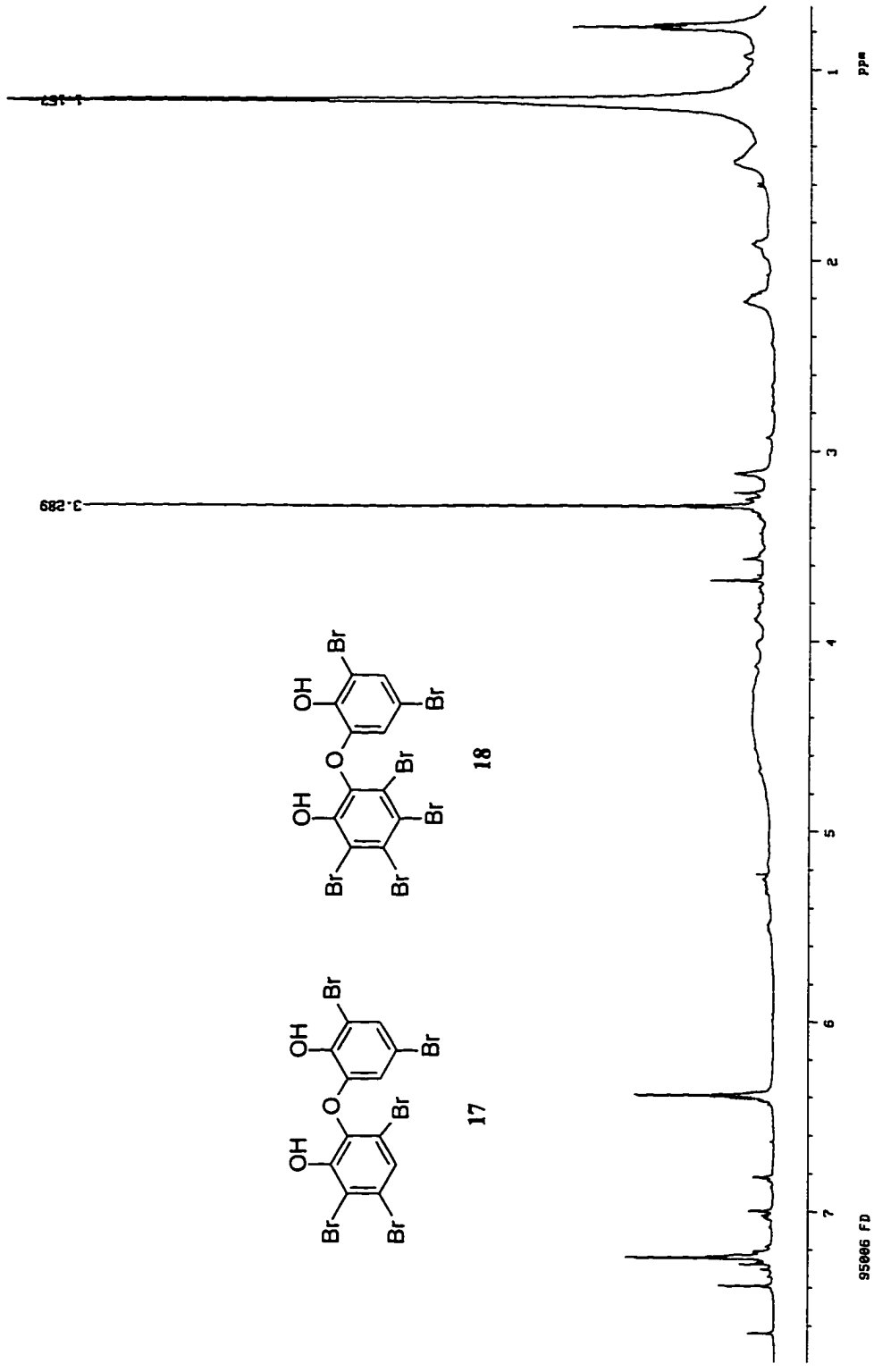


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Figure 5.18. ^{1}H-NMR Spectra-95007 ($CDCl_3$).

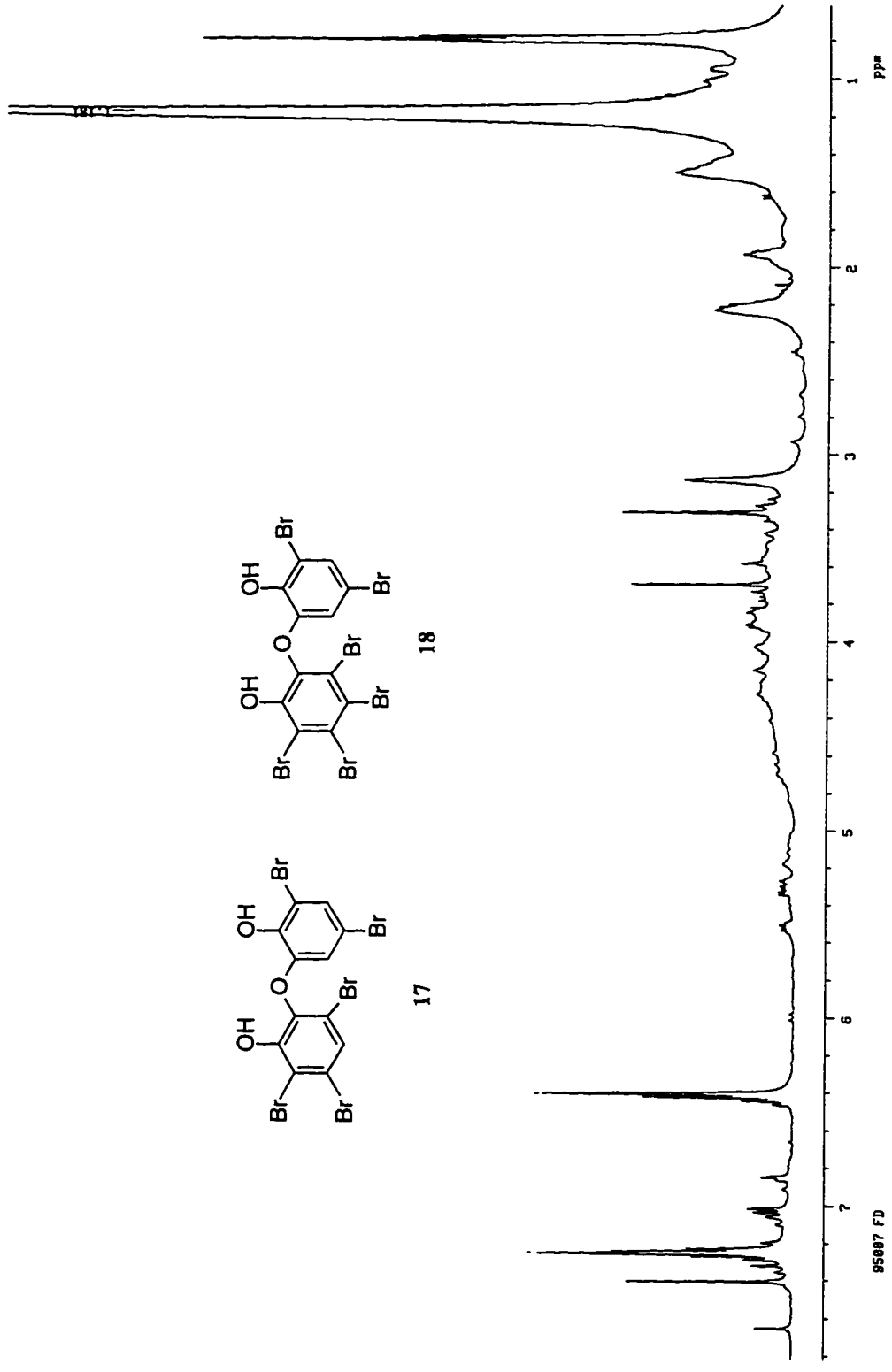

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Figure 5.19. LRESIM Spectra-95007.

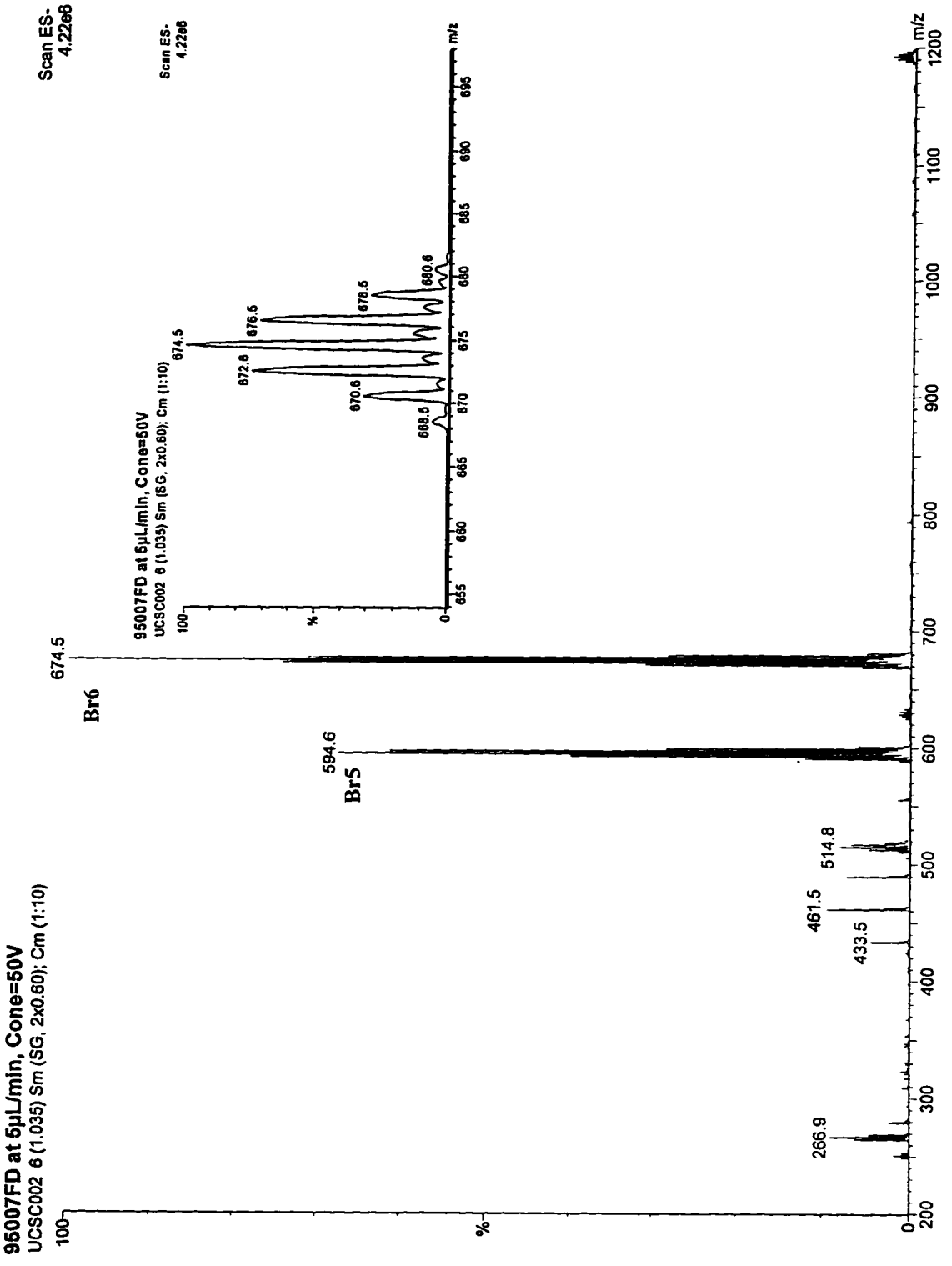

Figure 5.20. ^{1}H-NMR Spectra-95080 ($CDCl_3$).

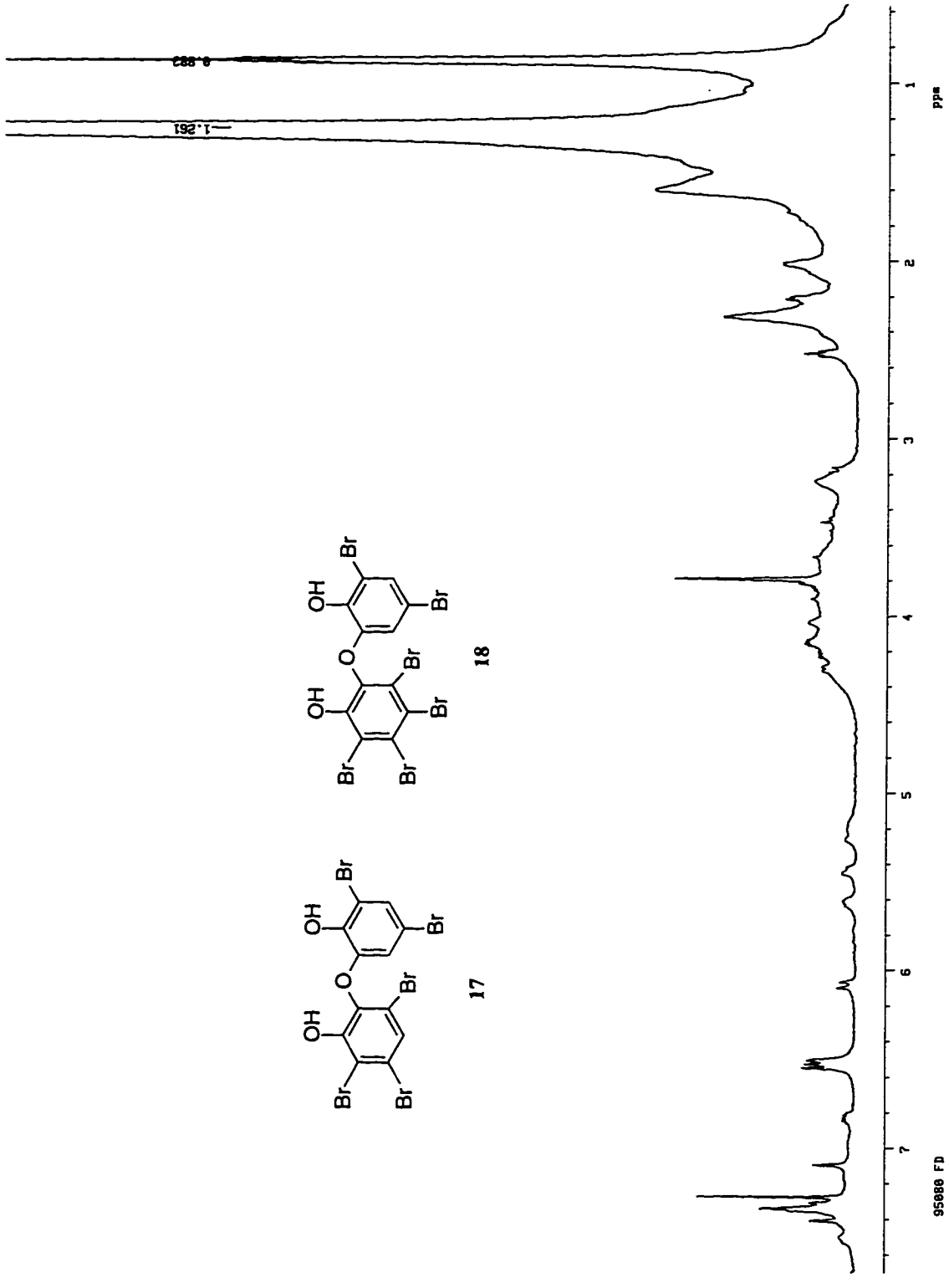

Figure 5.21. LRESIM Spectra-95080.

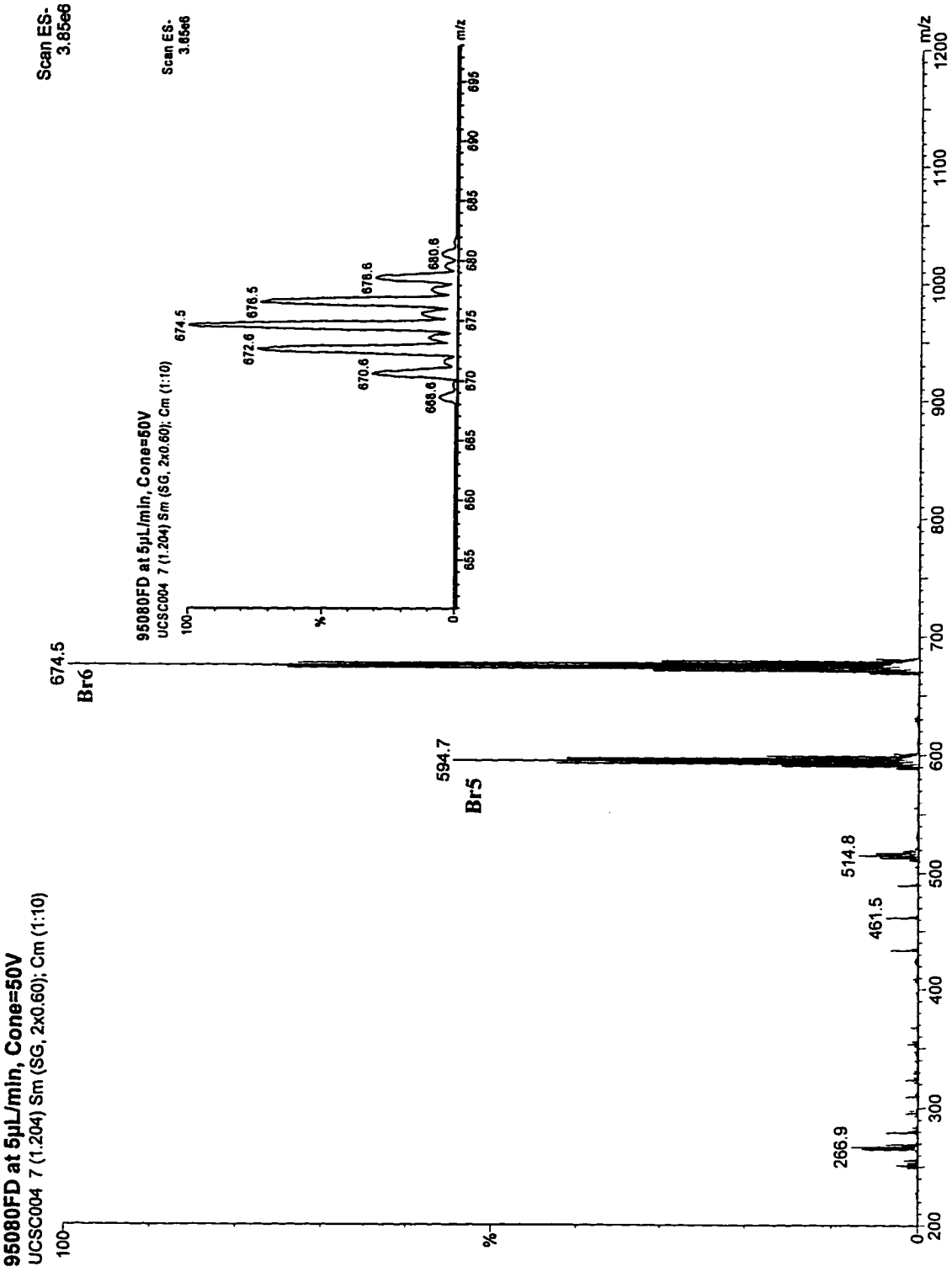

Figure 5.22. ^{1}H-NMR Spectra-95081 ($CDCl_3$).

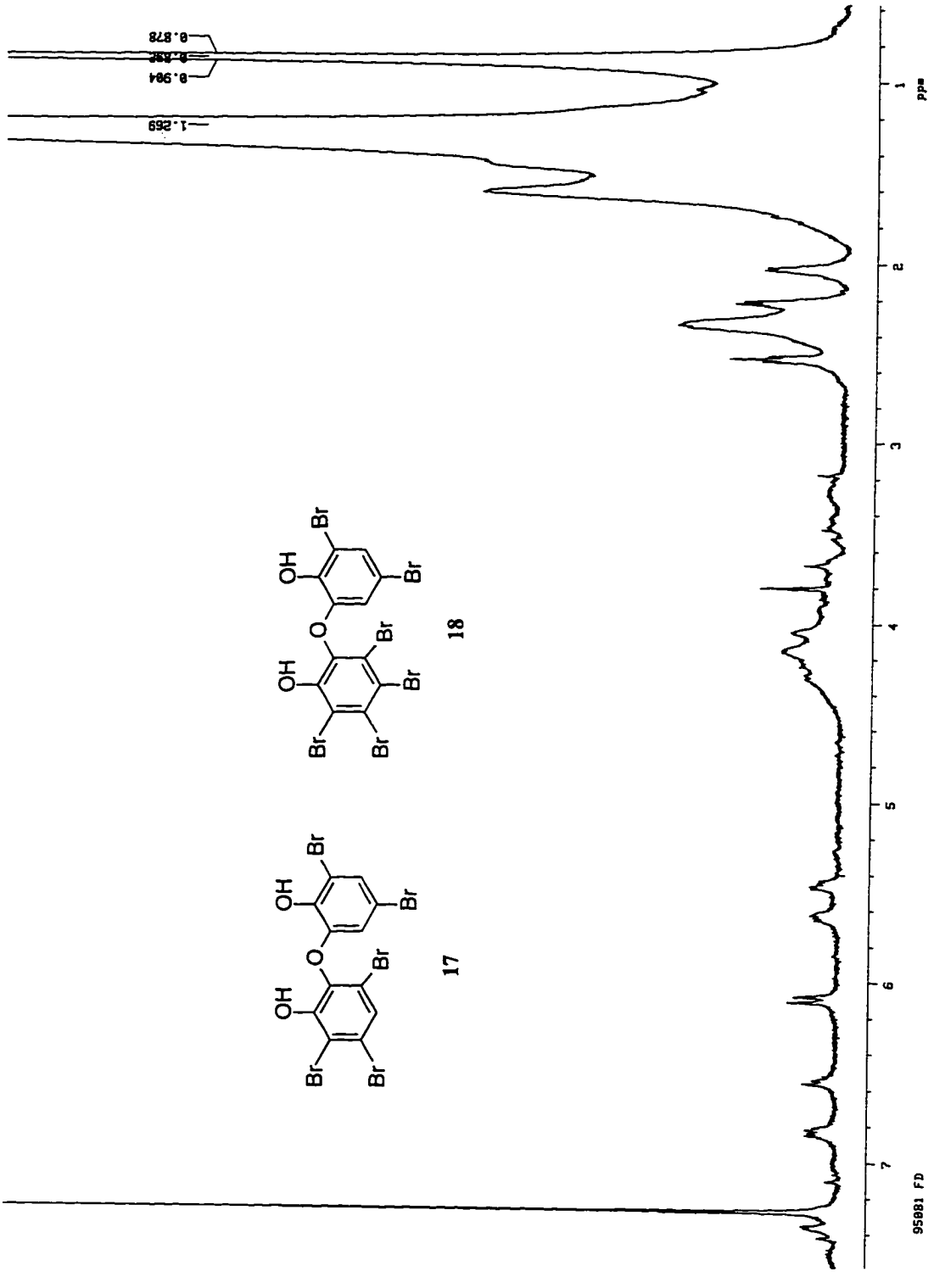

Figure 5.23. ^{1}H-NMR Spectra-95083 ($CDCl_3$).

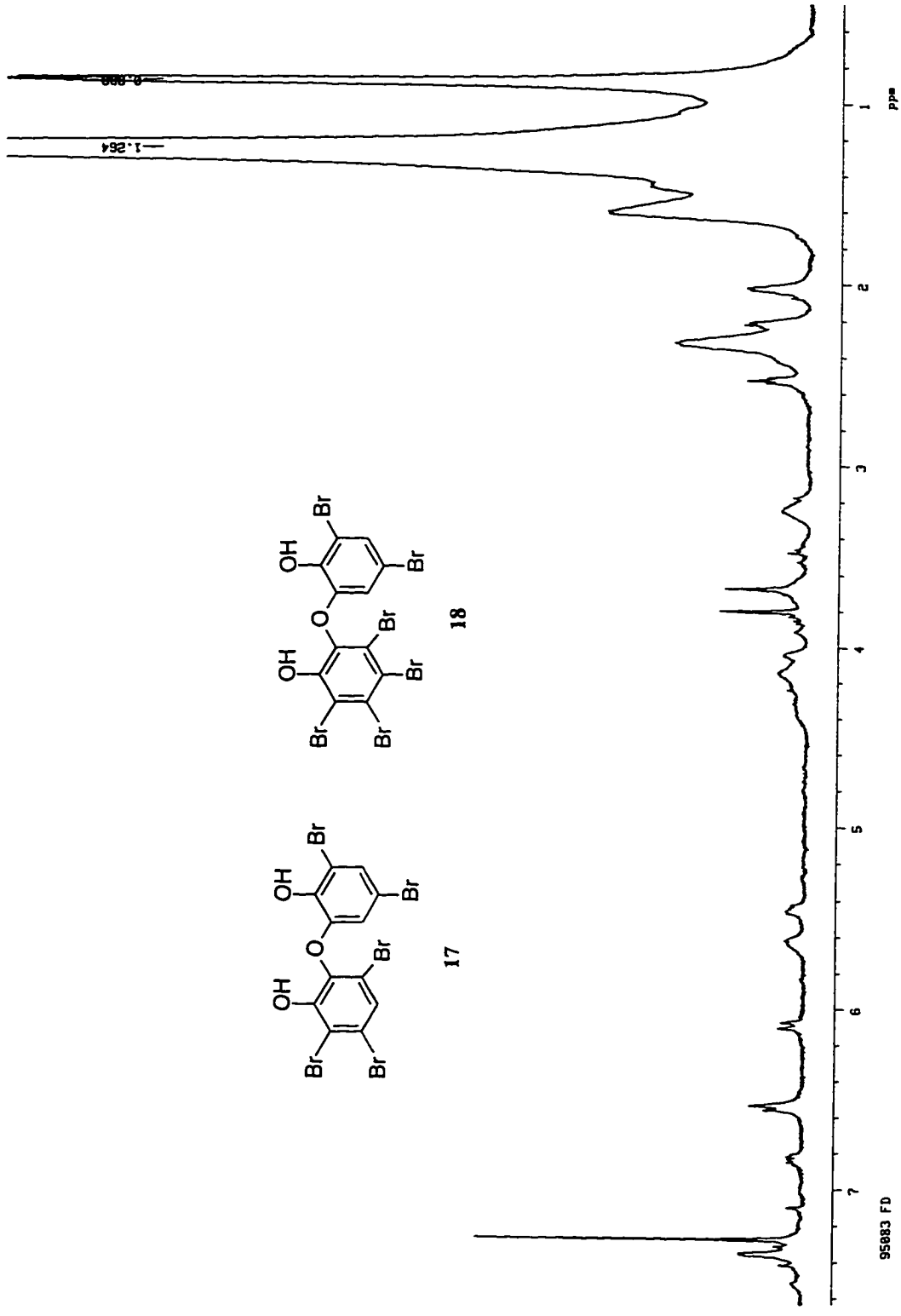

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Figure 5.24. ^{1}H-NMR Spectra-95084 ($CDCl_3$).

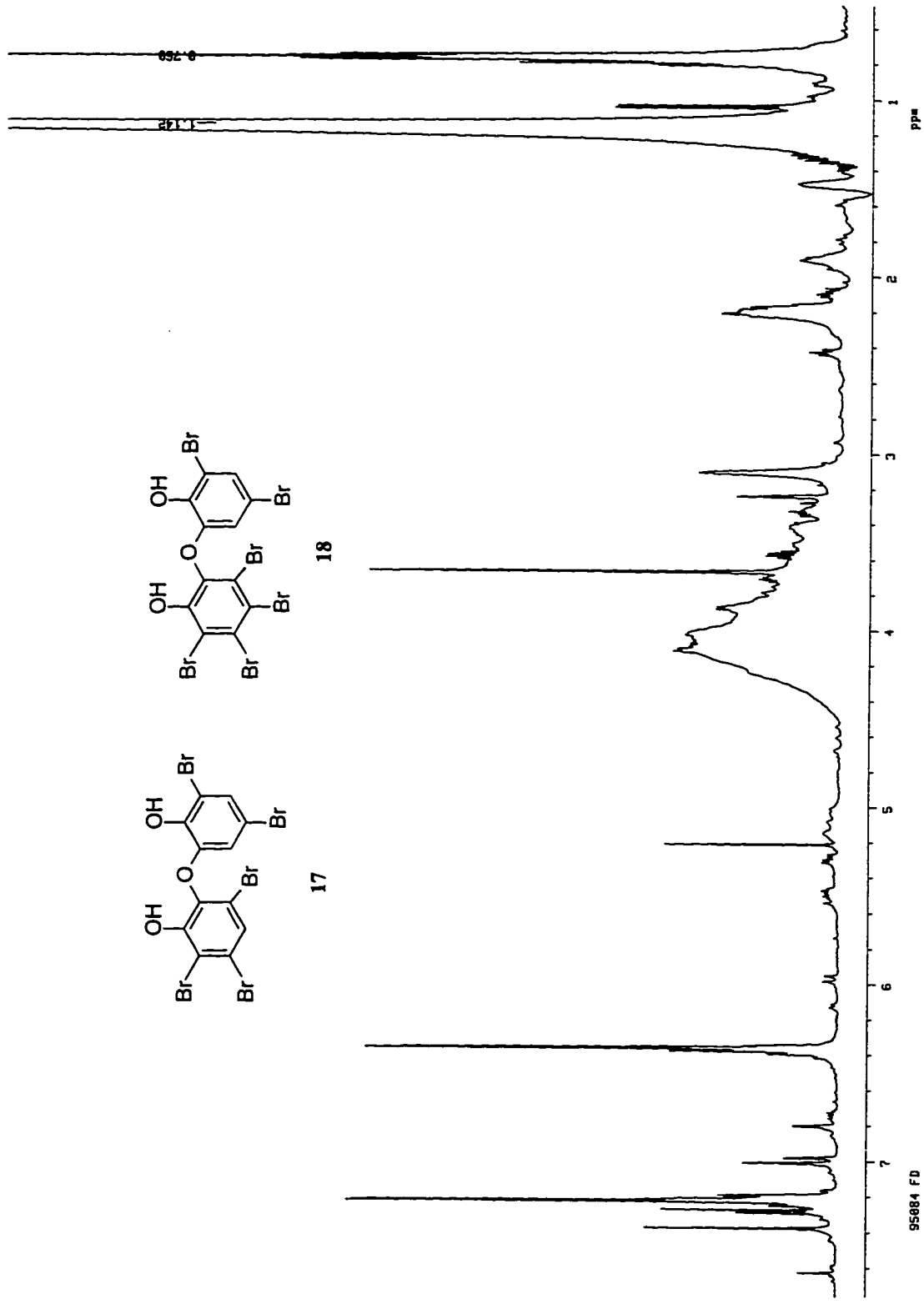

Figure 5.25. LRESIM Spectra-95084.

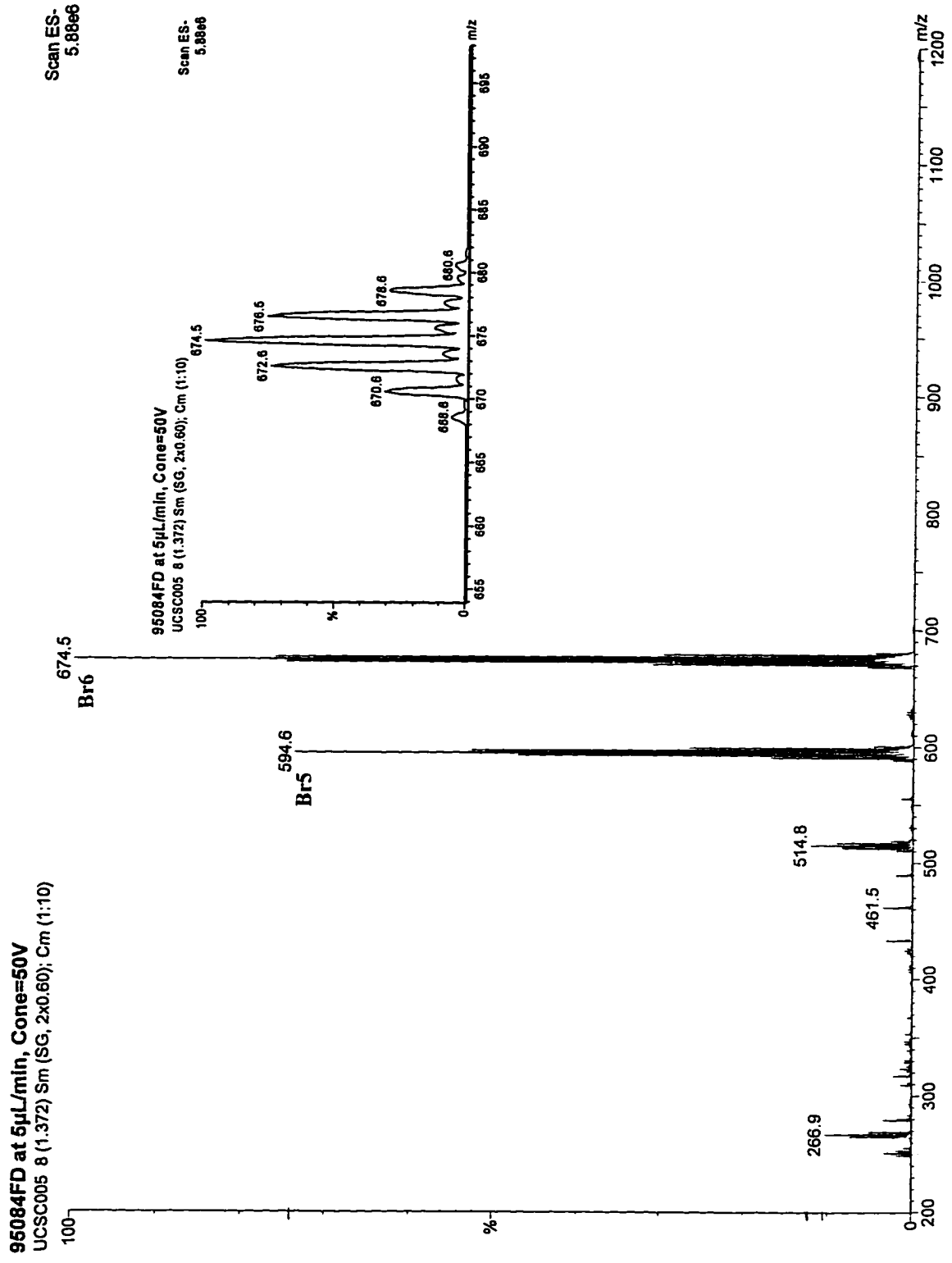

Figure 5.26. ^{1}H-NMR Spectra-95085 ($CDCl_3$).

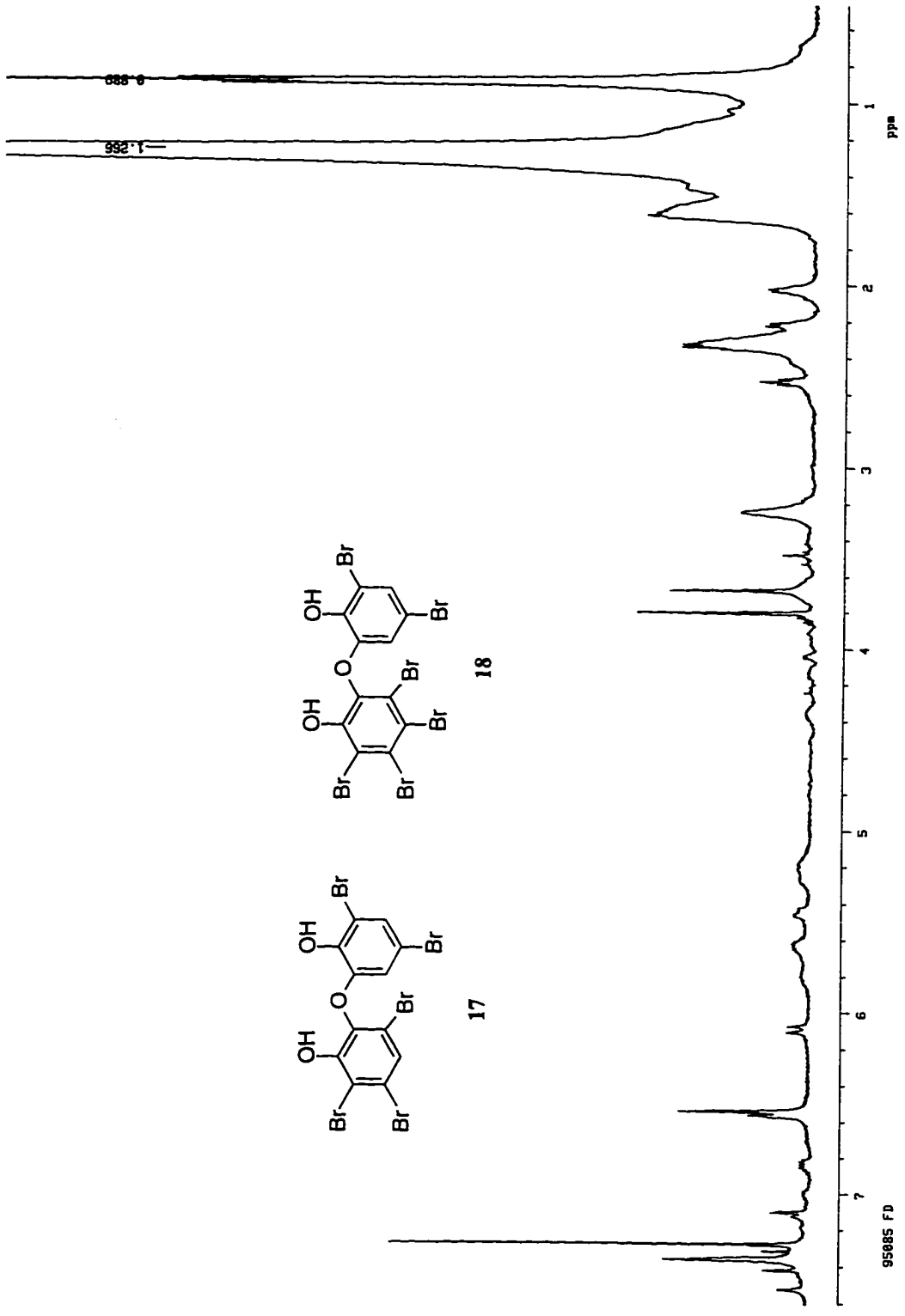

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Figure 5.27. ^{1}H-NMR Spectra-95098 ($CDCl_3$).

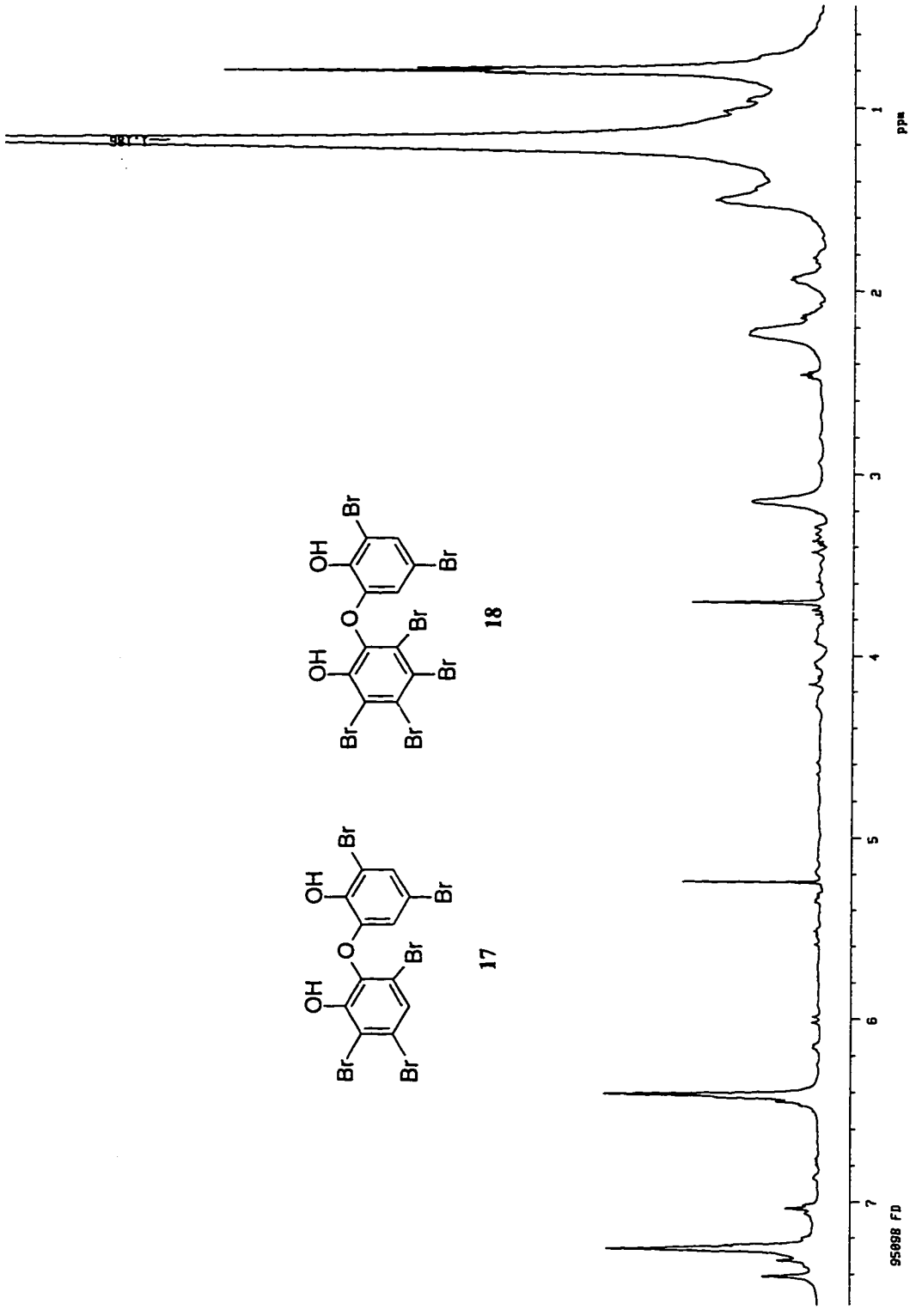

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Figure 5.28. LRESIM Spectra-95098.

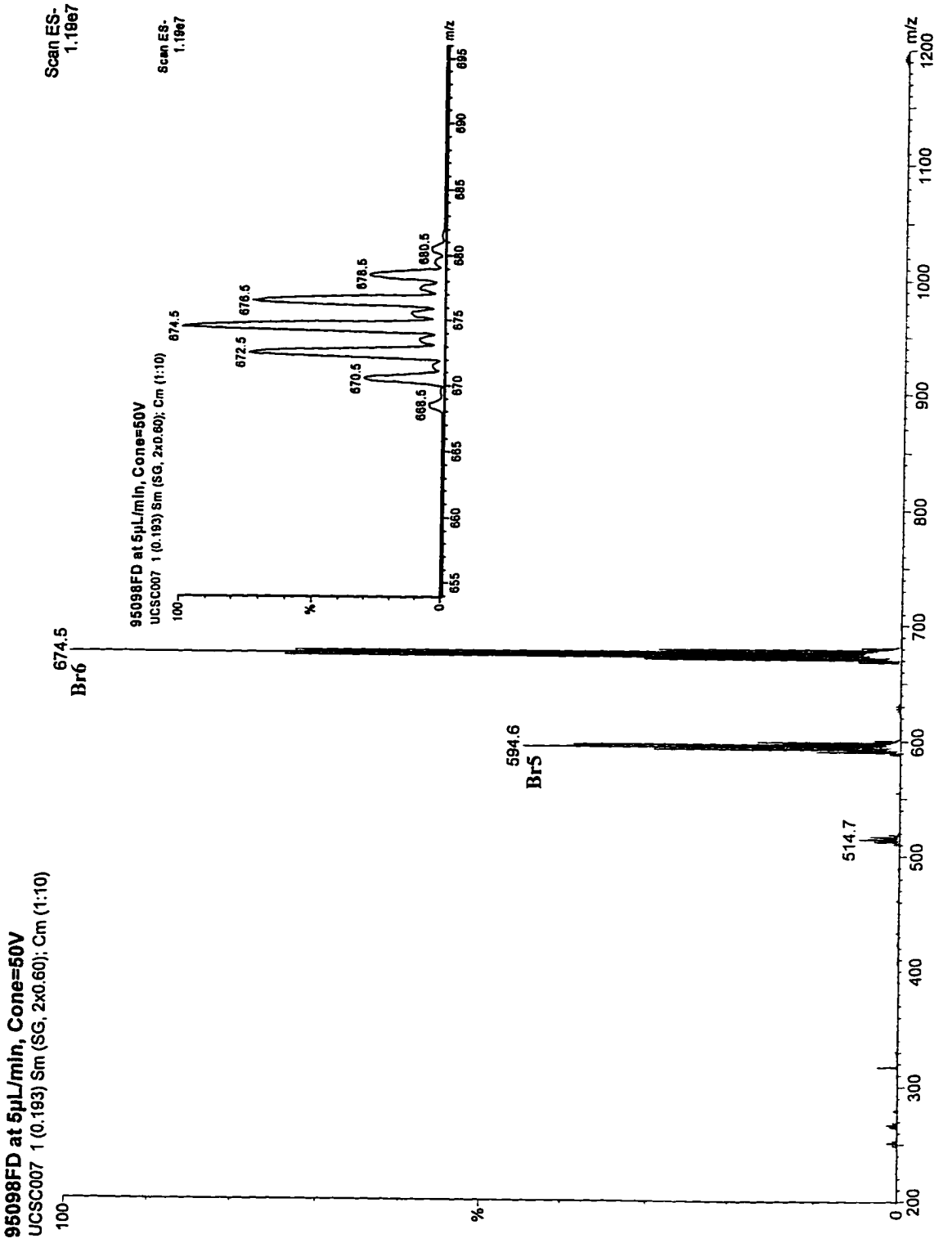


Figure 5.29. ^{1}H-NMR Spectra-95154 ($CDCl_3$).

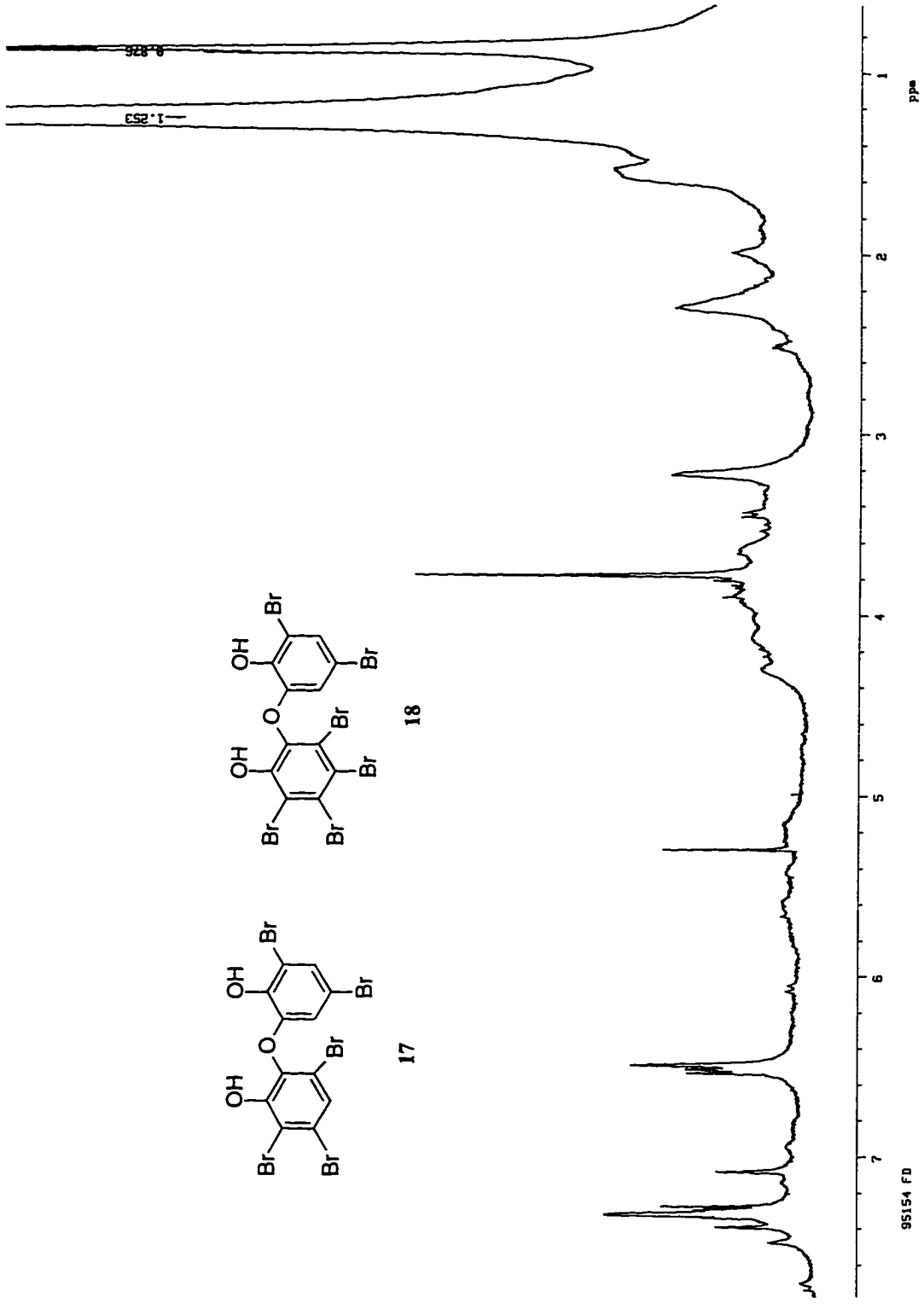


Figure 5.30. ^{1}H-NMR Spectra-95661 (CD_3OD).

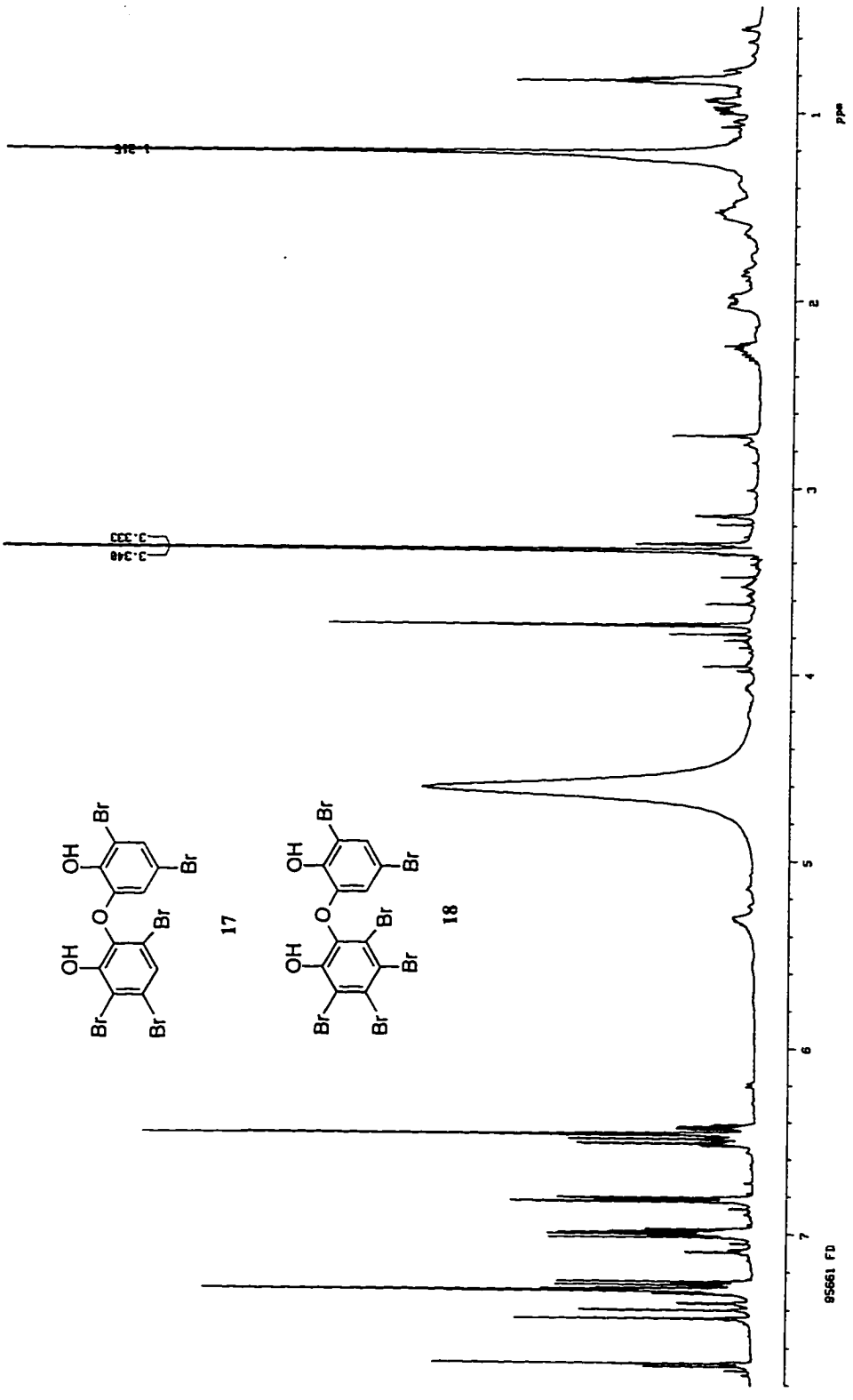
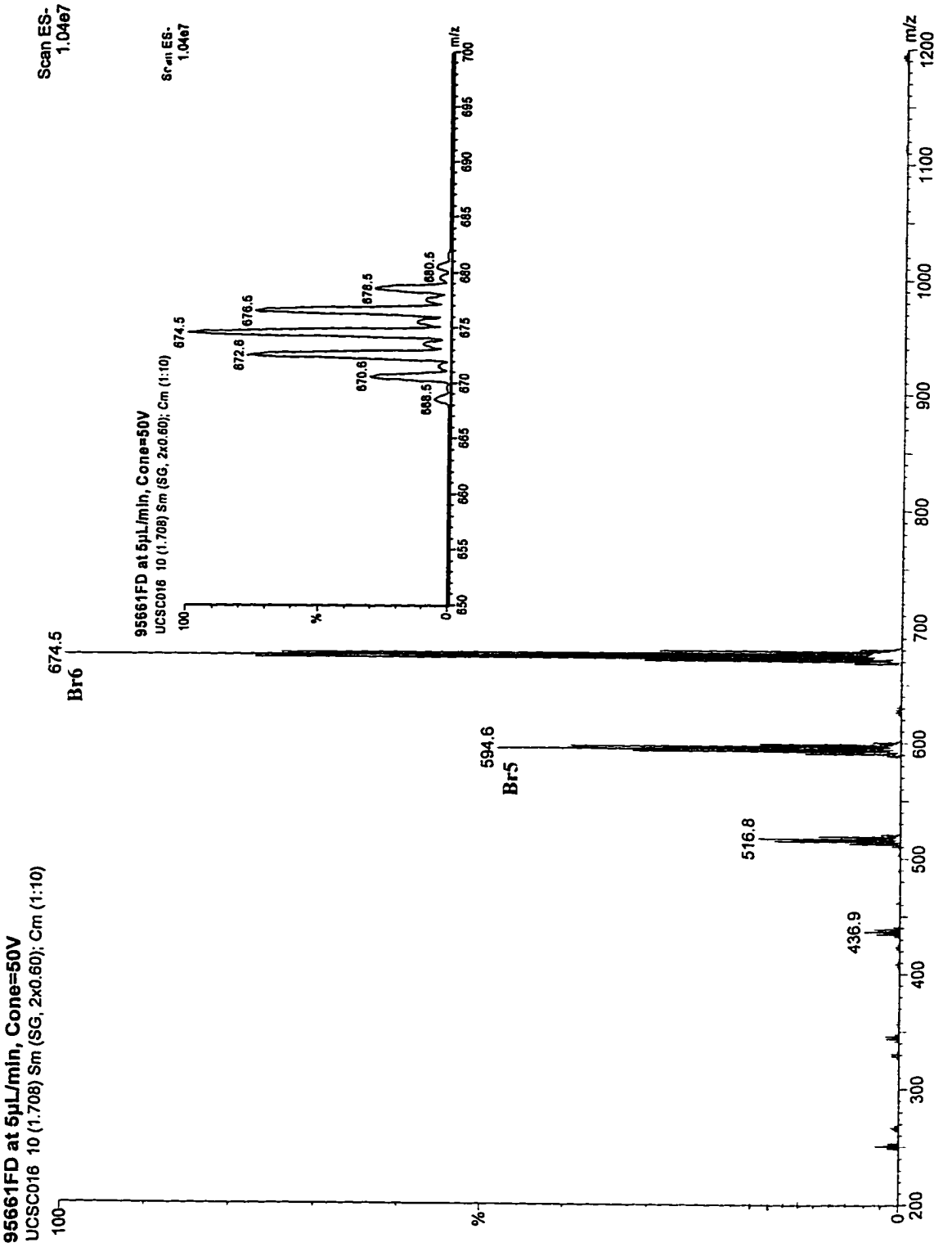


Figure 5.31. LRESIM Spectra-95661.



Supplementary Material
Morphotype II

Figure 5.32. ^{1}H-NMR Spectra-94601 ($CDCl_3$).

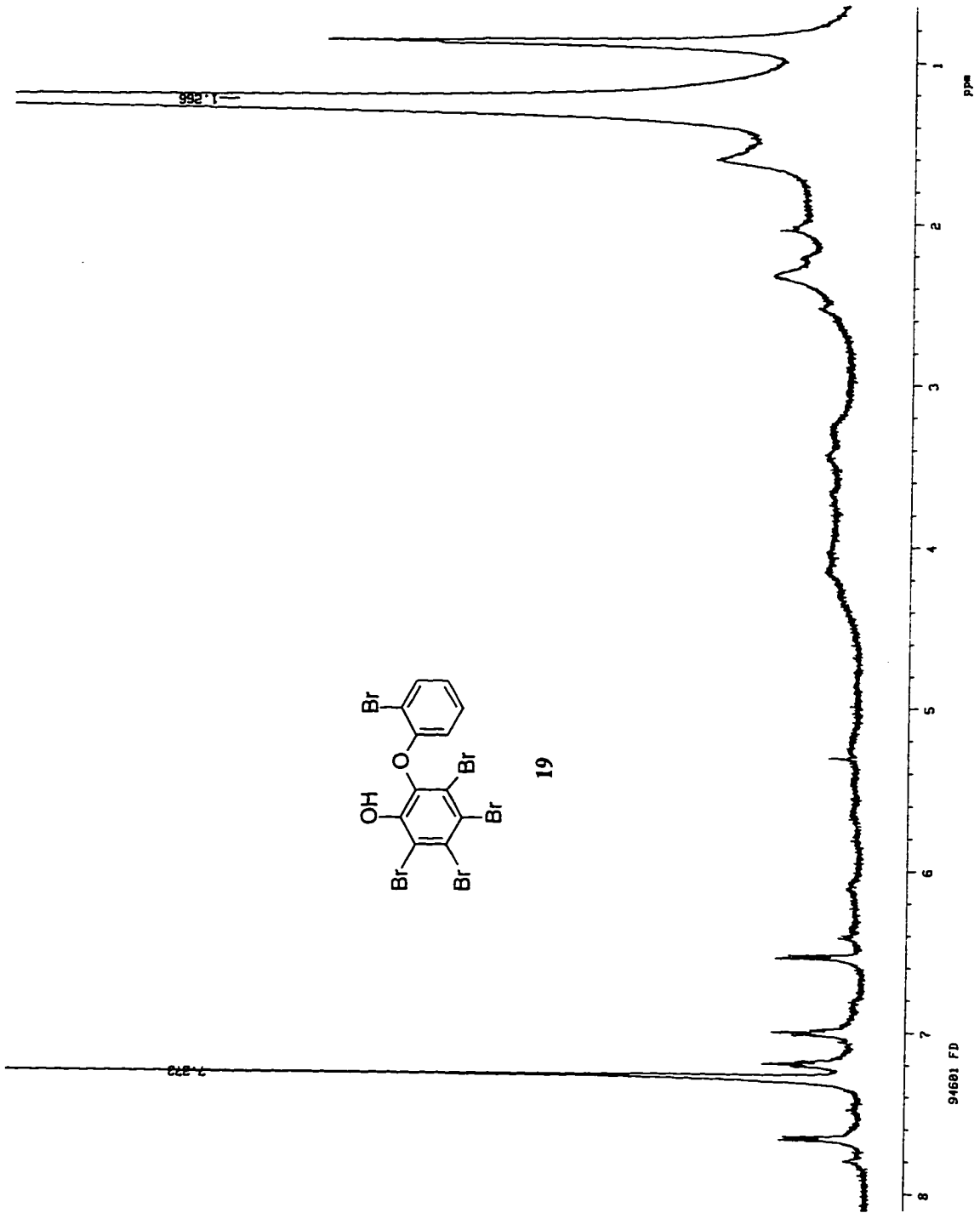


Figure 5.33. LRESIM Spectra-94601.

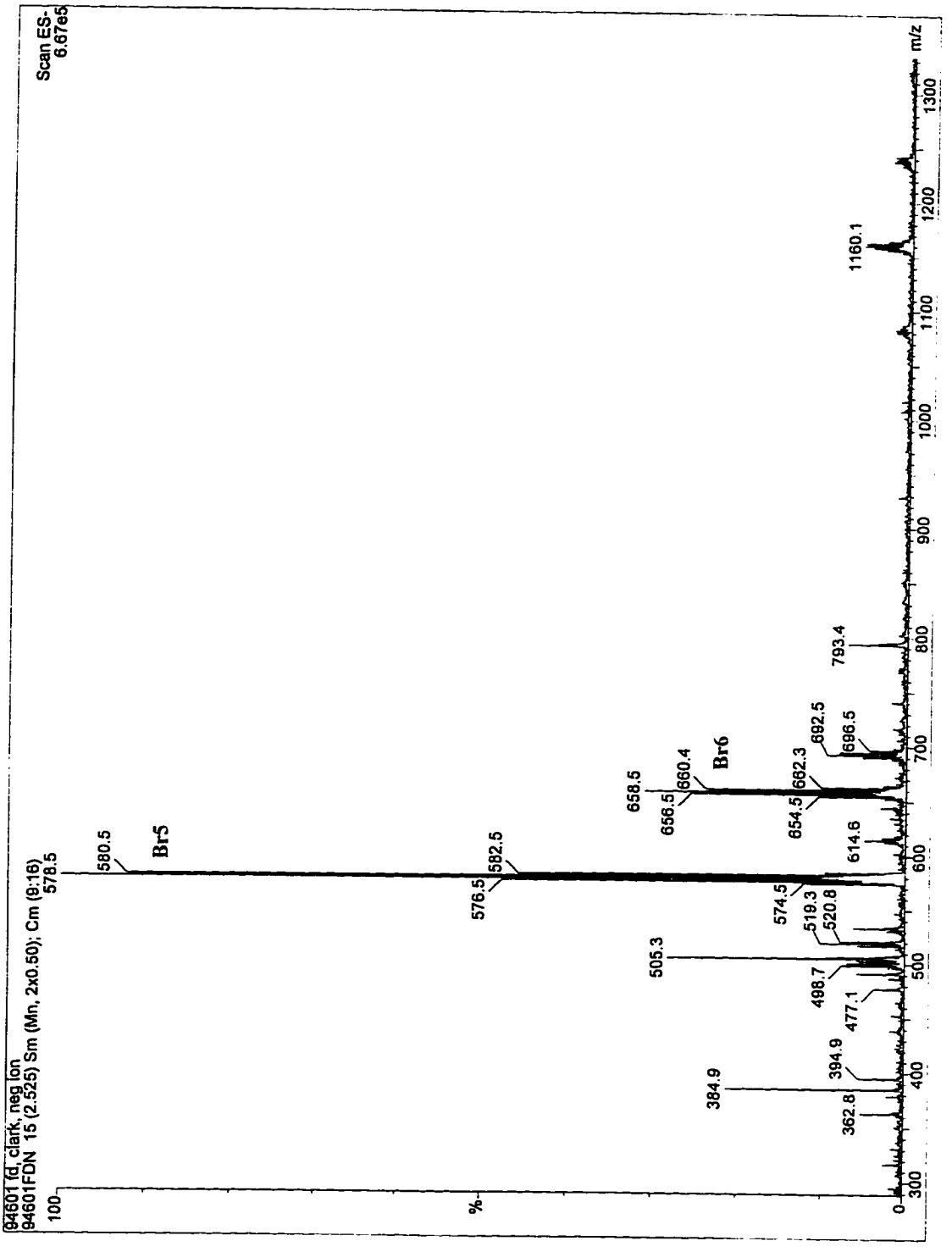


Figure 5.34. ^{1}H-NMR Spectra-94603 ($CDCl_3$).

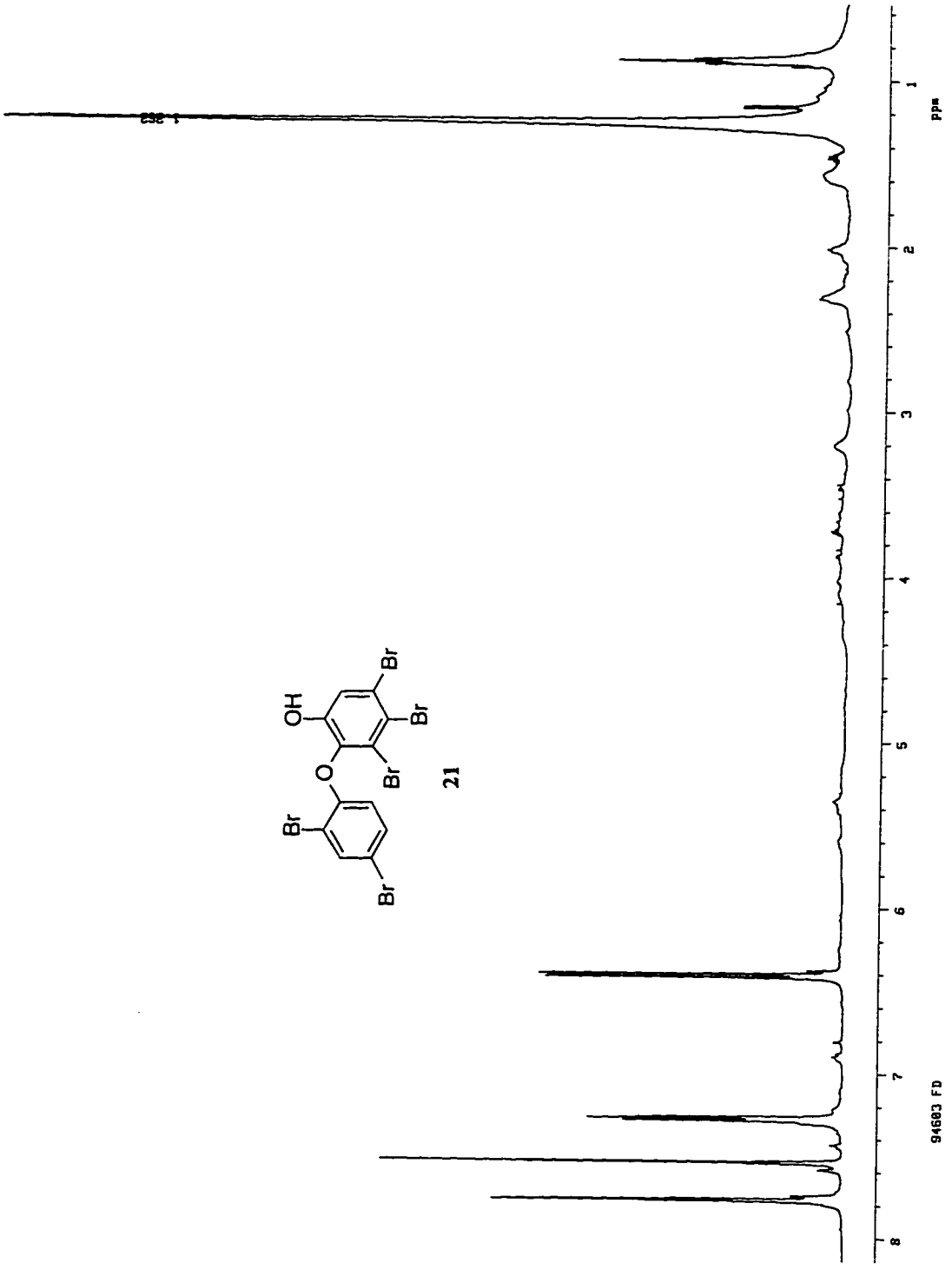


Figure 5.35. ^{1}H-NMR Spectra-94604 ($CDCl_3$).

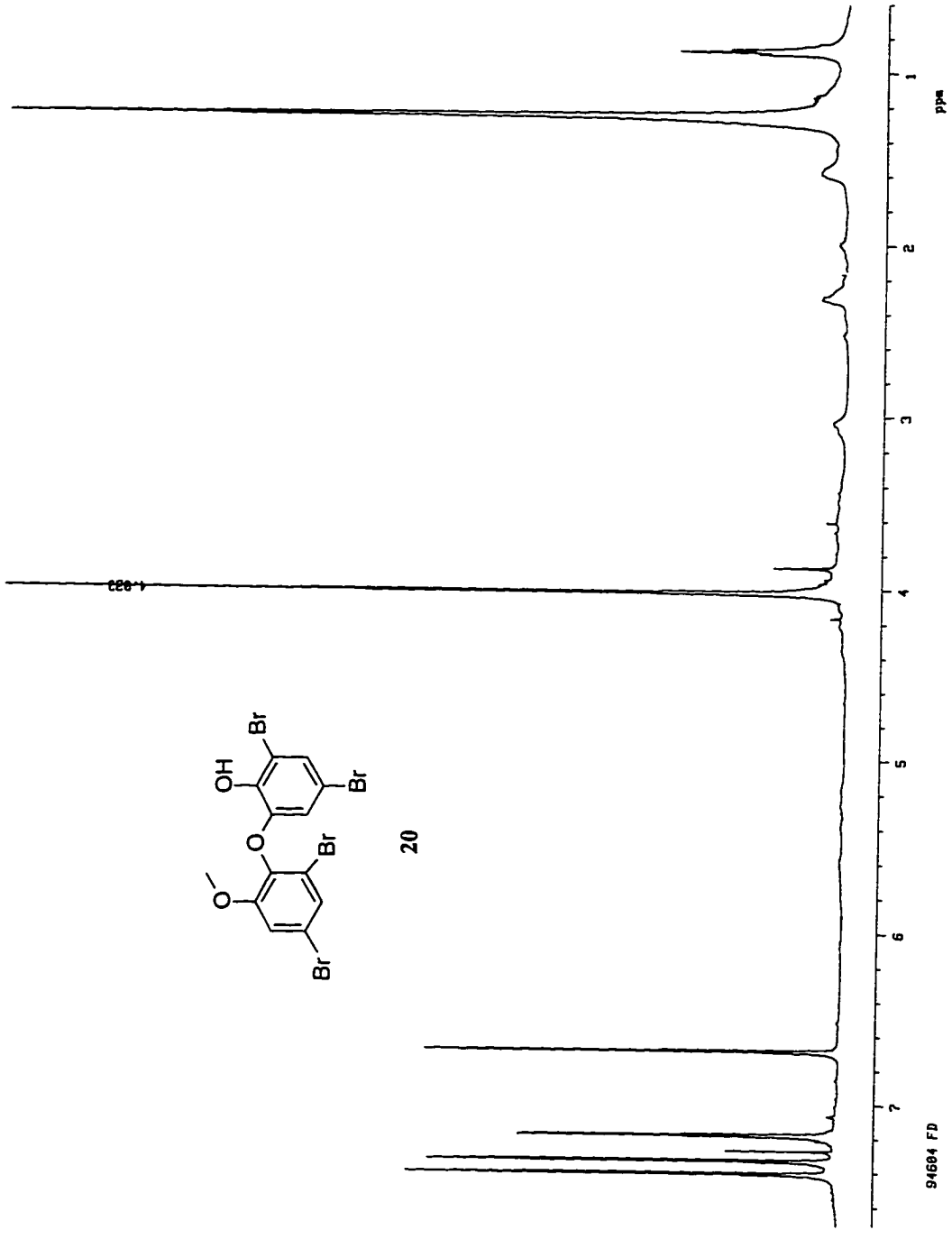


Figure 5.36. LRESIM Spectra-94604.

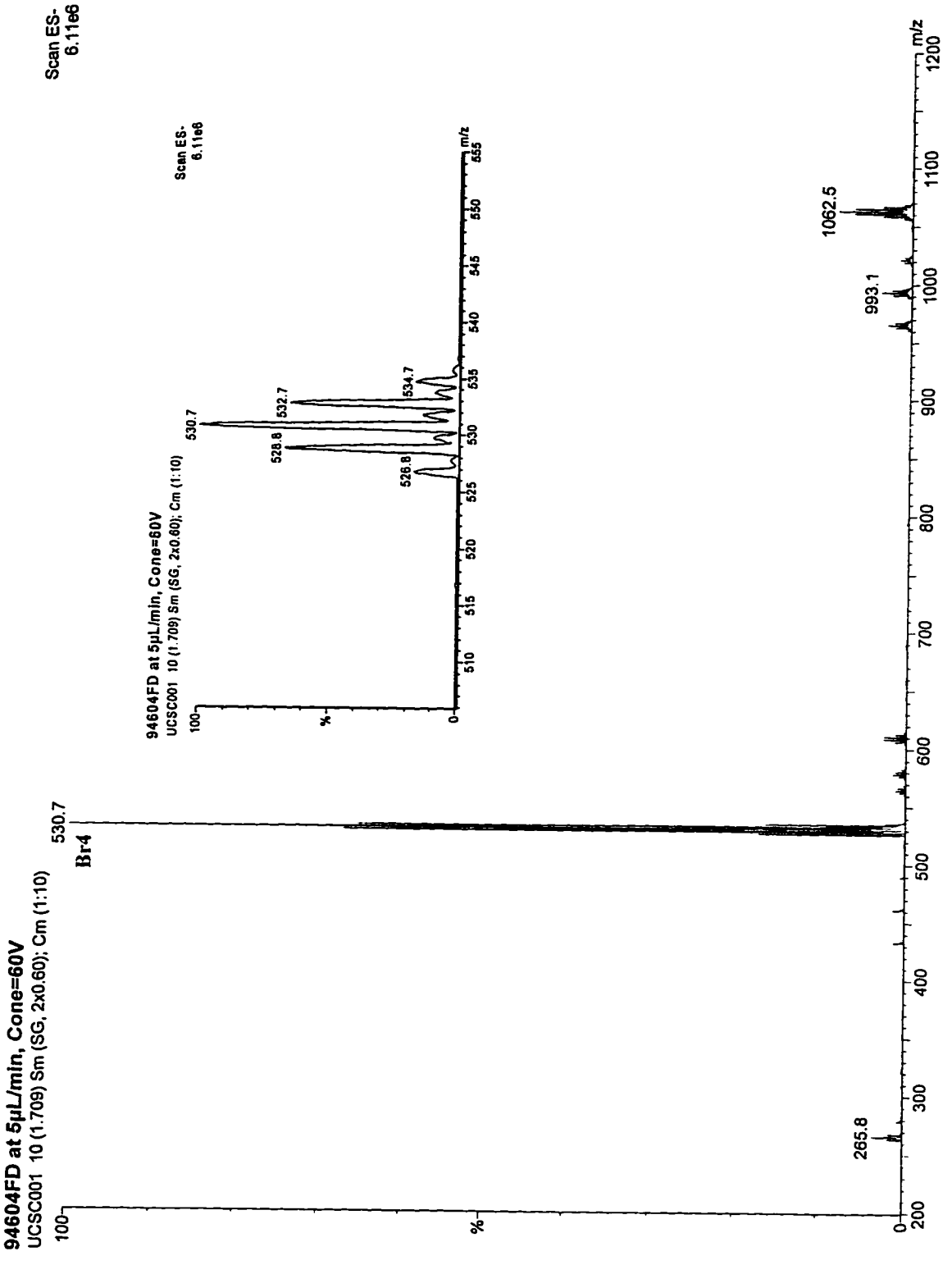


Figure 5.37. ^{1}H-NMR Spectra-94605 ($CDCl_3$).

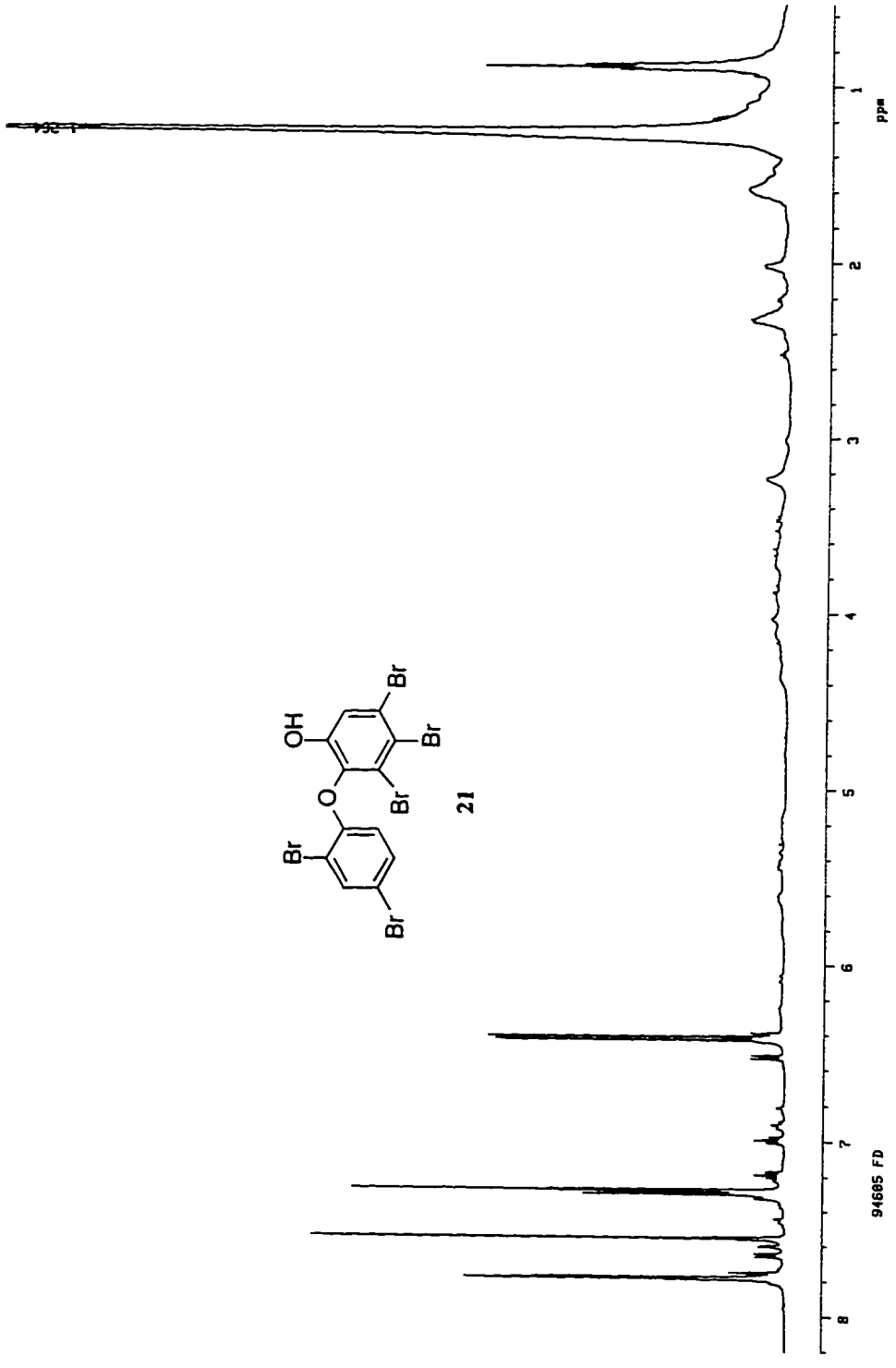


Figure 5.38. ^{1}H-NMR Spectra-94608 ($CDCl_3$).

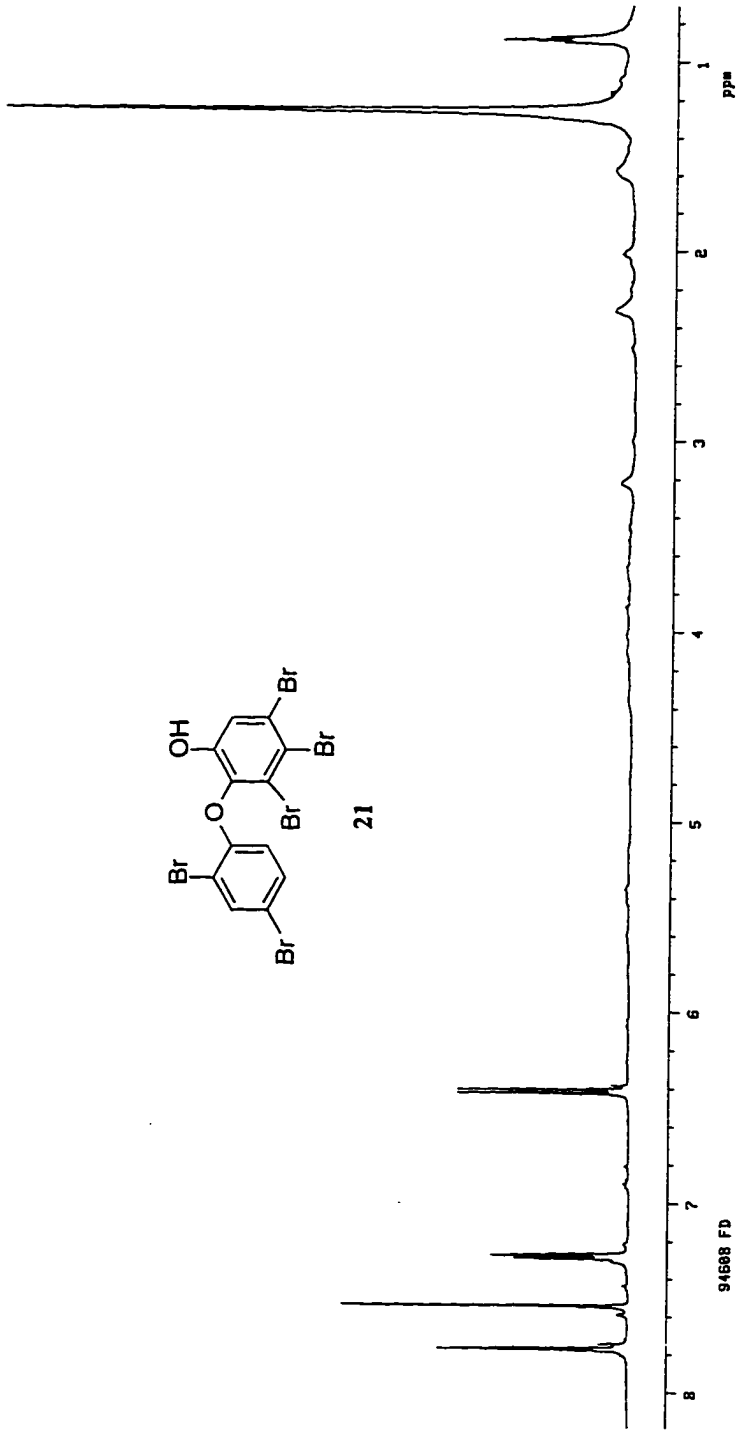


Figure 5.39. ^{1}H-NMR Spectra-94626 ($CDCl_3$).

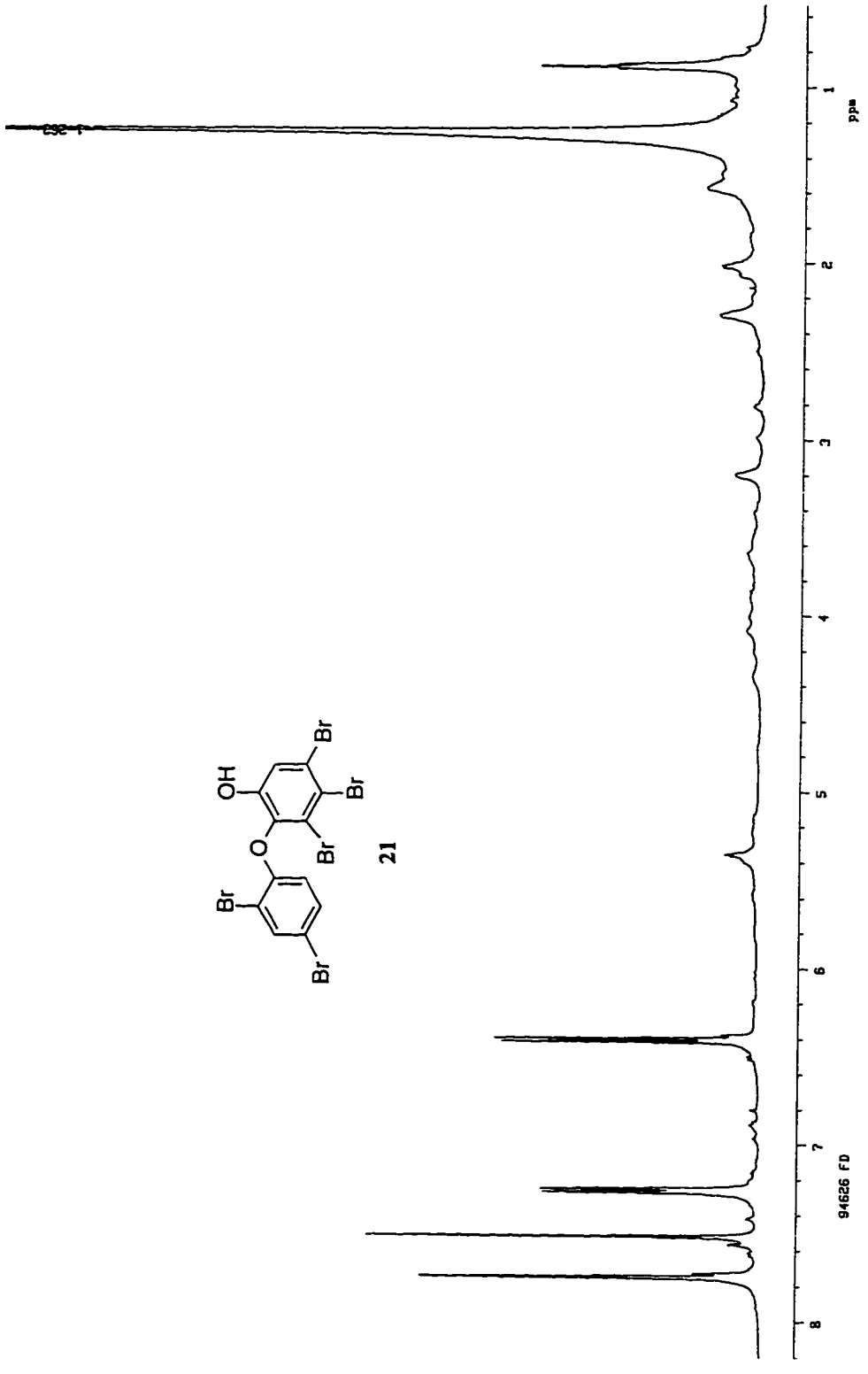


Figure 5.40. LRESIM Spectra-94626.

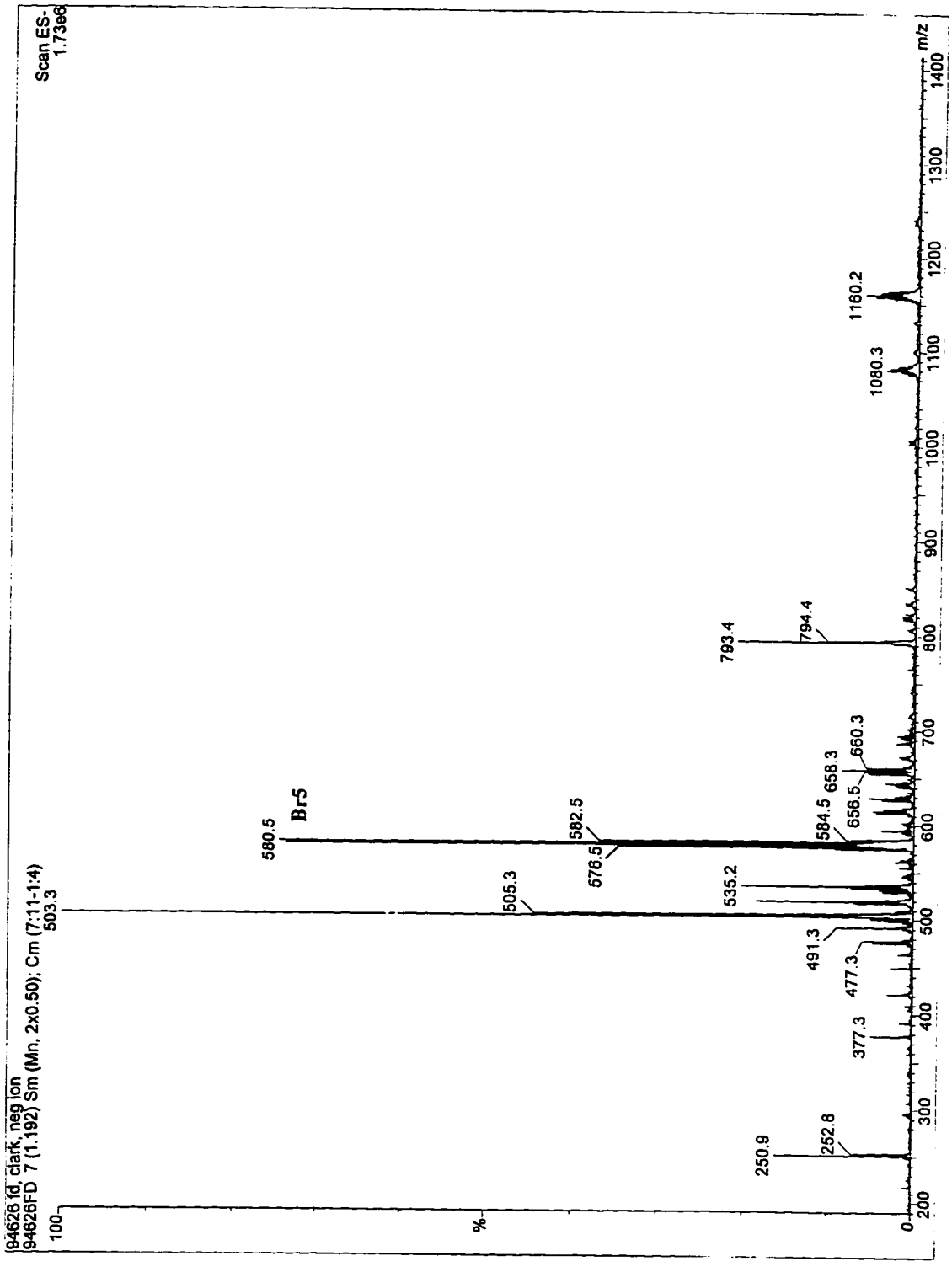


Figure 5.41. ^{1}H-NMR Spectra-94631 ($CDCl_3$).

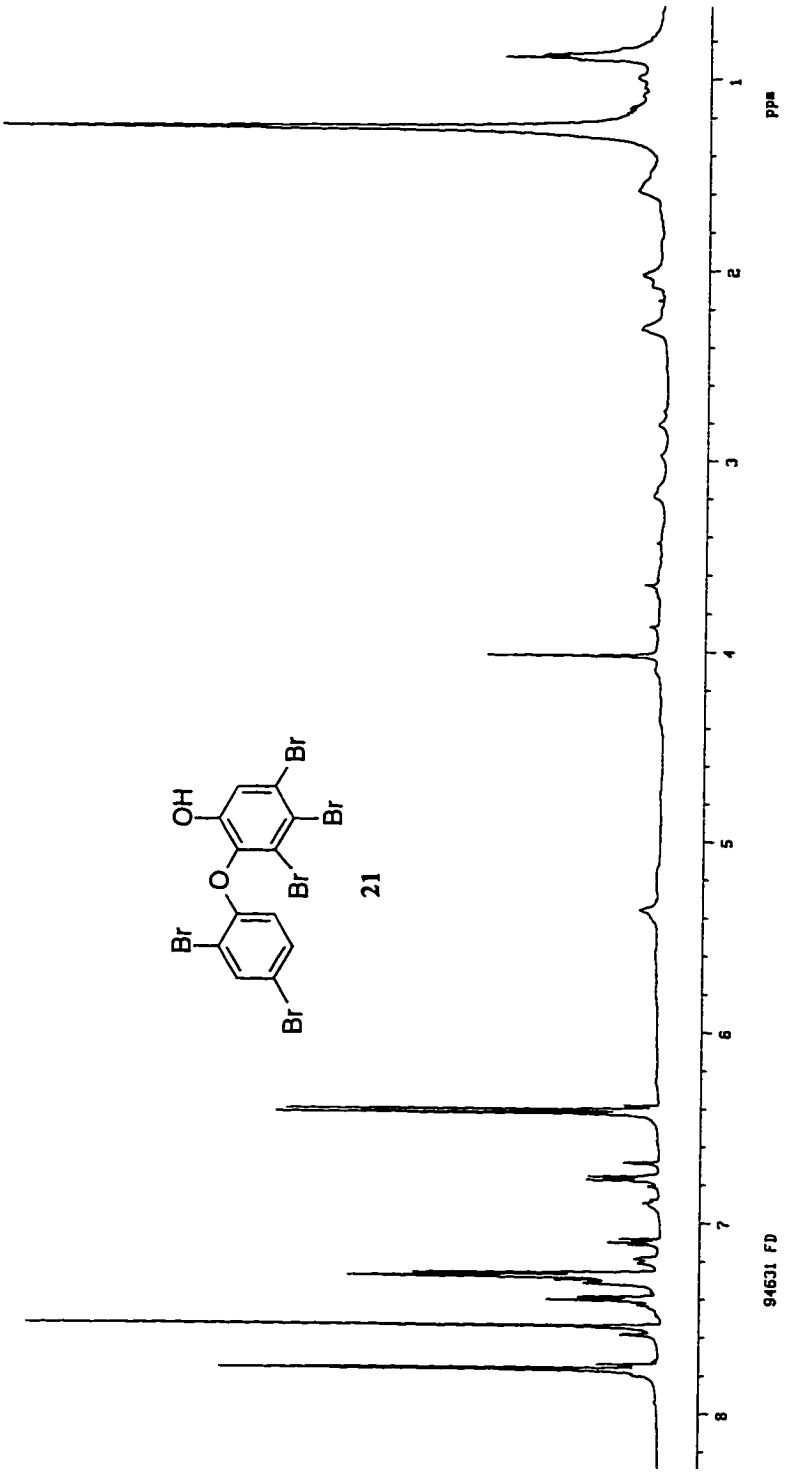


Figure 5.42. ^{1}H-NMR Spectra-94632 ($CDCl_3$).

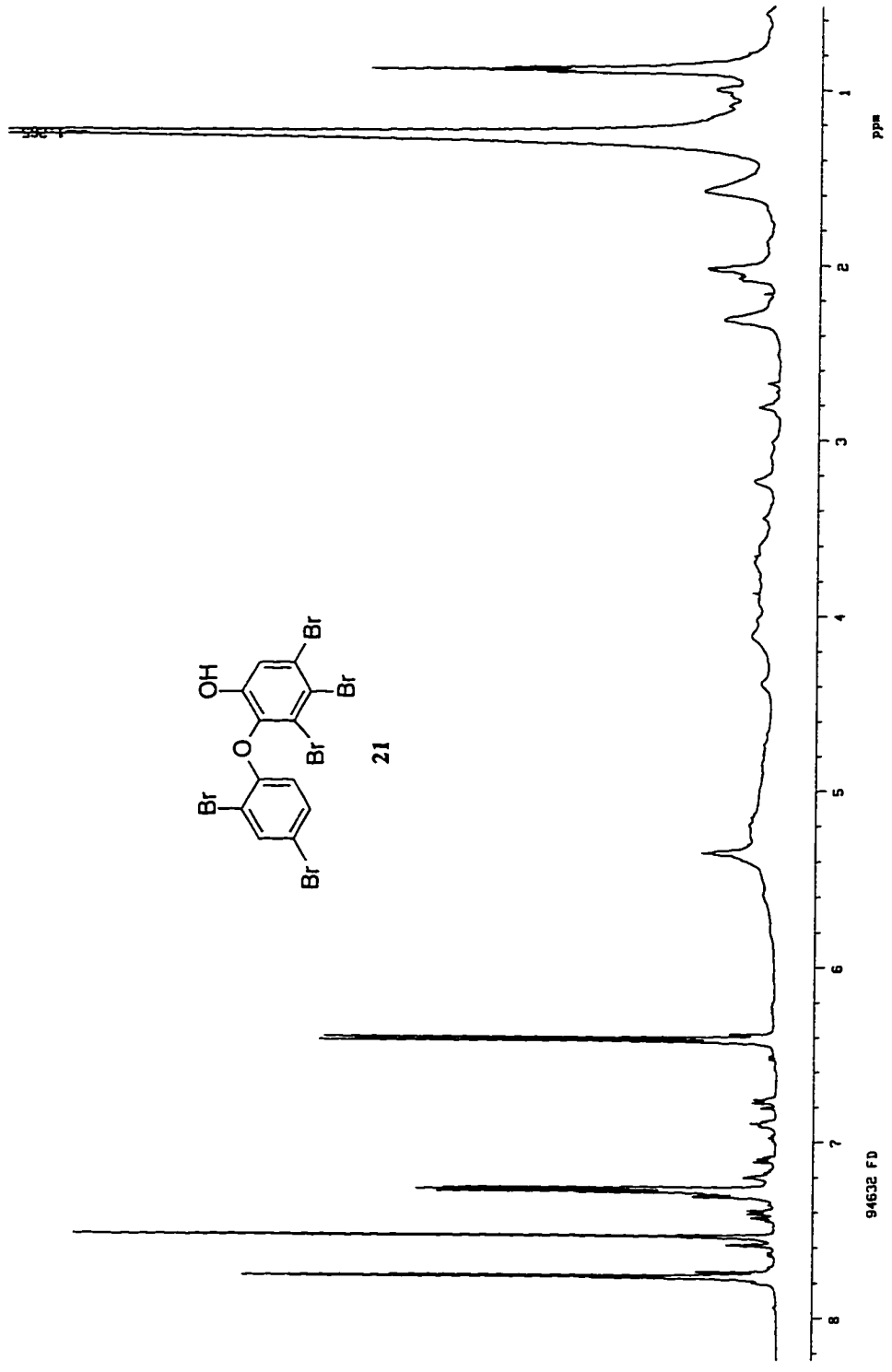


Figure 5.43. ^{1}H-NMR Spectra-95594 ($CDCl_3$).

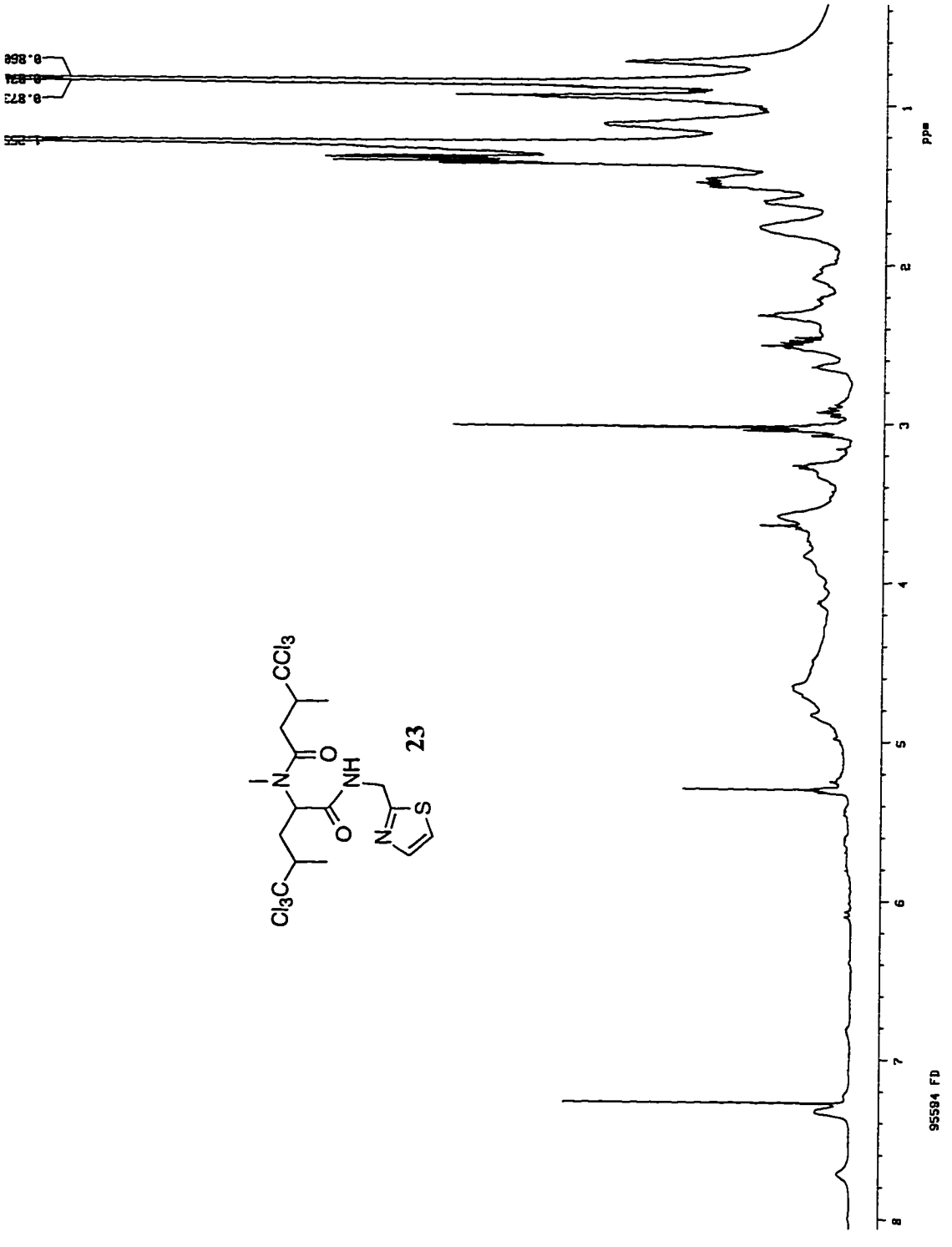


Figure 5.44. LRESIM Spectra-95594.

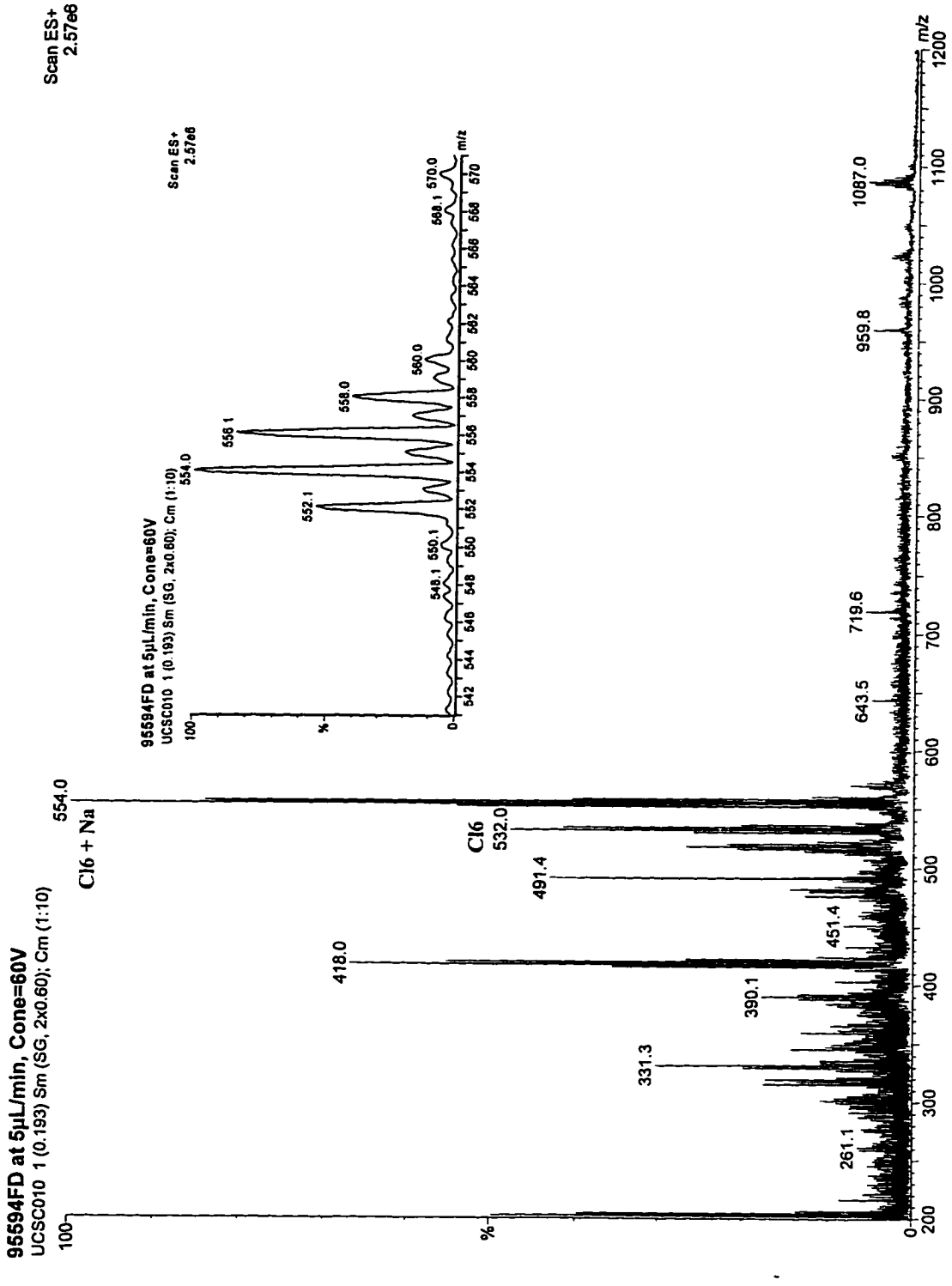


Figure 5.45. ^{1}H-NMR Spectra-95595 (CD_3OD).

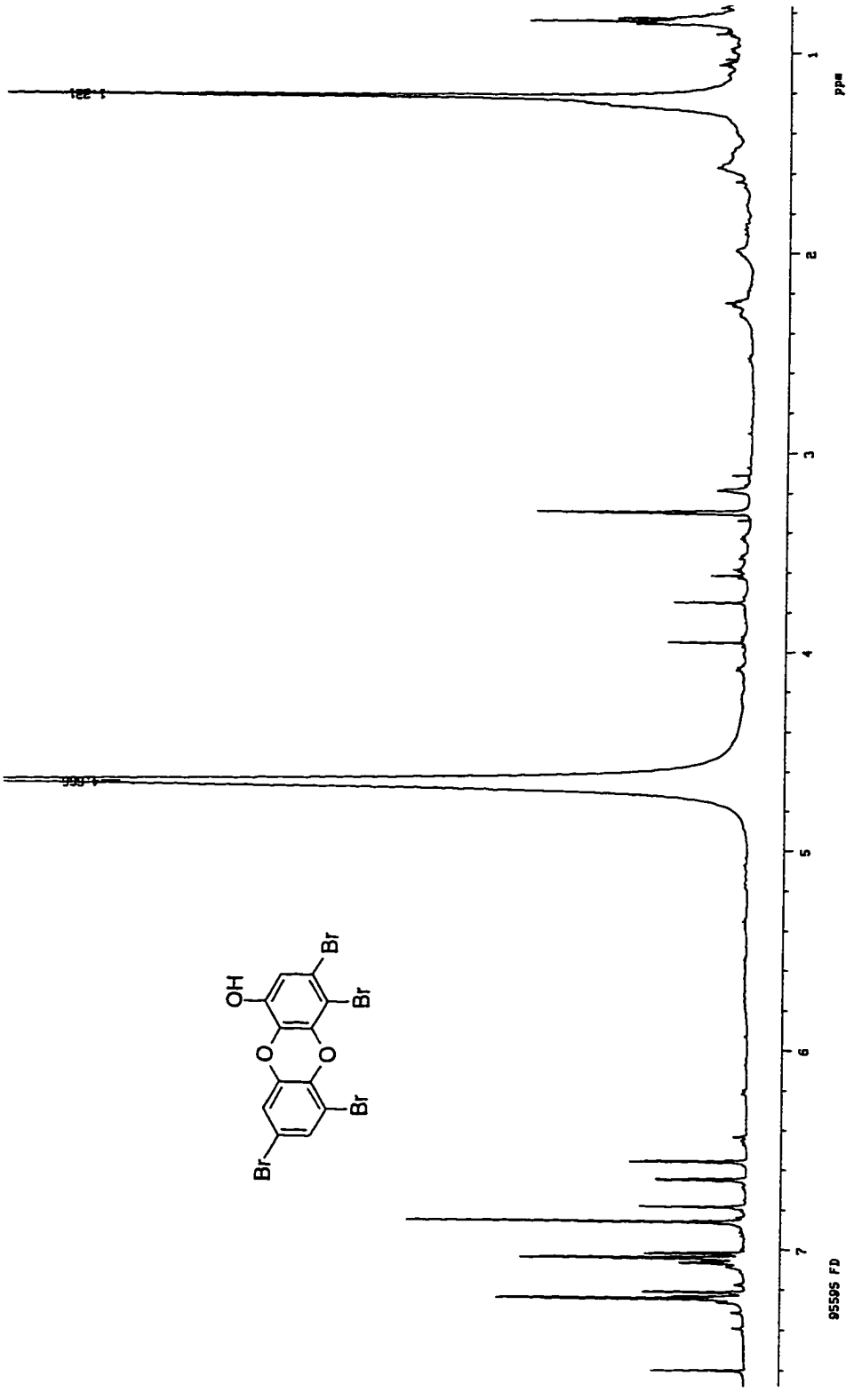


Figure 5.46. LRESIM Spectra-95595.

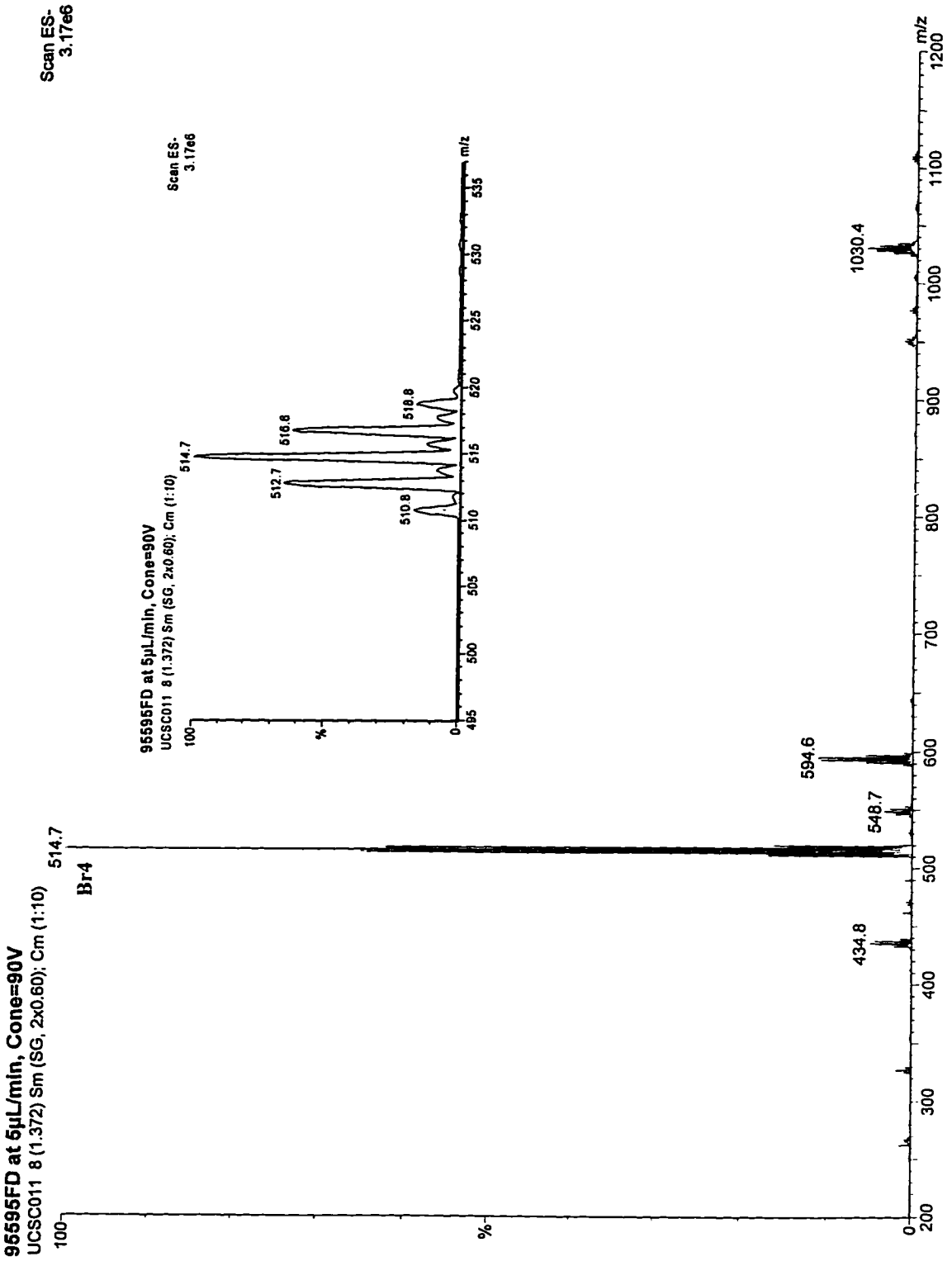


Figure 5.47. ^{1}H-NMR Spectra-95596 ($CDCl_3$).

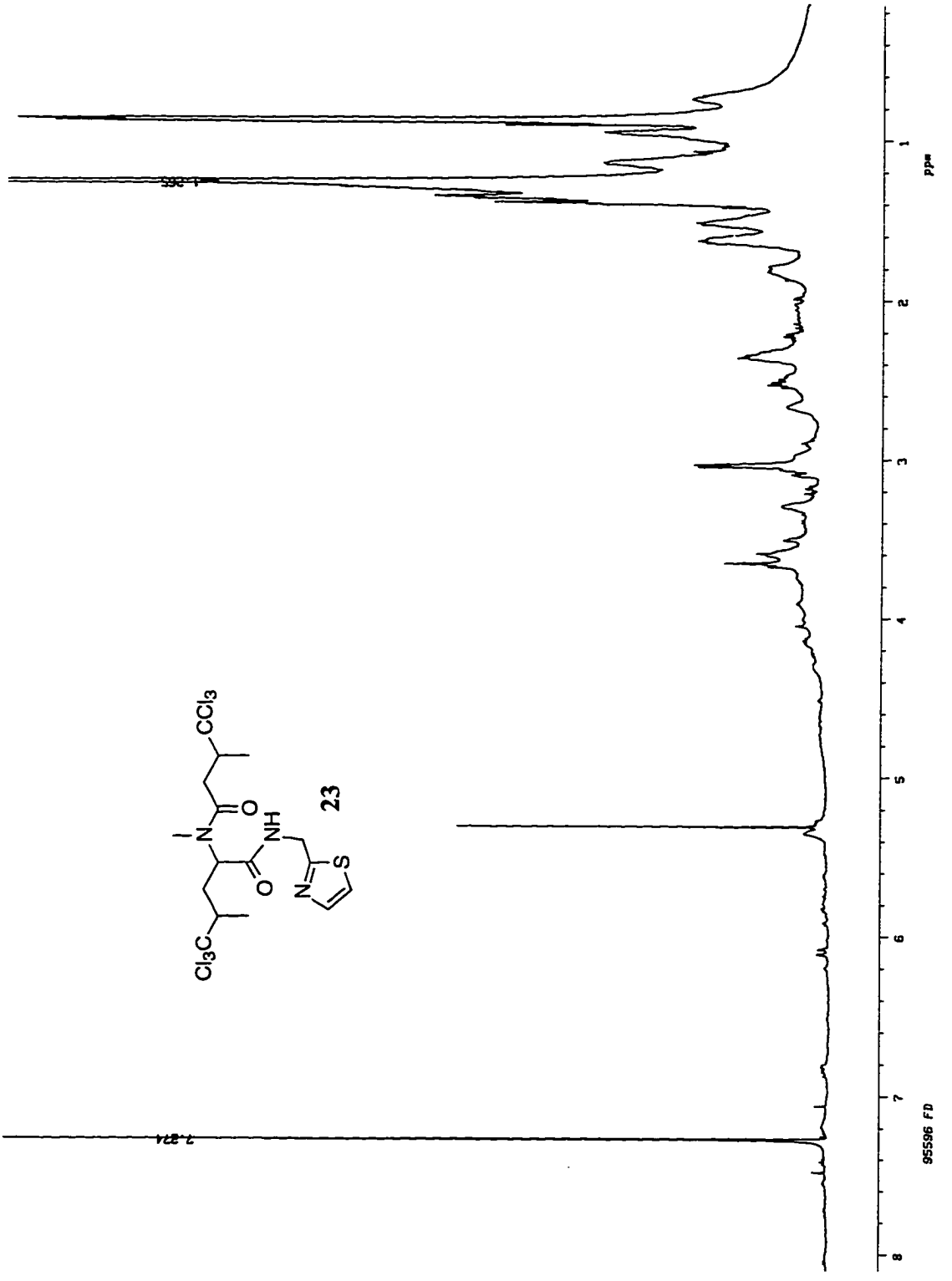
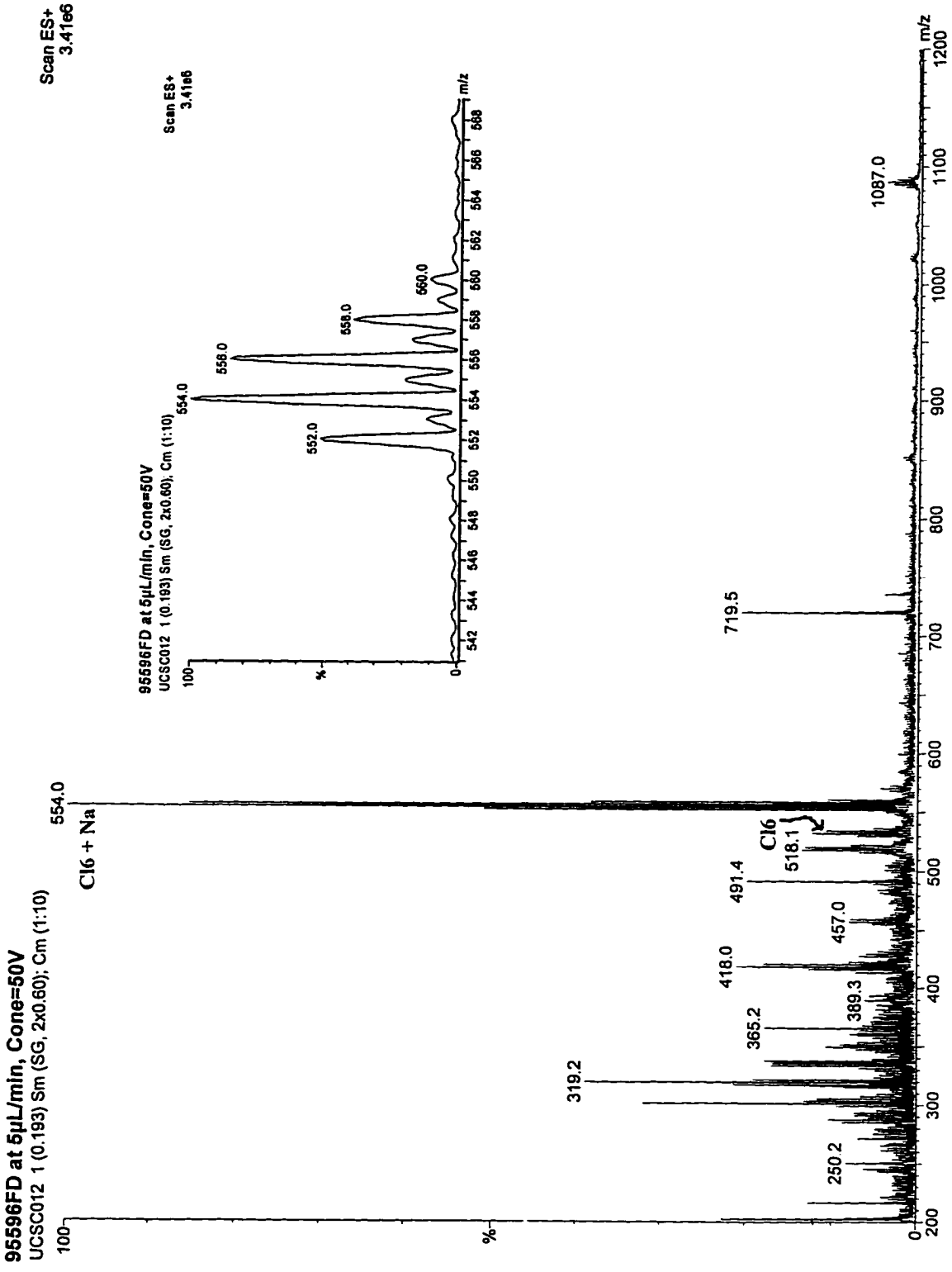


Figure 5.48. LRESIM Spectra-95596.



Supplementary Material
Morphotype III

Figure 5.49. ^{1}H-NMR Spectra-94555 ($CDCl_3$).

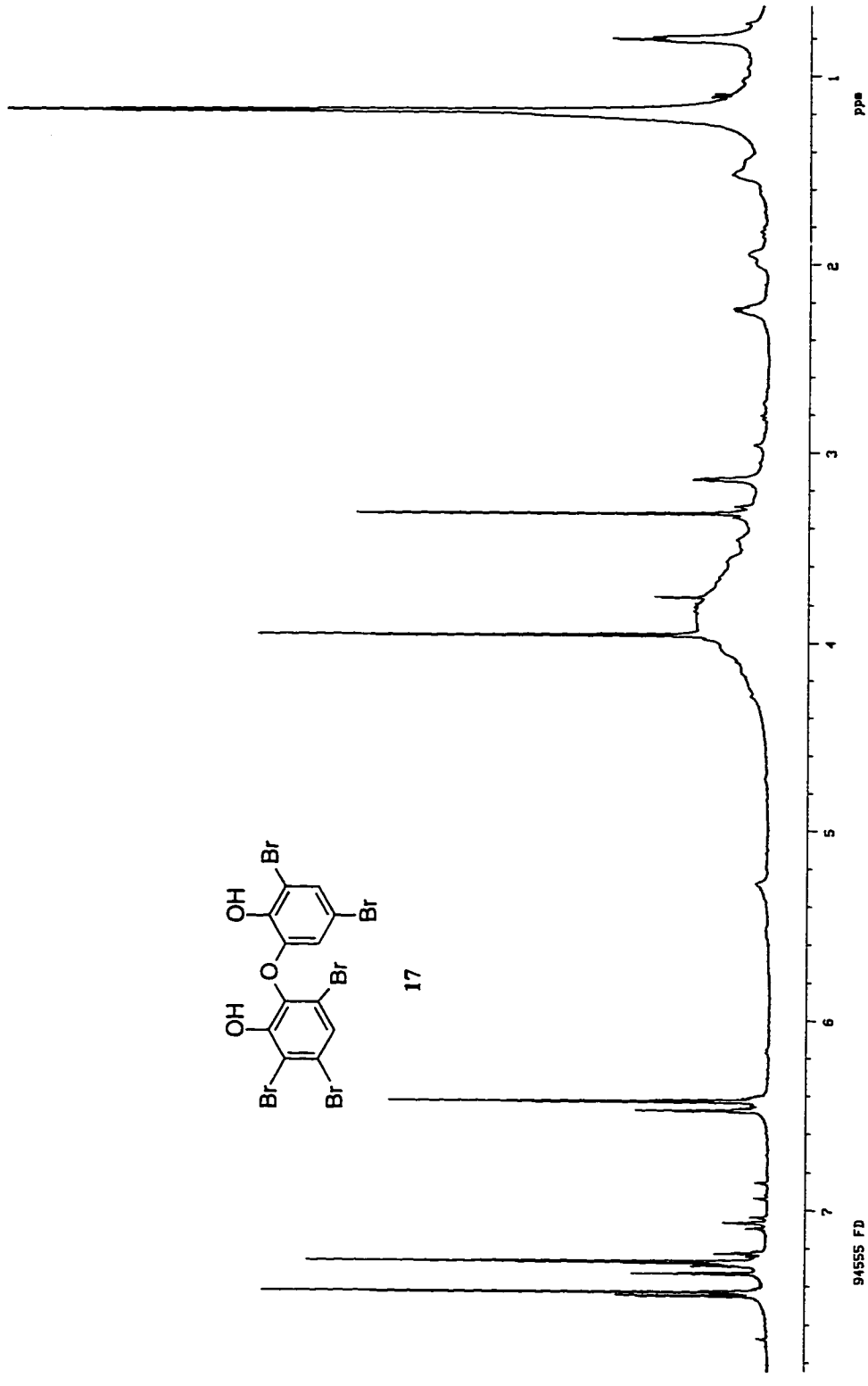


Figure 5.50. LRESIM Spectra-94555.

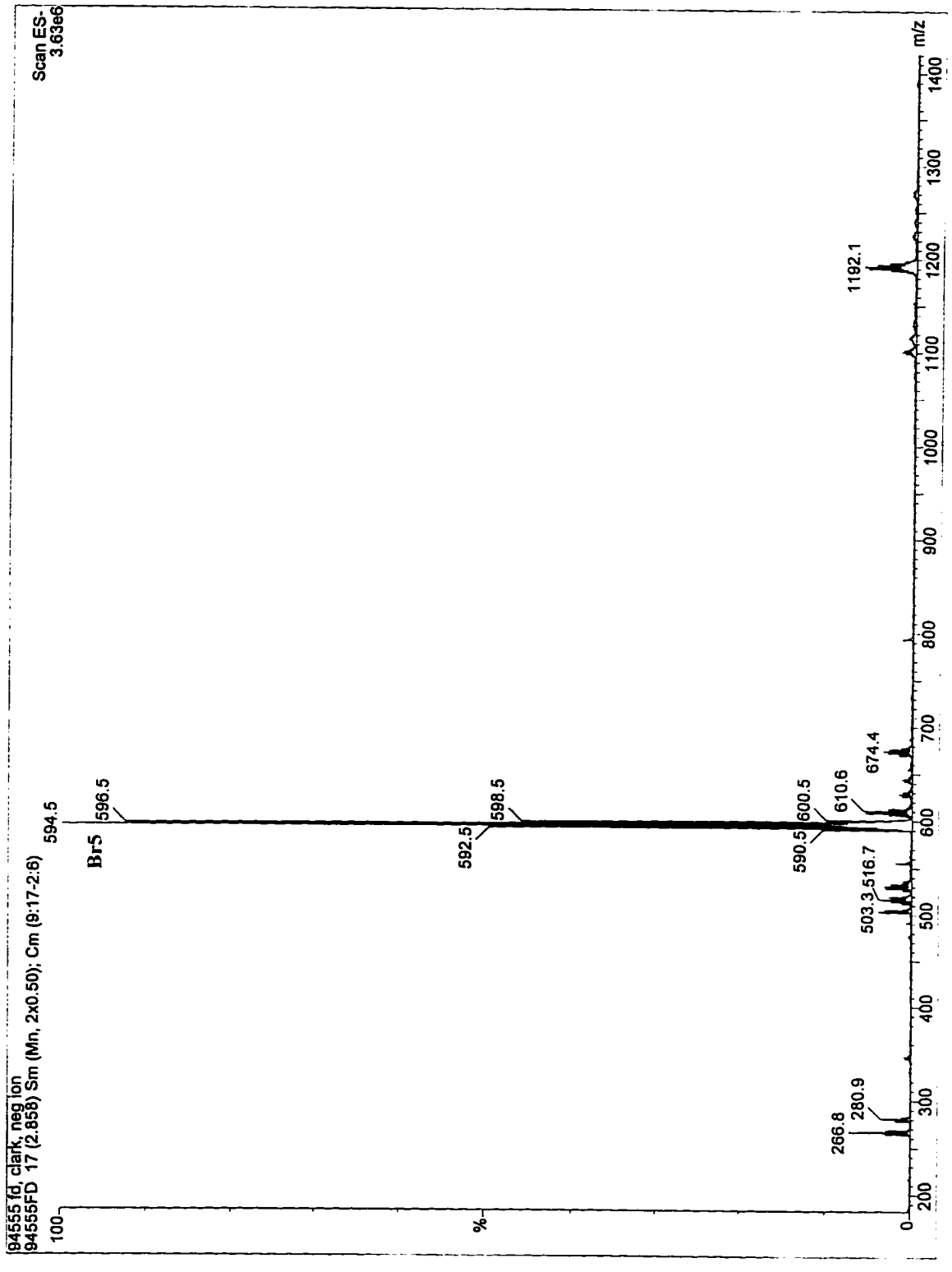


Figure 5.51. ^{1}H-NMR Spectra-94602 ($CDCl_3$).

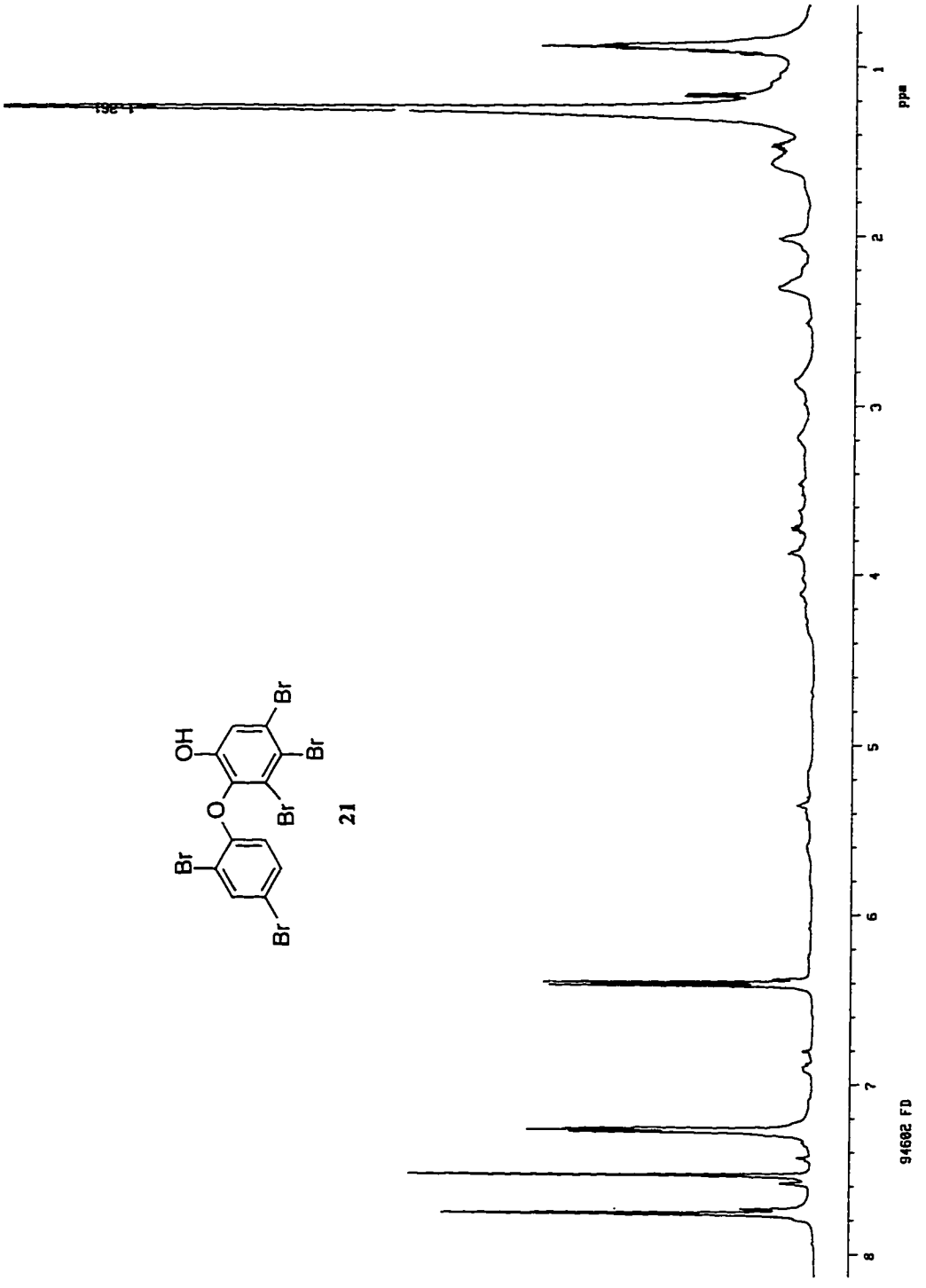


Figure 5.52. ^{1}H-NMR Spectra-94607 ($CDCl_3$).

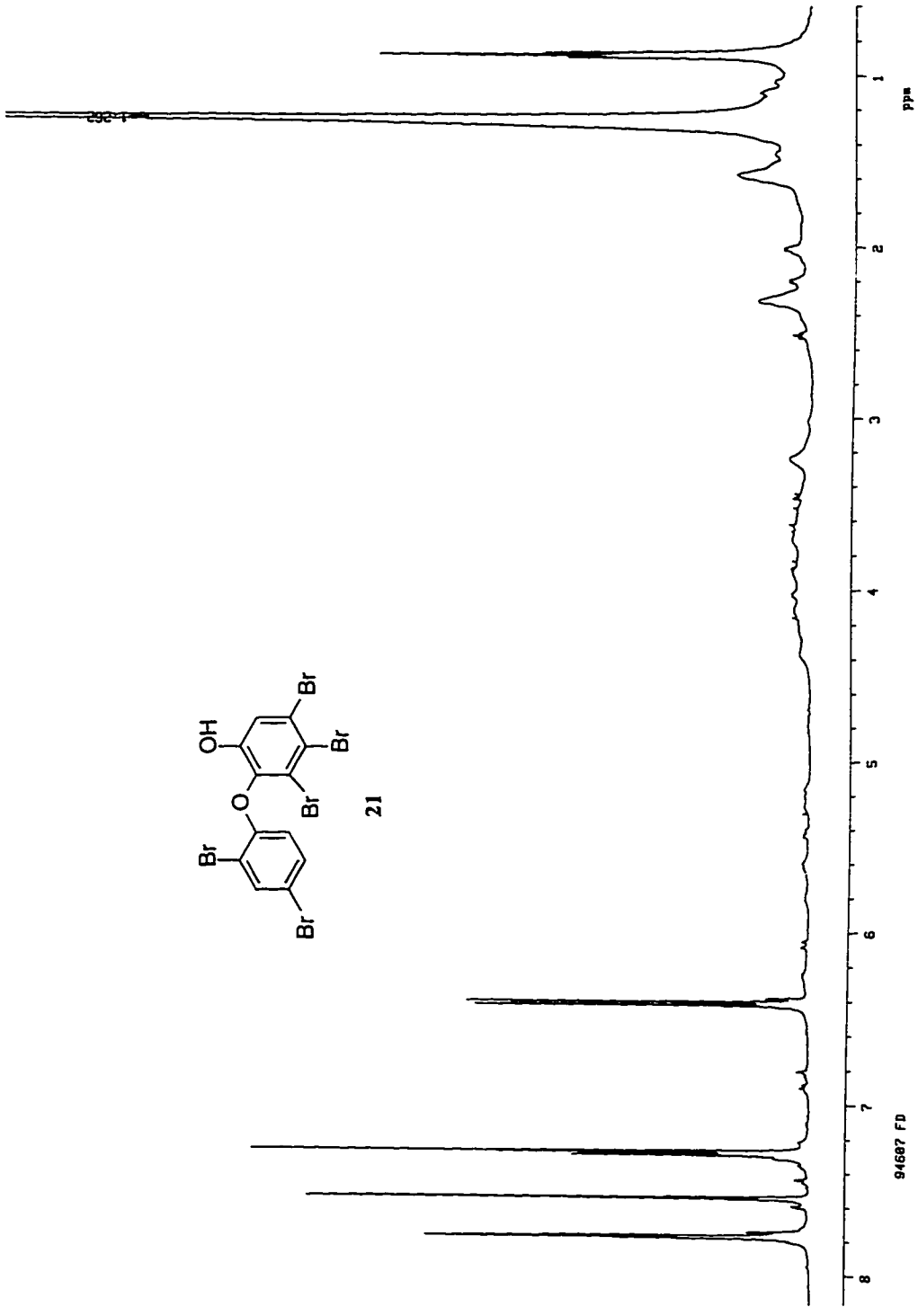


Figure 5.53. ^{1}H-NMR Spectra-95612 ($CDCl_3$).

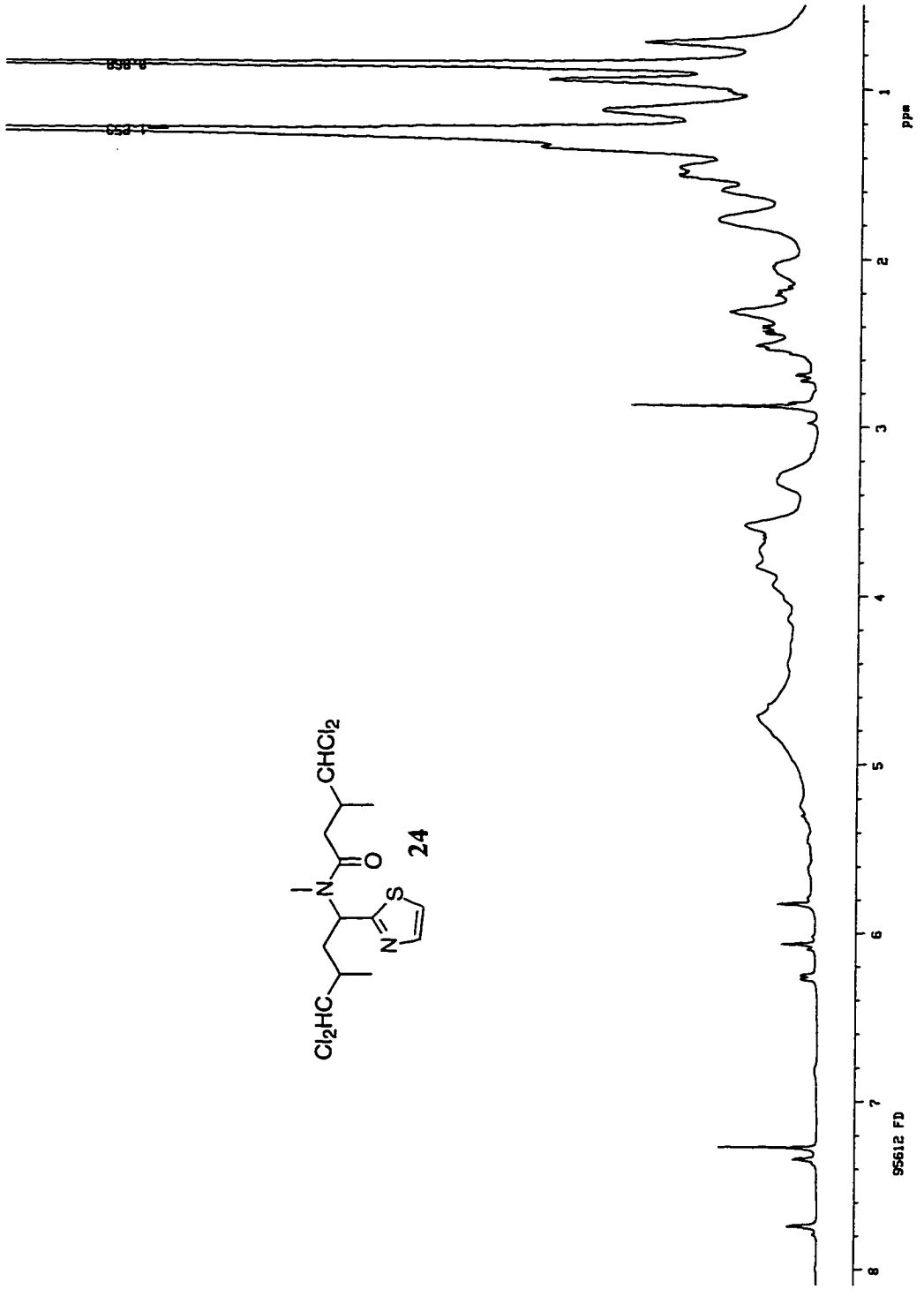


Figure 5.54. ^{1}H-NMR Spectra-95616 ($CDCl_3$).

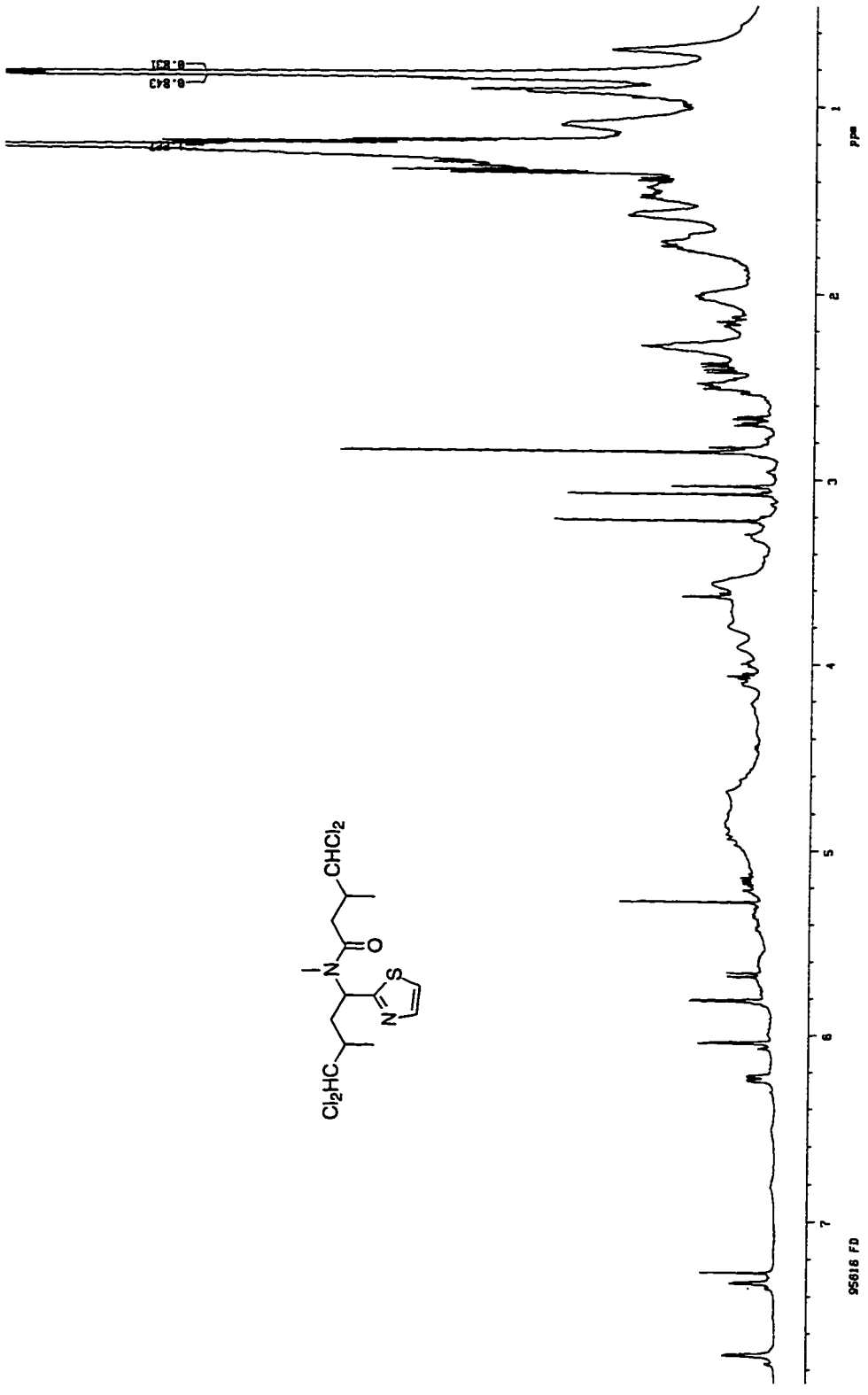
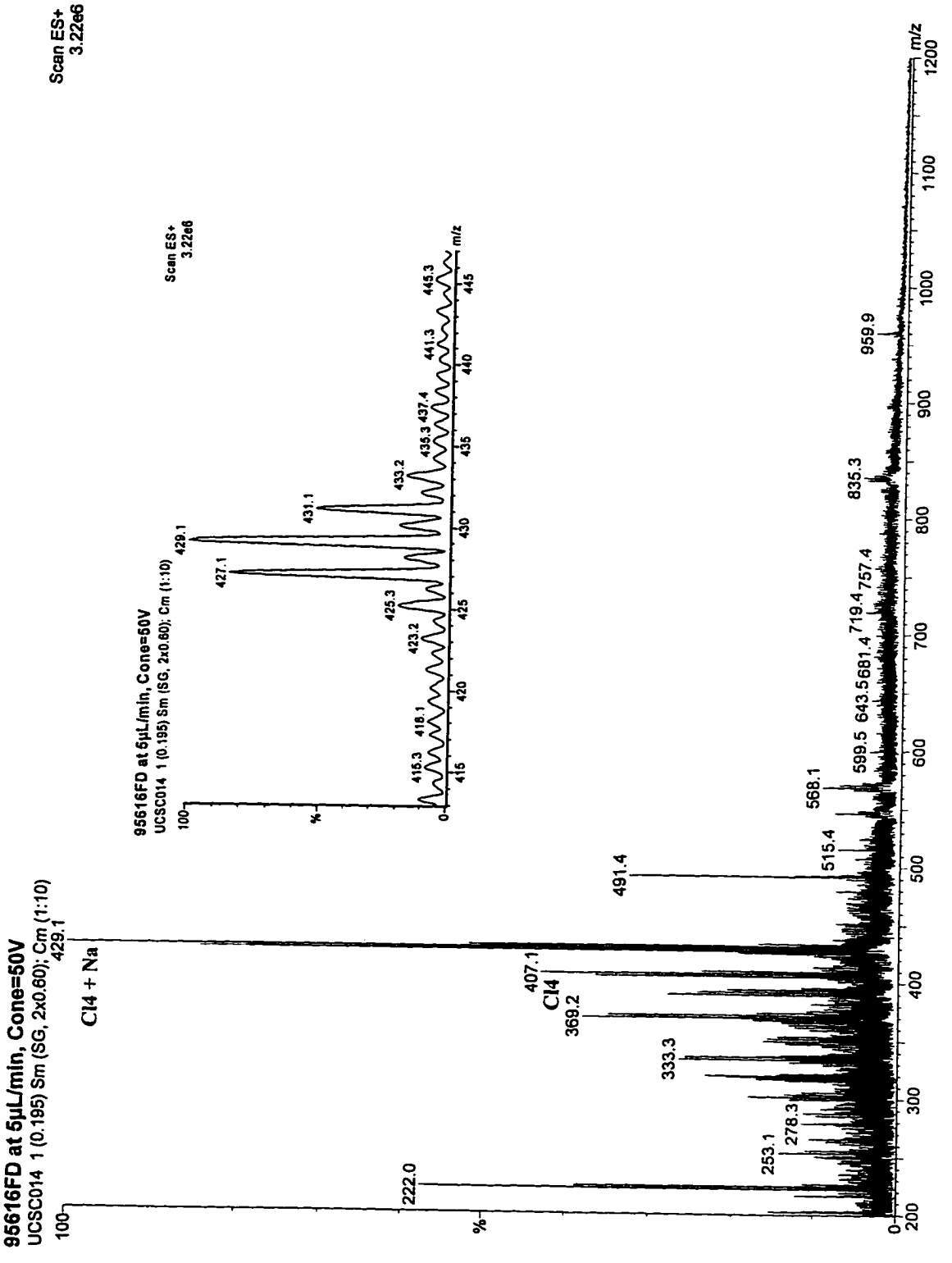


Figure 5.55. LRESIM Spectra-95616.



Supplementary Material
Morphotype IV

Figure 5.56. ^{1}H-NMR Spectra-94557 ($CDCl_3$).

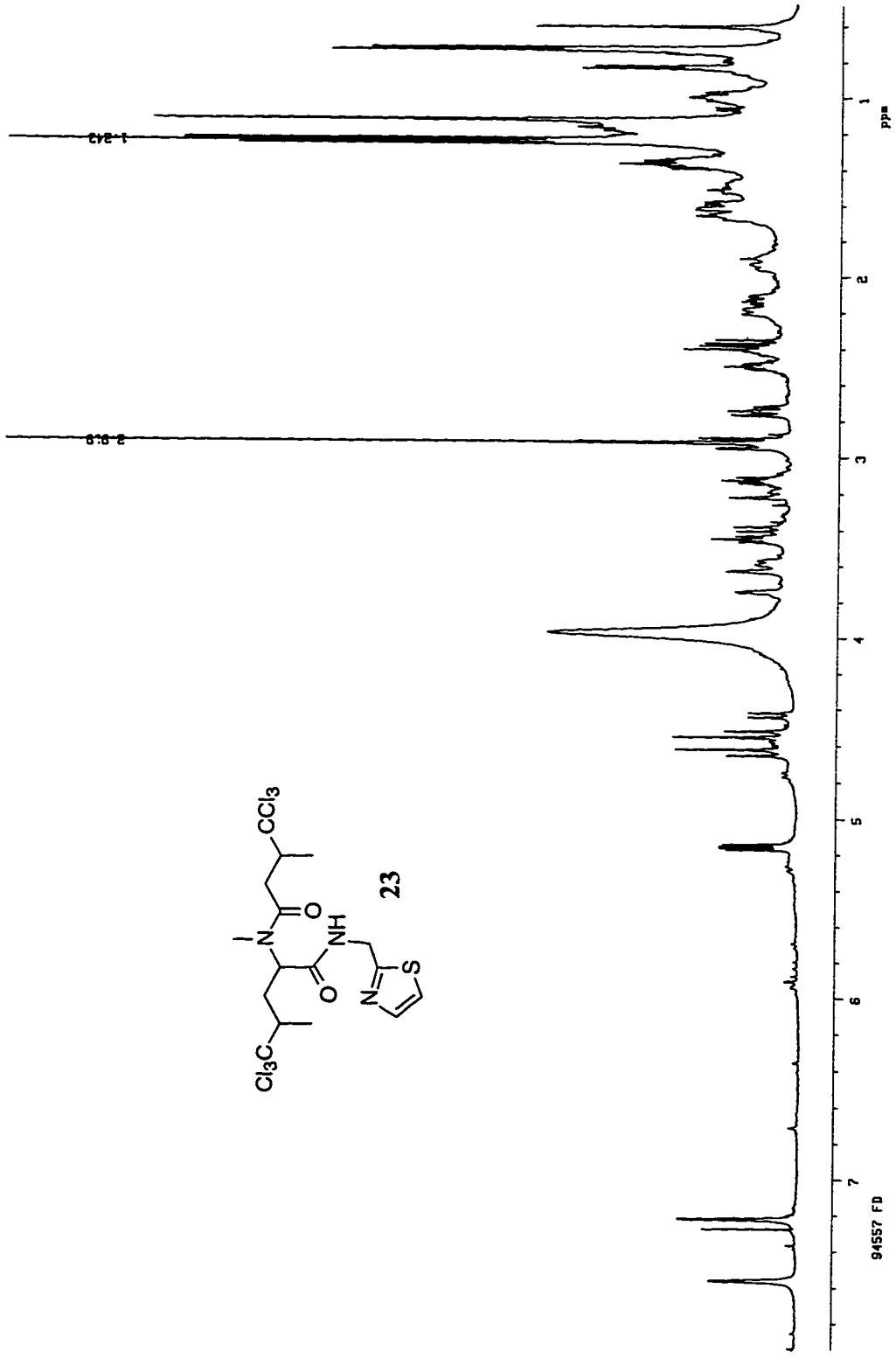


Figure 5.57. LRESIM Spectra-94557.

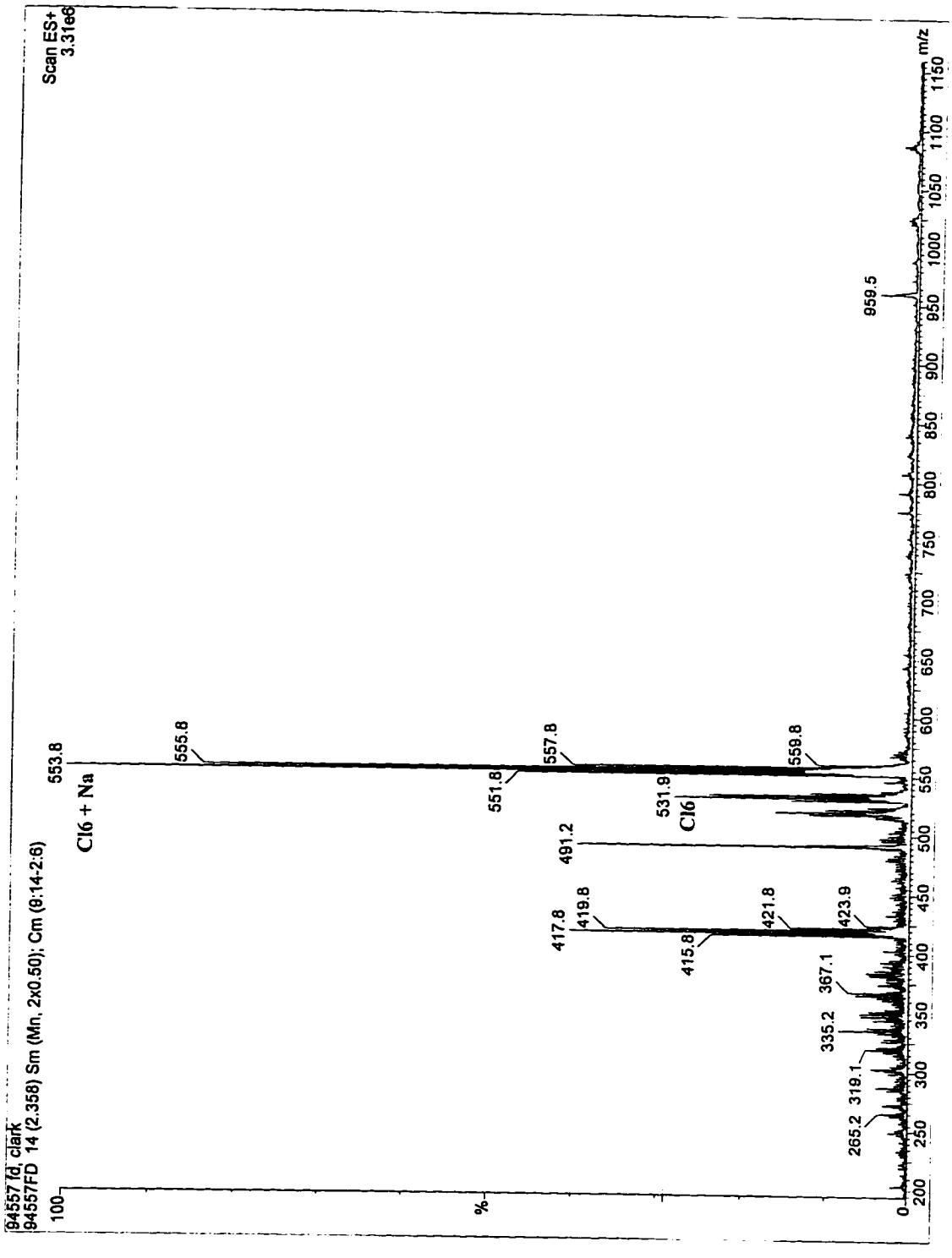


Figure 5.58. ^{1}H-NMR Spectra-94558 ($CDCl_3$).

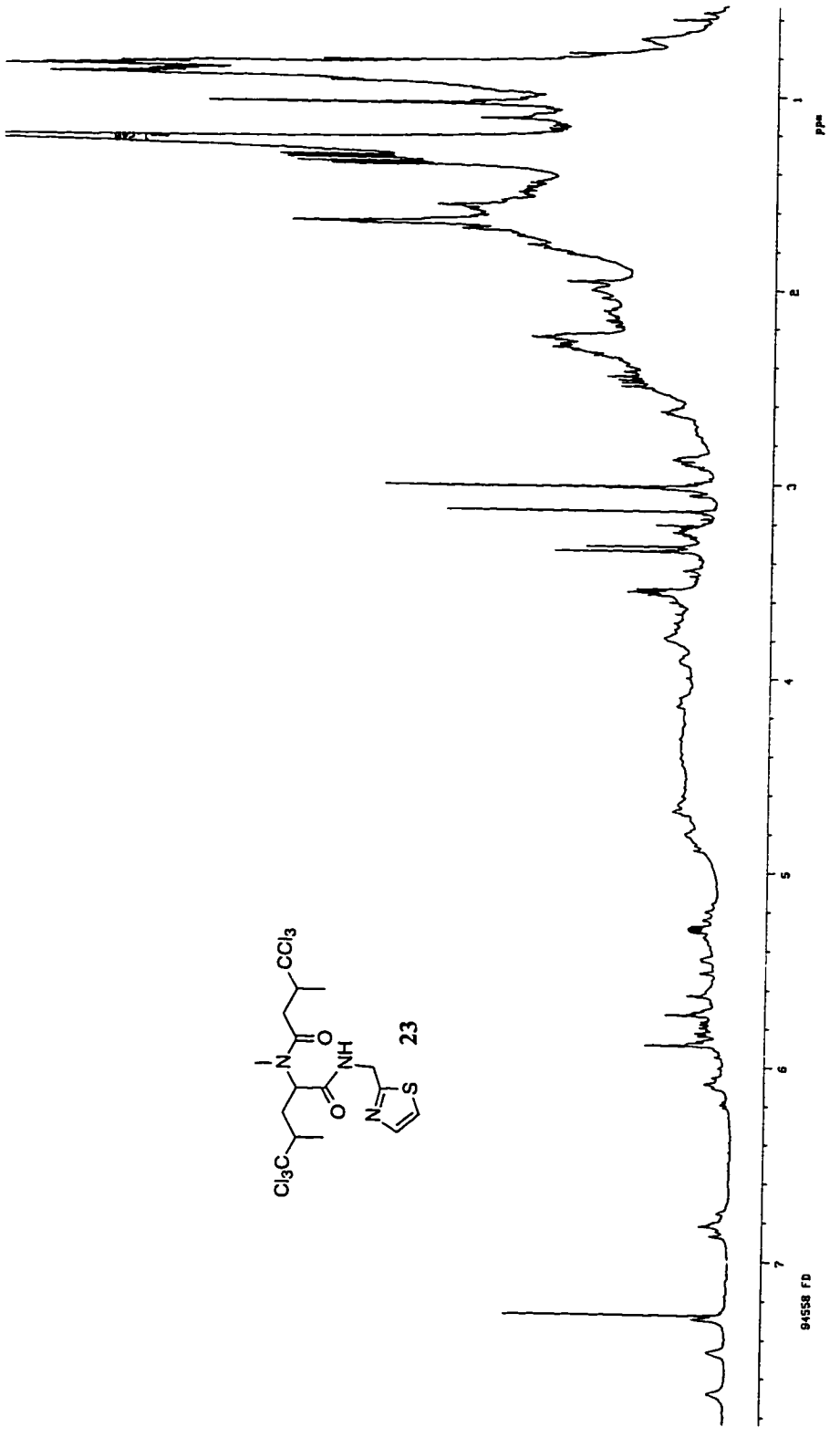


Figure 5.59. LRESIM Spectra-94558.

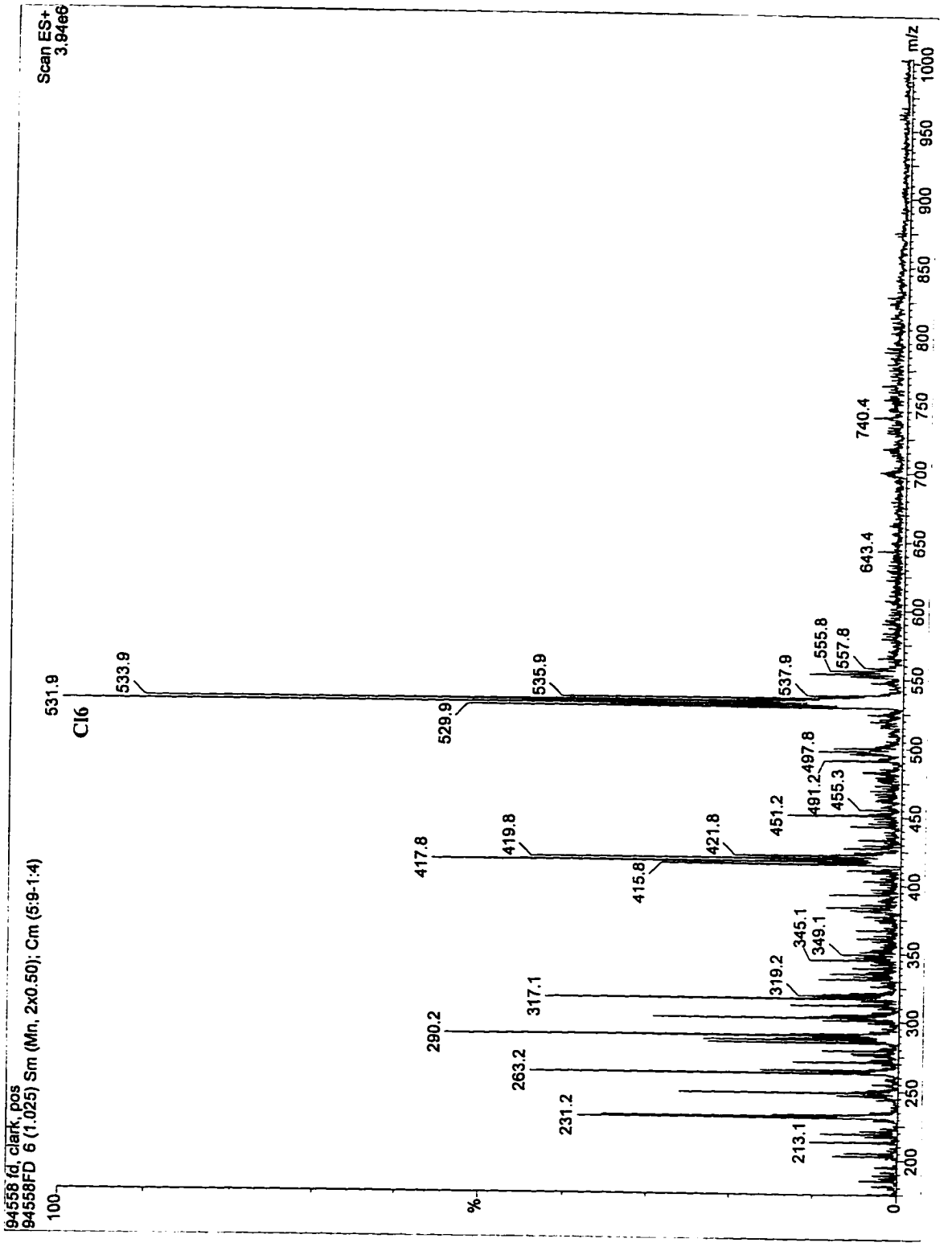


Figure 5.60. ^{1}H-NMR Spectra-94635 ($CDCl_3$).

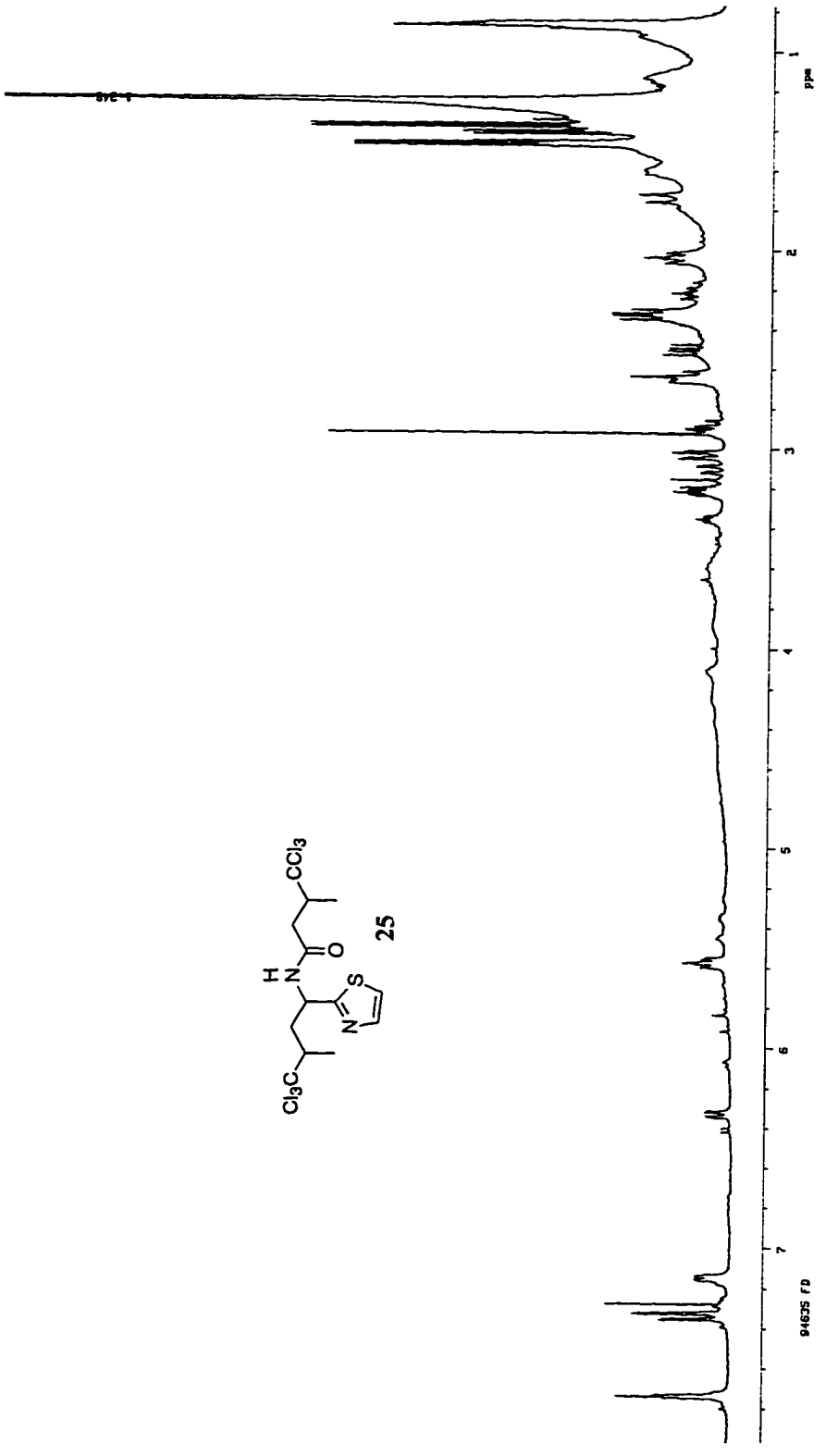


Figure 5.61. LRESIM Spectra 94635.

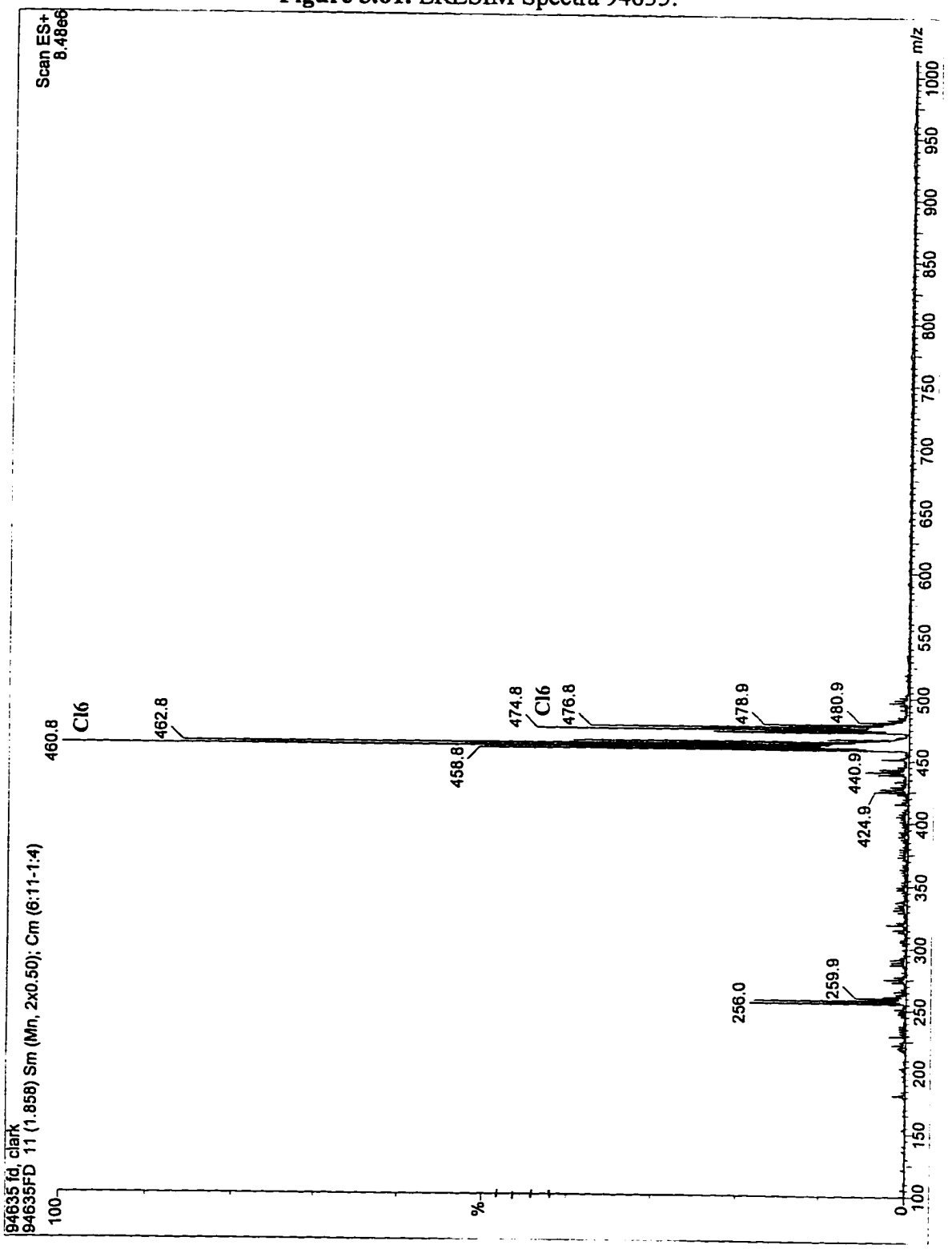


Figure 5.62. ^{1}H-NMR Spectra-95078 ($CDCl_3$).

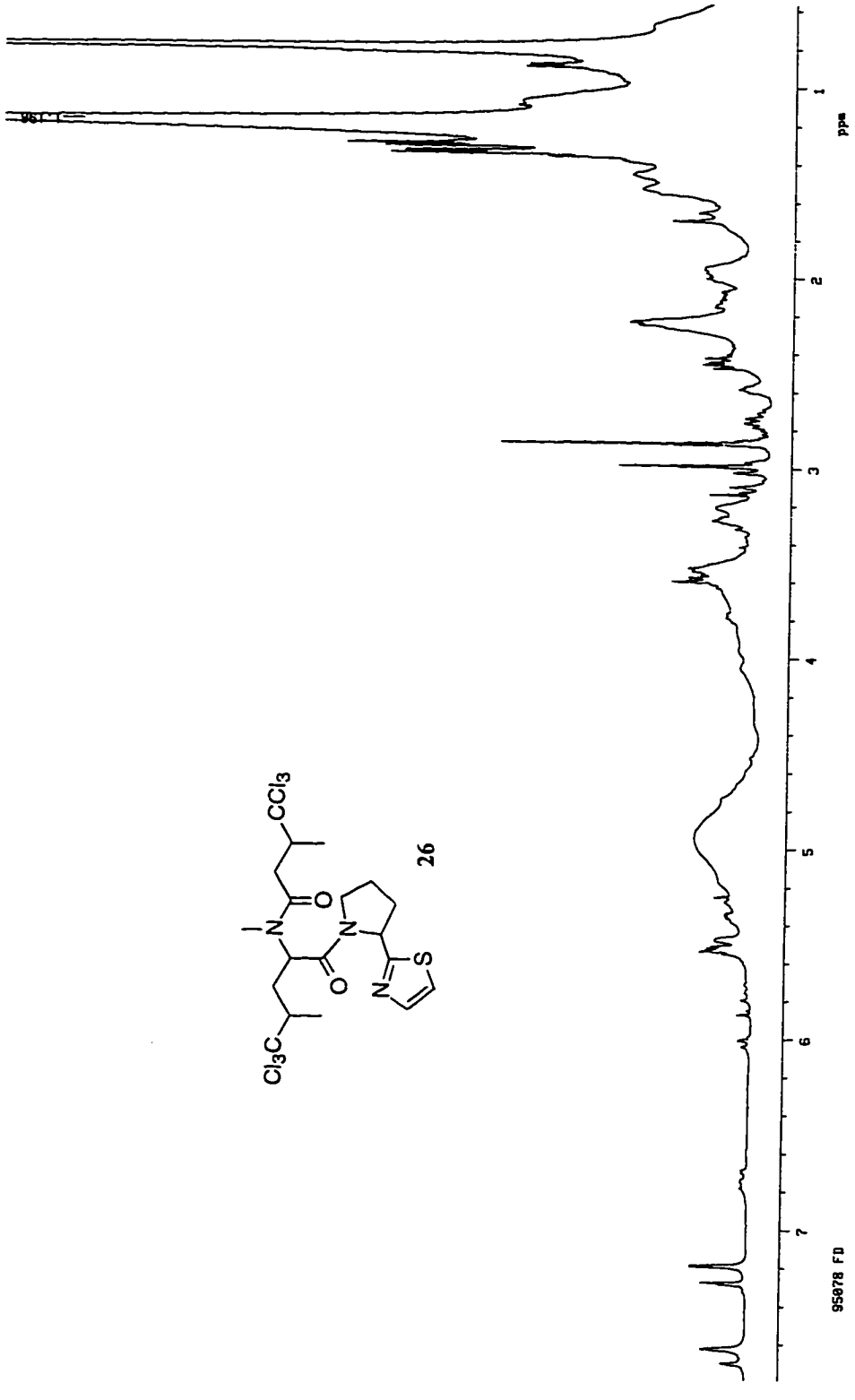


Figure 5.63. LRESIM Spectra-95078.

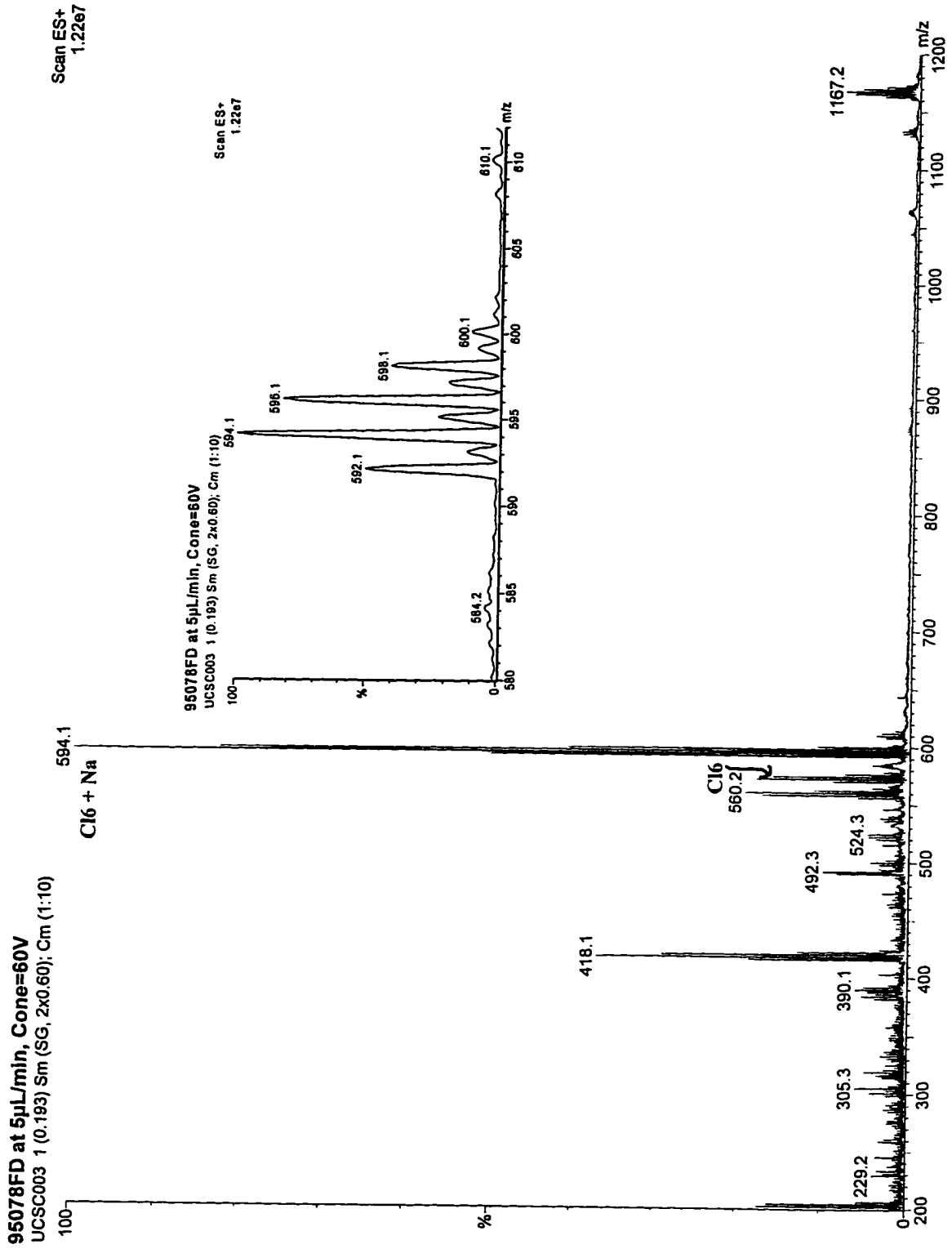


Figure 5.64. ^{1}H-NMR Spectra-95082 ($CDCl_3$).

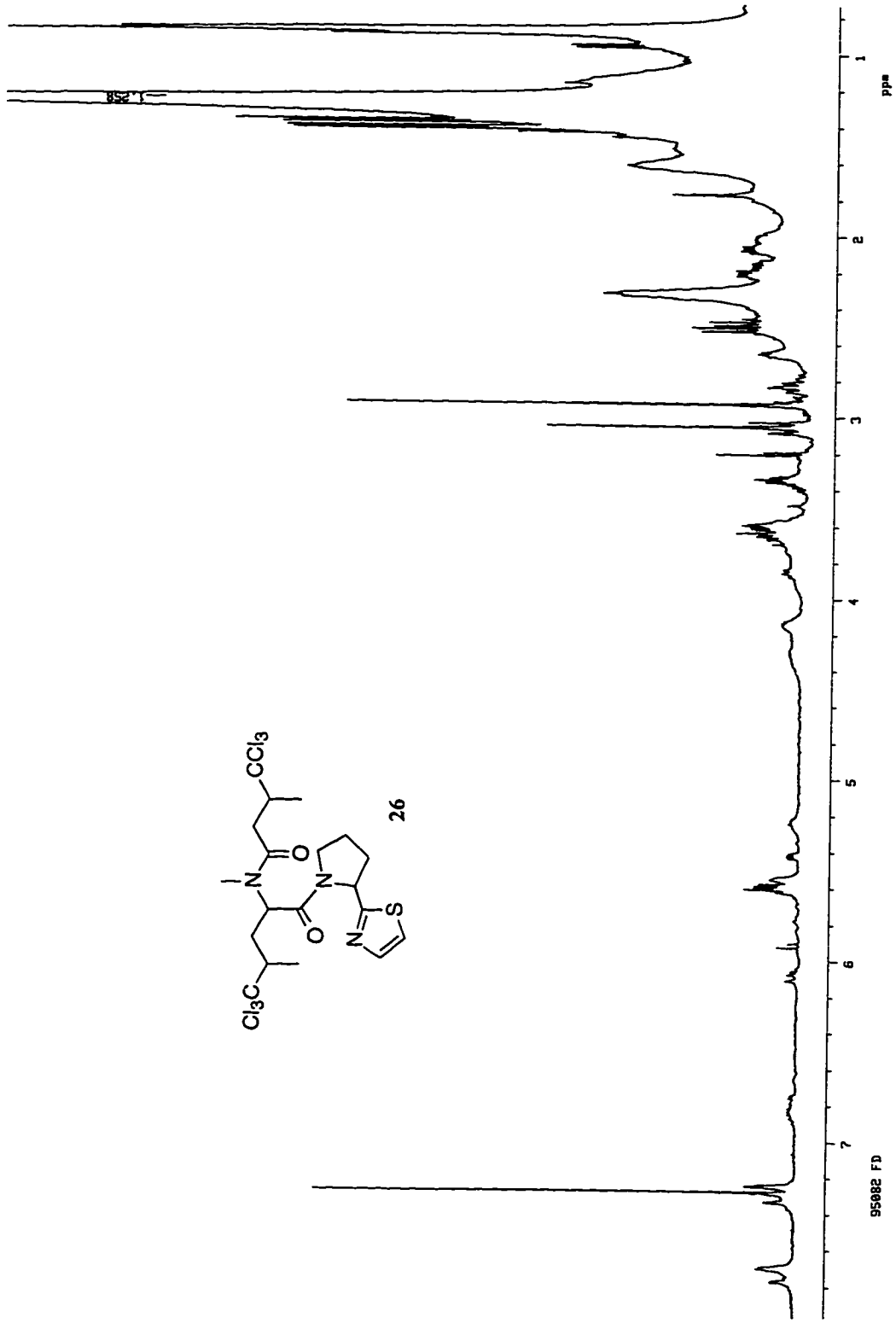


Figure 5.65. ^{1}H-NMR Spectra-95095 ($CDCl_3$).

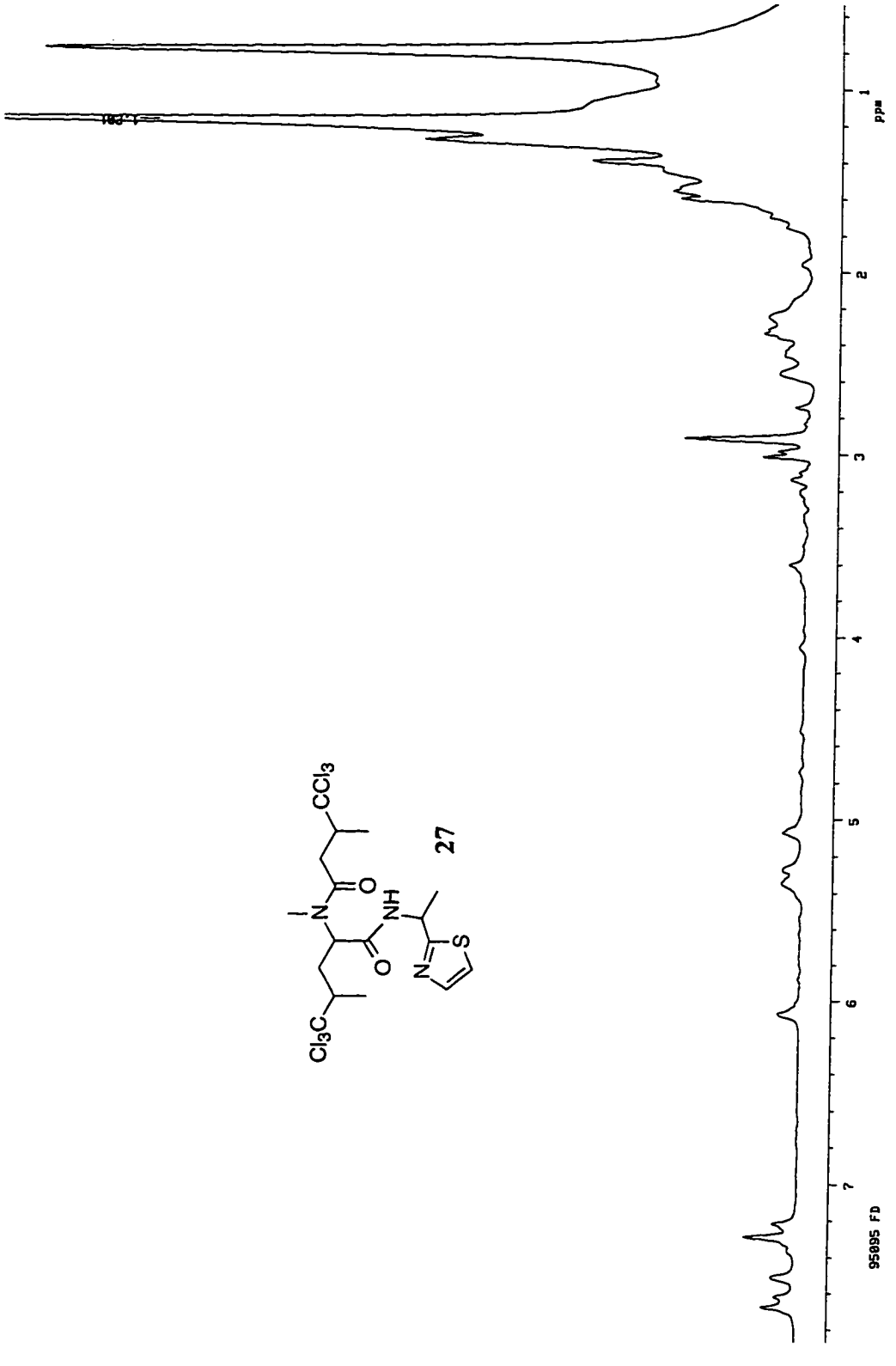


Figure 5.66. LRESIM Spectra-95095.

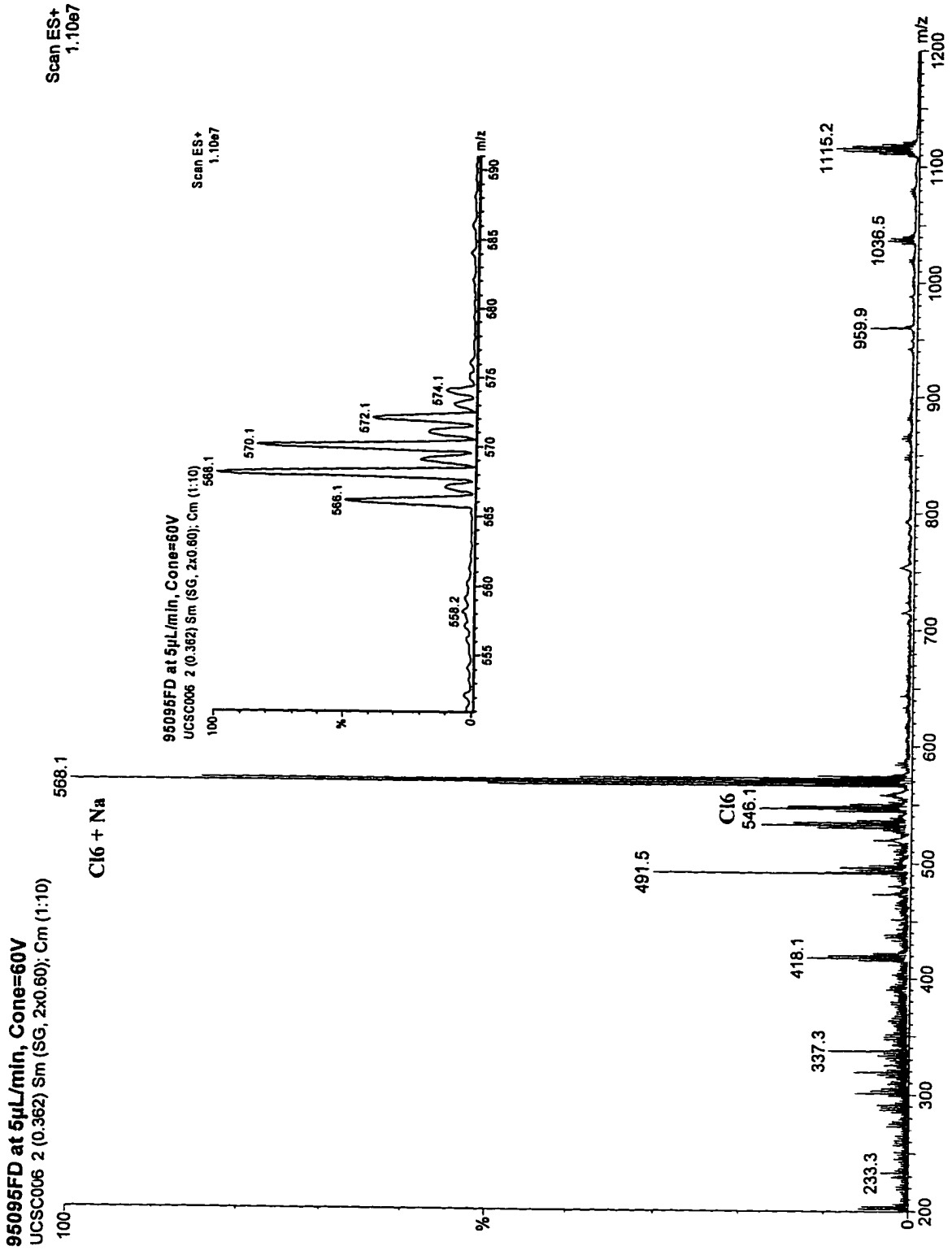


Figure 5.67. ^{1}H-NMR Spectra-95152 ($CDCl_3$).

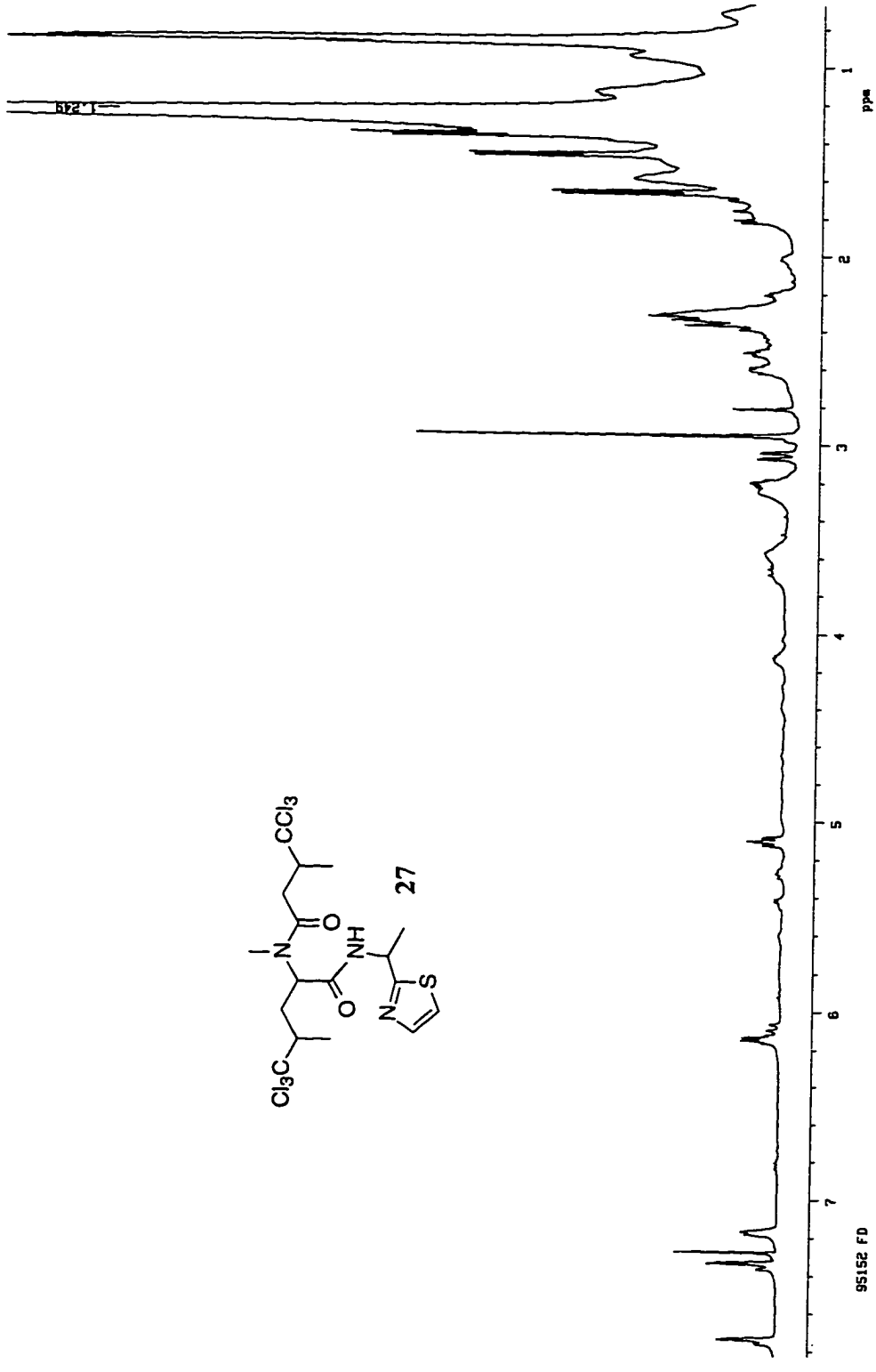


Figure 5.68. LRESIM Spectra-95152.

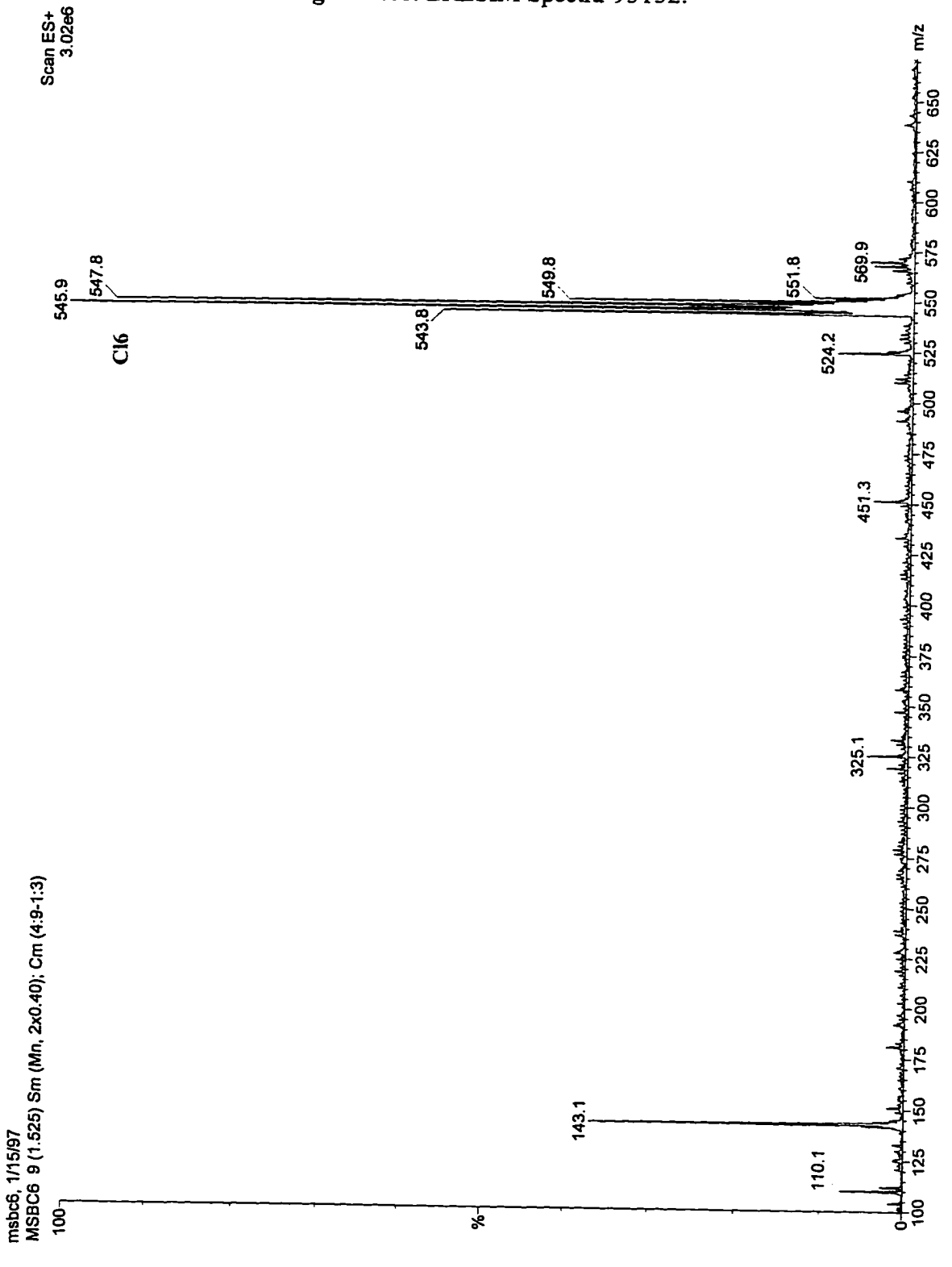


Figure 5.69. ^{1}H-NMR Spectra-95643 ($CDCl_3$).

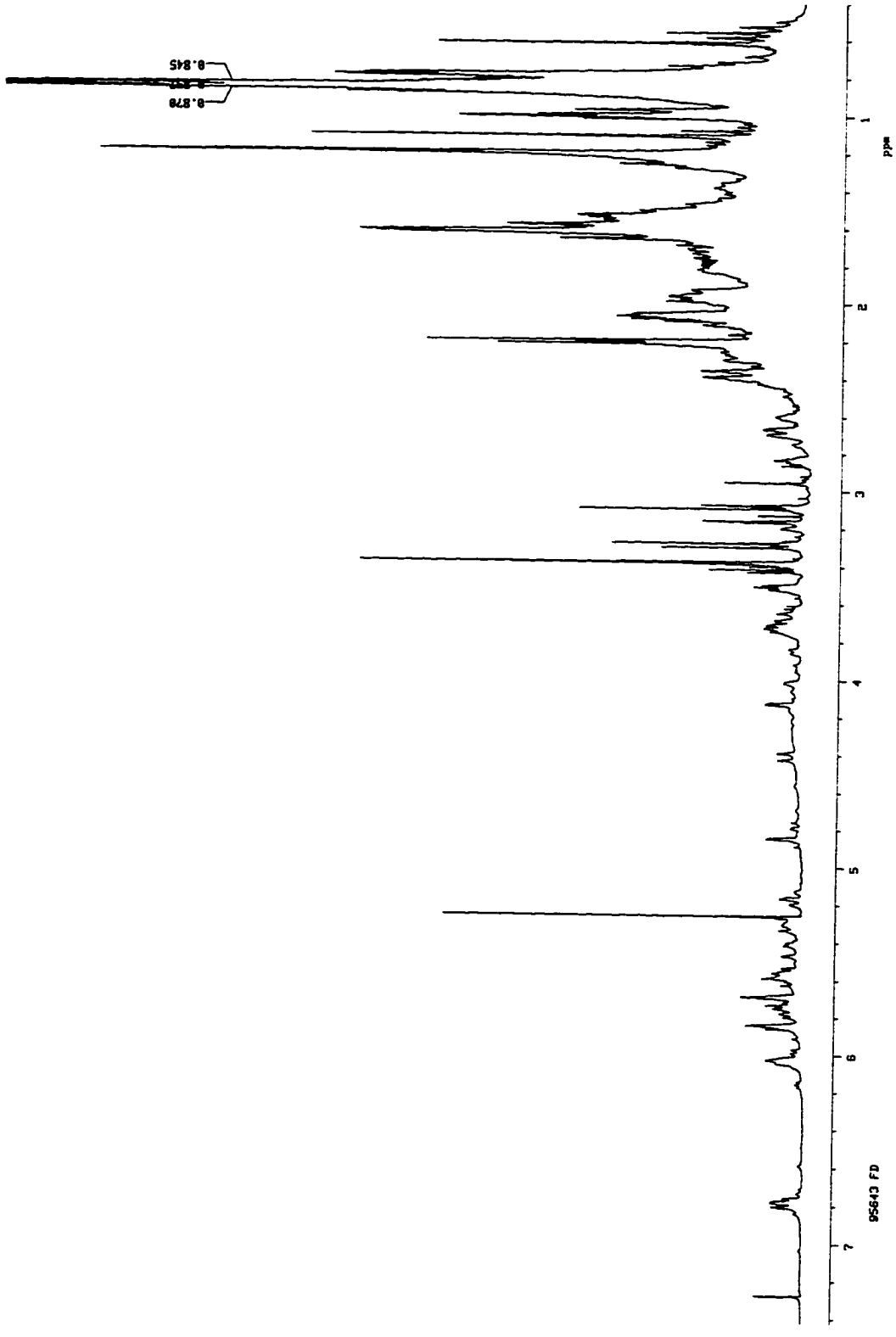


Figure 5.70. LRESIM Spectra-95643.

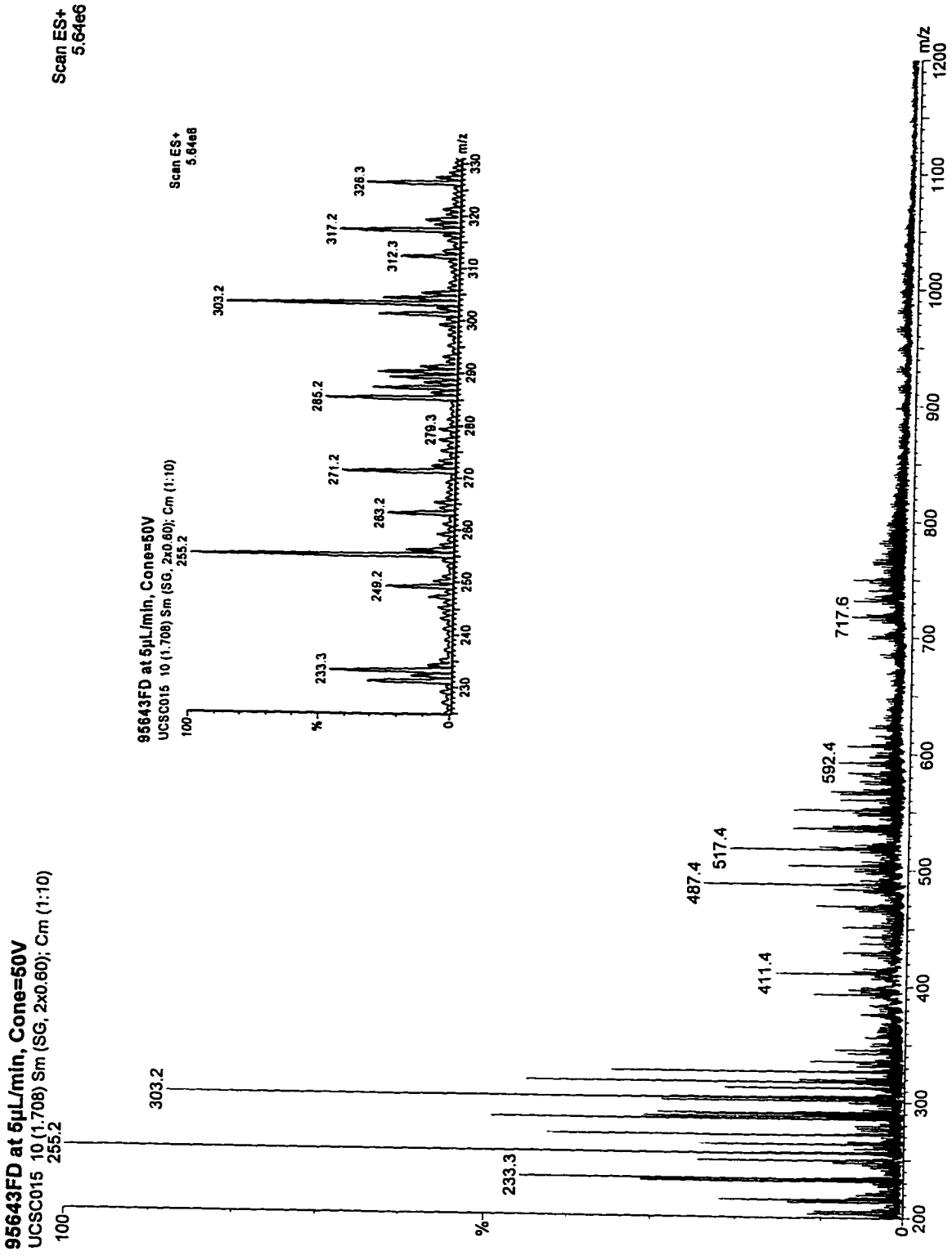


Figure 5.71. APT NMR Spectra-95643.

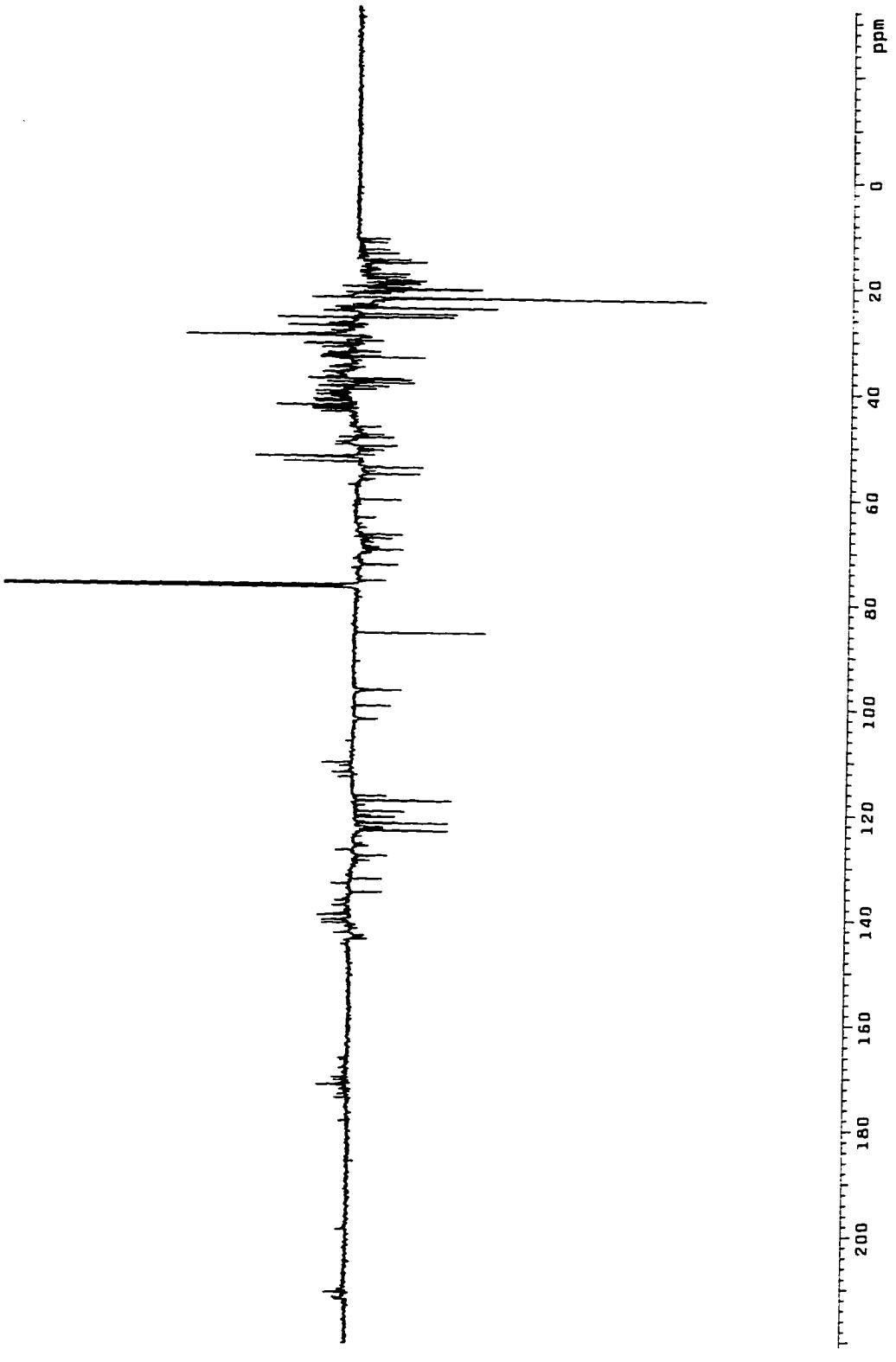
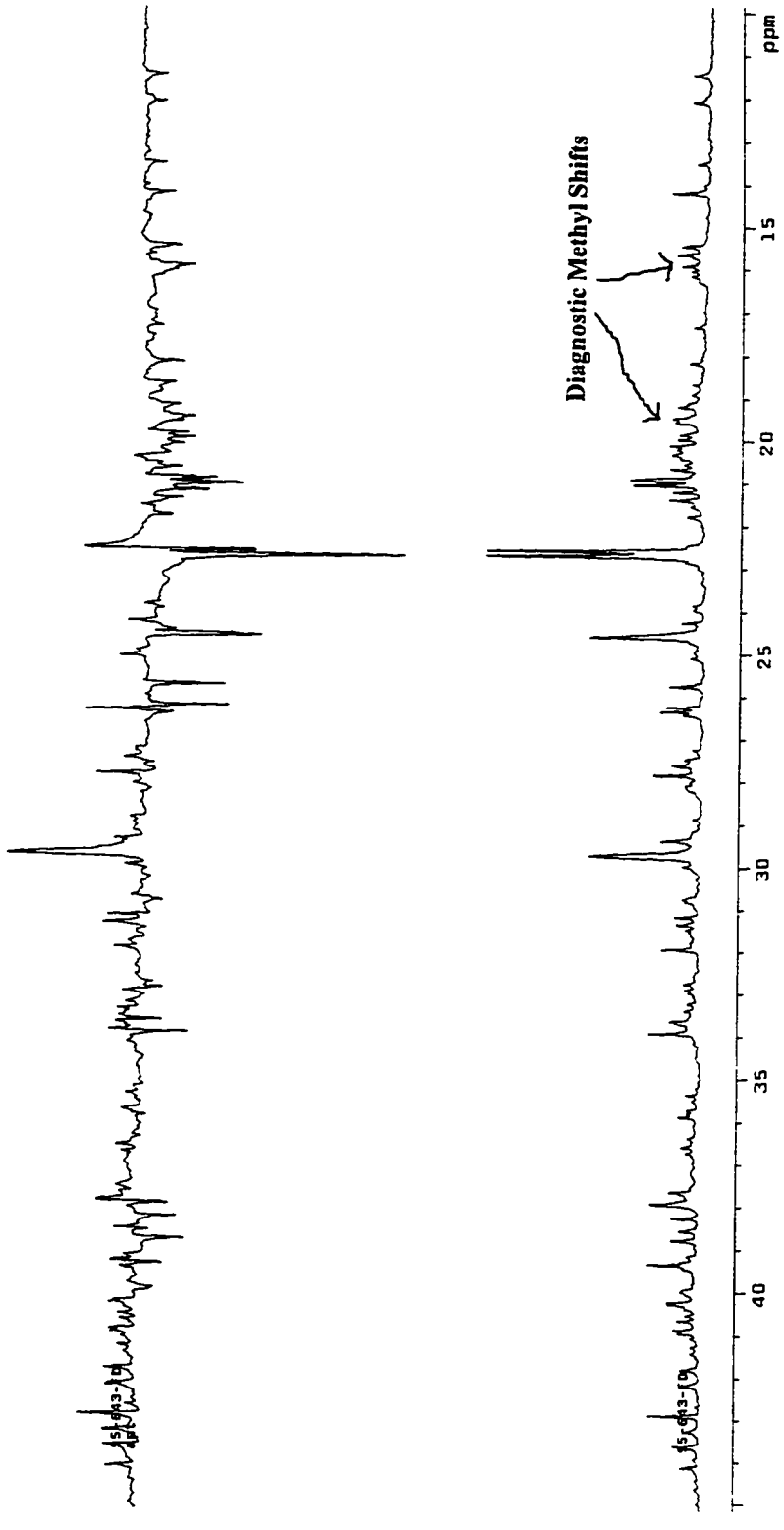


Figure 5.72. APT Expansion NMR Spectra-95643.



Supplementary Material
Morphotype V

Figure 5.73. ^{1}H-NMR Spectra-95153 ($CDCl_3$).

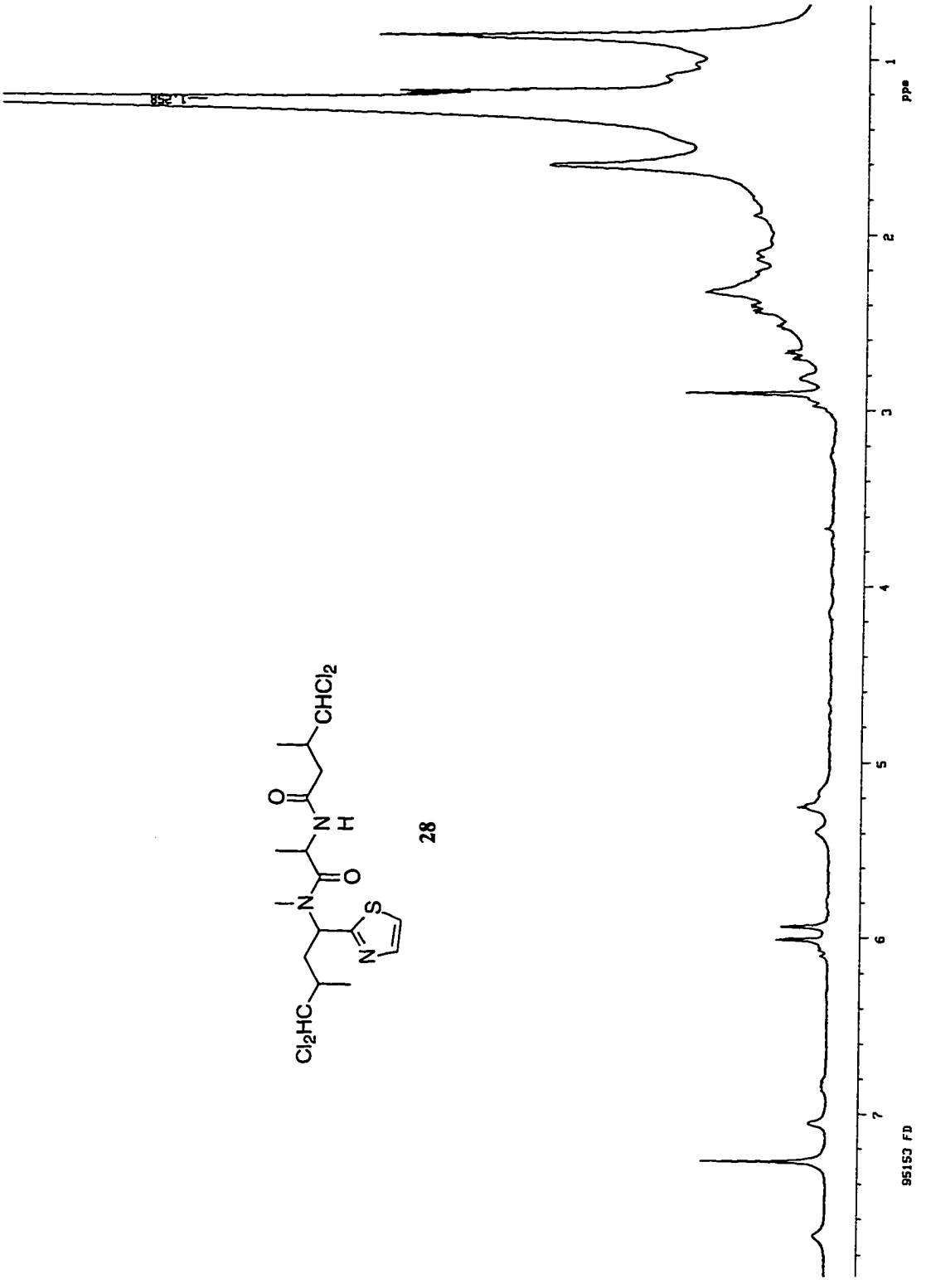


Figure 5.74. LRESIM Spectra-95153.

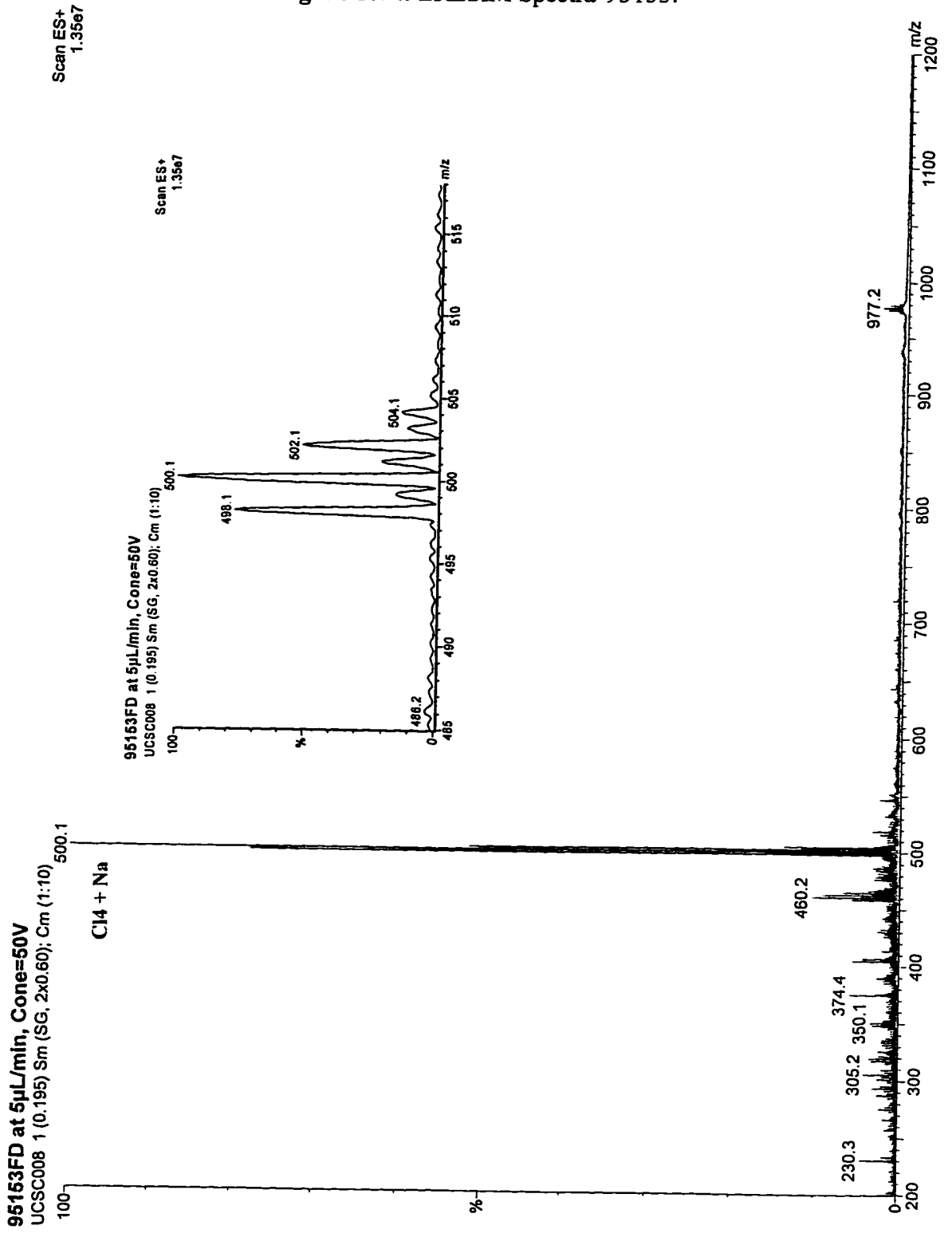


Figure 5.75. ^{1}H-NMR Spectra-95589 ($CDCl_3$).

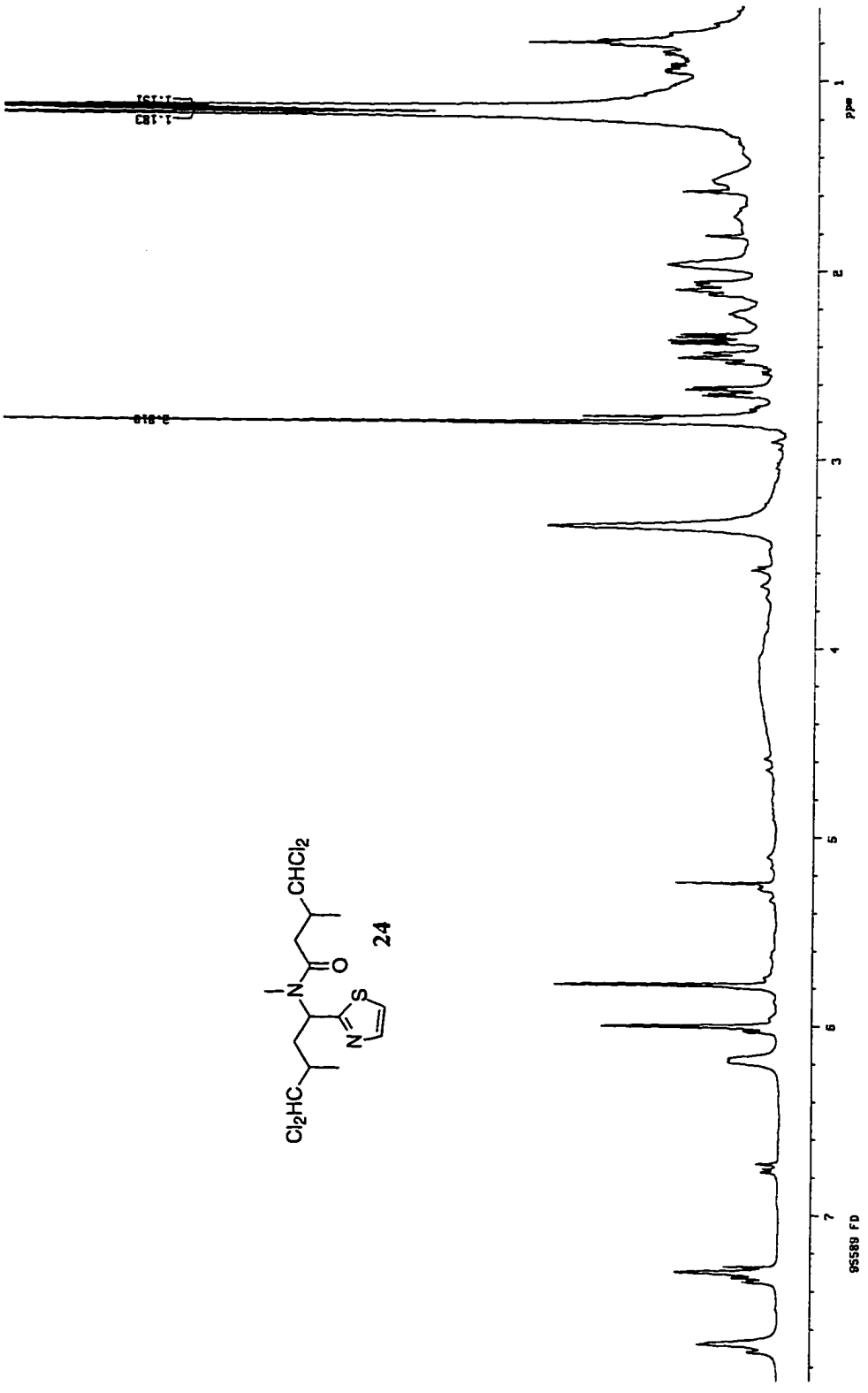


Figure 5.76. LRESIM Spectra-95589.

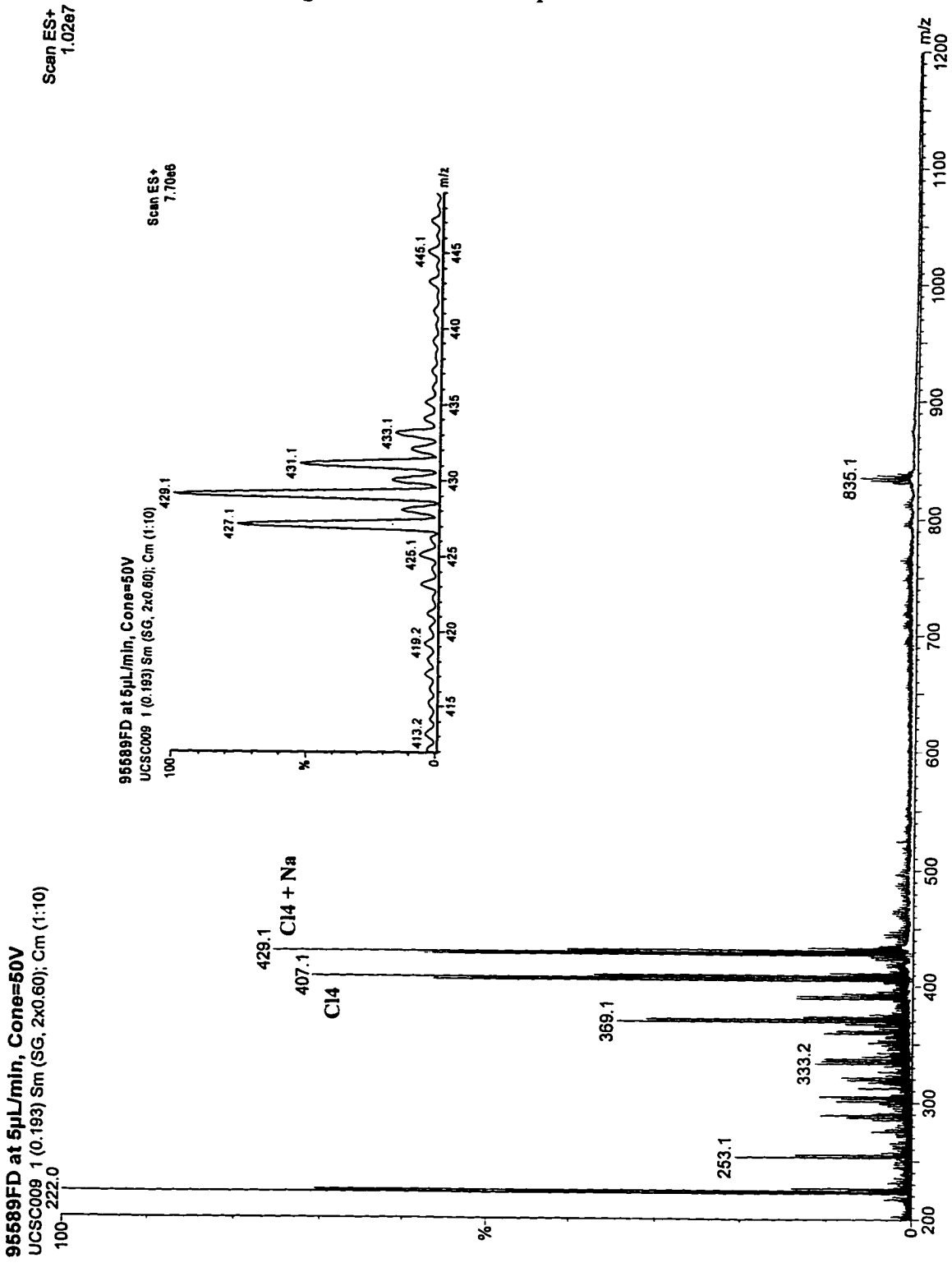


Figure 5.77. ^{1}H-NMR Spectra-95590 ($CDCl_3$).

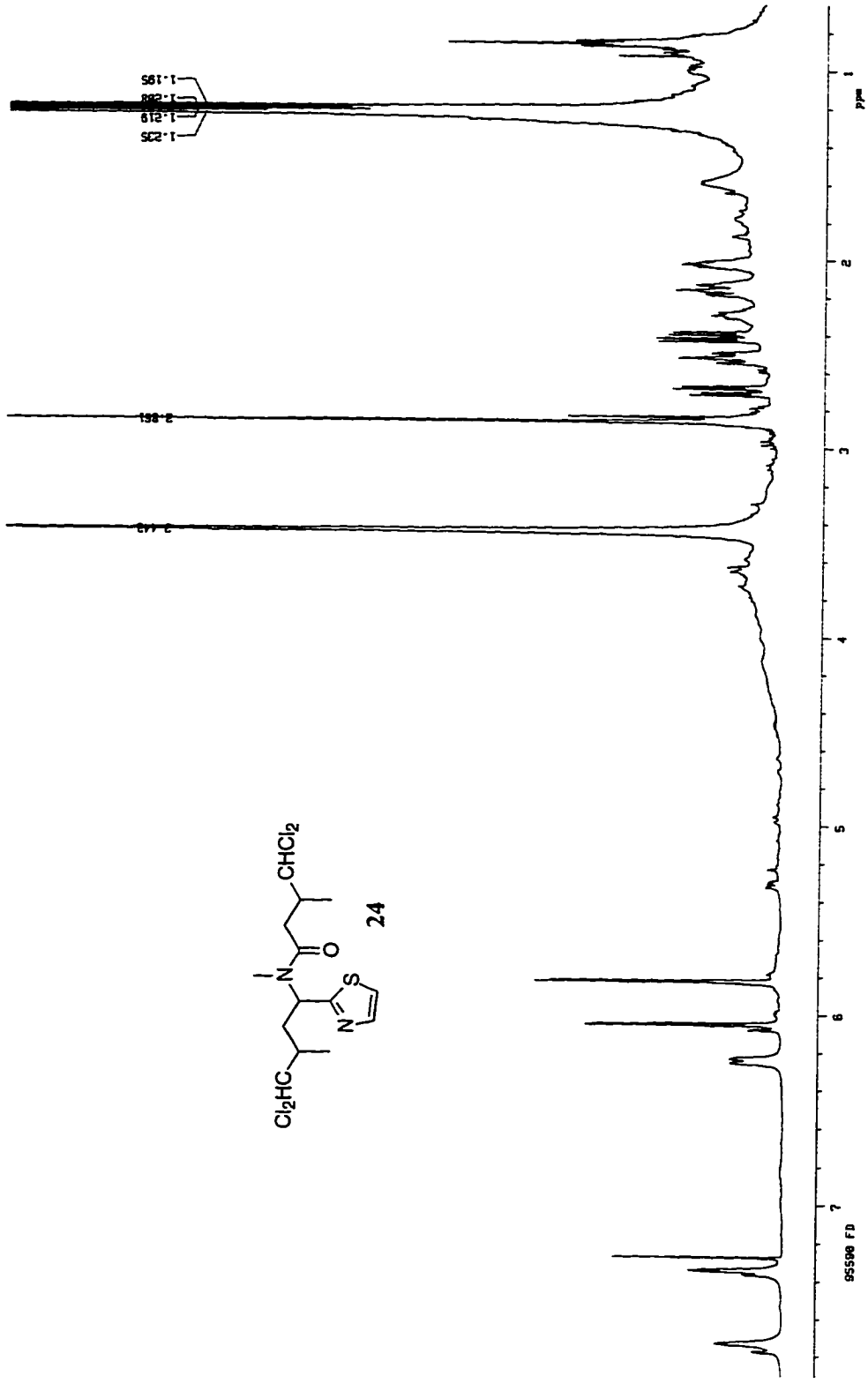
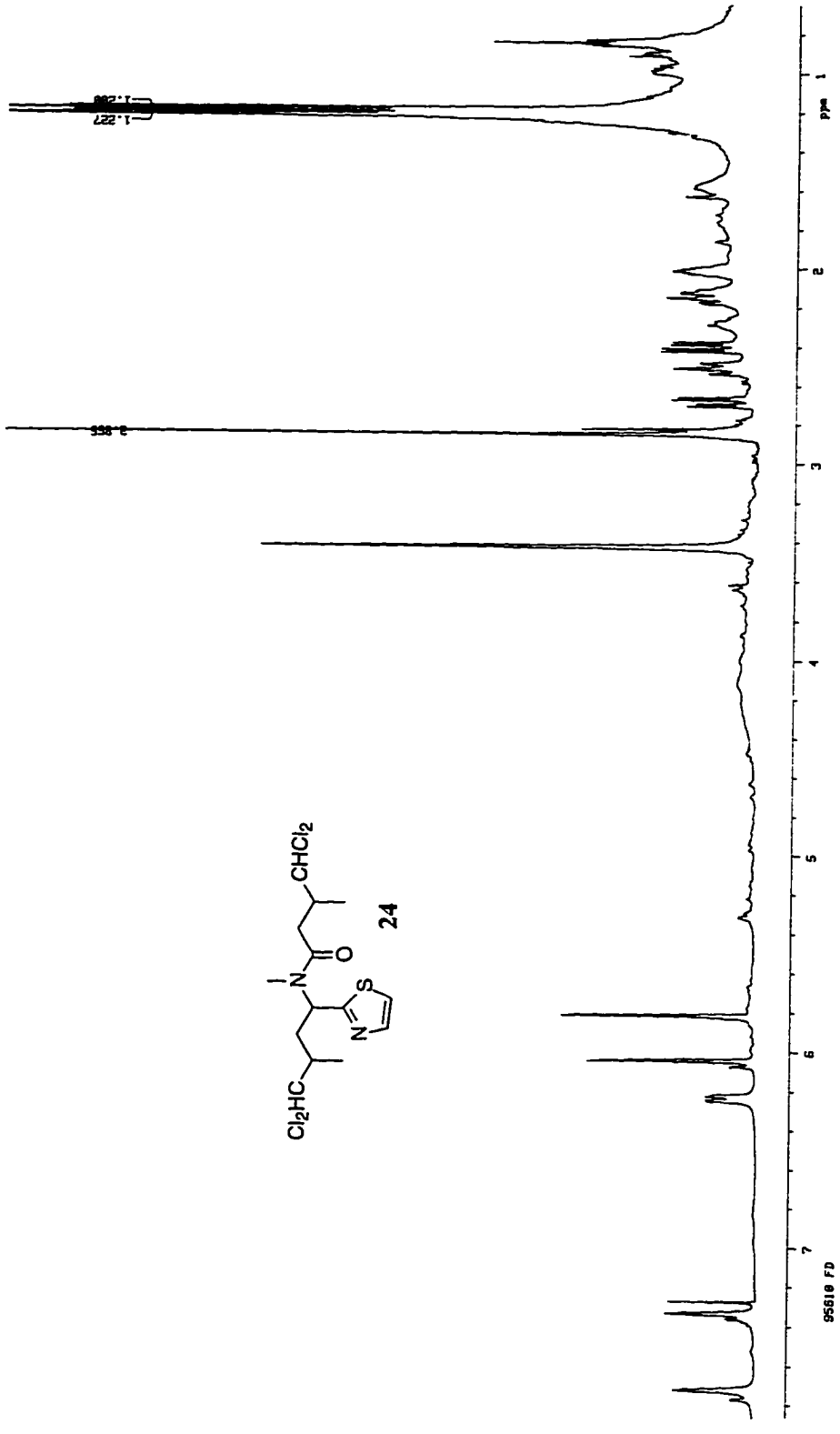


Figure 5.78. ^{1}H-NMR Spectra-95610 ($CDCl_3$).



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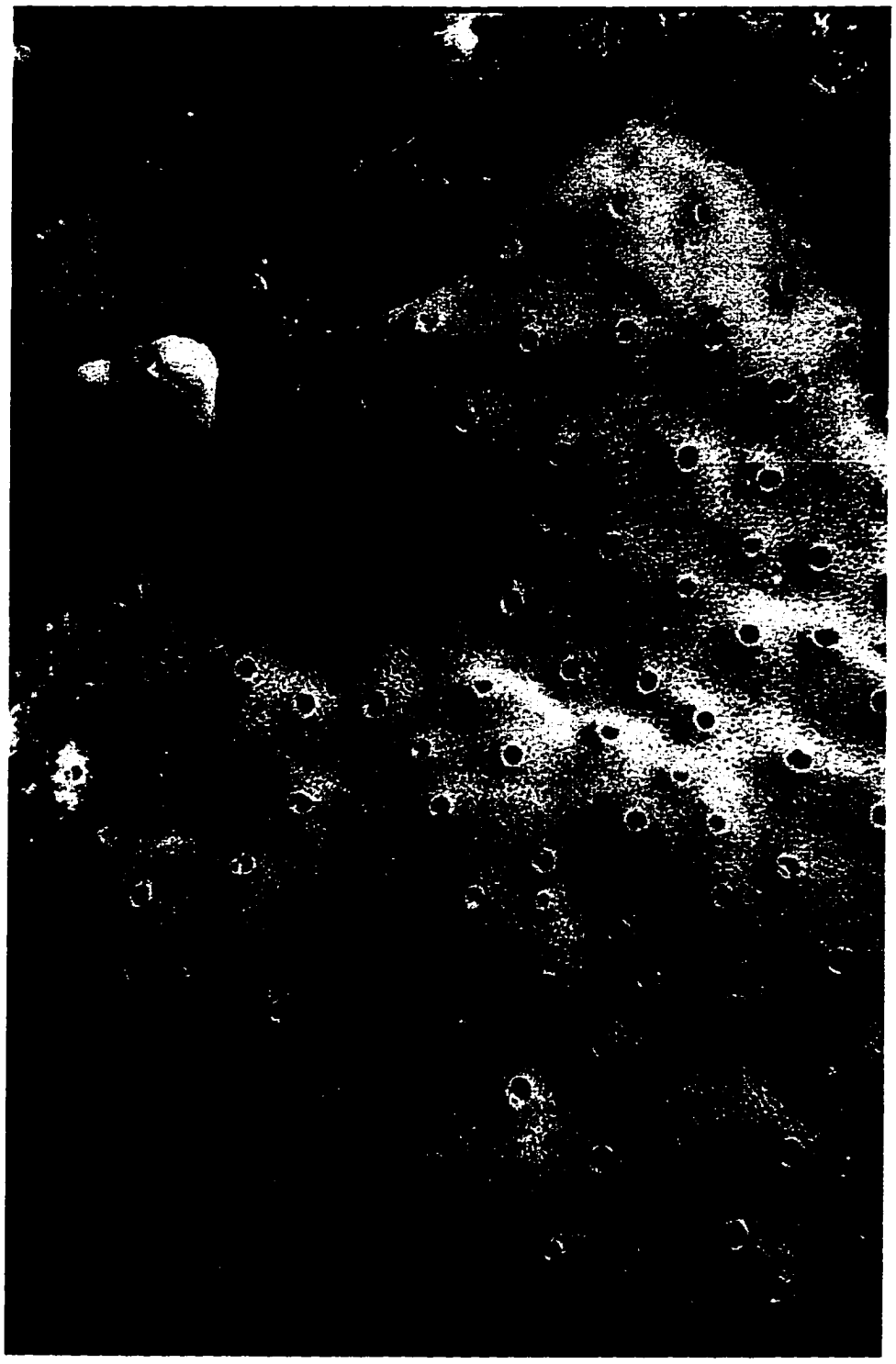
Chapter 6-Conclusions



Marine Sponge-Papua New Guinea Photo Courtesy of Jay Burreson, Ph.D.

Conclusions

In summary the work of this dissertation demonstrates that marine sponges of the Dictyoceratida order are viable resources of new and interesting secondary metabolites. As well, these compounds are halogenated, containing either chlorine or bromine or both which adds to the list of constituents with this functionality. This is best illustrated by the plethora of bromophenols and chlorinated peptides that continue to emerge from *Dysidea*. This Indo-Pacific sponge represents a unique example in the realm of marine natural products chemistry and is responsible for a major portion of the halogenated metabolites identified in this order.

The direction of the UCSC marine natural products program has been focused mainly on marine organisms in other orders of the Porifera due to the abundance of sesqui- and sesterterpene compounds in the order Dictyoceratida. These metabolites have been well-studied over the years and are of little interest. However, the identification of the unusual cytotoxic hexapeptide from *Psammocinia* (Thorectidae: Dictyoceratida) clearly illustrates the need for further study of marine sponges of this order, especially those sponges that appear to contain cyanobacteria. It is possible that these organisms are in fact the producers of these metabolites due to the minimal quantities isolated and by the fact that literature in the field is increasingly identifying circumstantial evidence in support of this theory.

The identification of this cytotoxic constituent also demonstrates that the chemistry of Thorectidae sponges, which have been little studied, deserves more attention. It should be noted that several of the extracts from the *Psammocinia* sponge exhibited potent activity and further efforts are underway to identify these other bioactive components. In terms of bioactivity another point emerges from the

cyclocinamide story; the importance of the brine shrimp assay in early identification of bioactive fractions during workup. In this case at least, it does what it is supposed to do and that is to quickly focus attention on the isolation of bioactive constituents and to minimize false leads that waste valuable time and resources. This assay coupled with high-field NMR is especially important for new incoming students to the field and is one reason the UCSC marine natural products program is using these valuable tools. It increases the chances of finding anticancer active compounds like cyclocinamide A which otherwise might go undiscovered.

As presented in this work, there is now a more definitive picture of the association of symbiotic cyanobacteria with marine sponges and specifically with the Indo-Pacific sponge *Dysidea* and the filamentous cyanobacteria *Oscillatoria*. Whether there is a evolutionary link between these organisms and marine sponges is still not fully understood due to the complexity of the situation. More studies will be needed to better understand this relationship. However, in the context of this dissertation on this matter there is now a broader understanding. The growing field of molecular biology may hold the key with the DNA sequencing of *Oscillatoria*. It could definitively identify the respective chemotypes or strains that inhabit this unique marine sponge. This research also lays the foundation for future chemotaxonomic work using the tools of NMR and ESIMS in the evaluation of other marine sponges. The Indo-Pacific sponge *Phyllospongia*, morphologically similar to *Dysidea* and containing cyanobacteria, is currently in need of evaluation. Work in this area is currently ongoing in the UCSC marine natural products program.

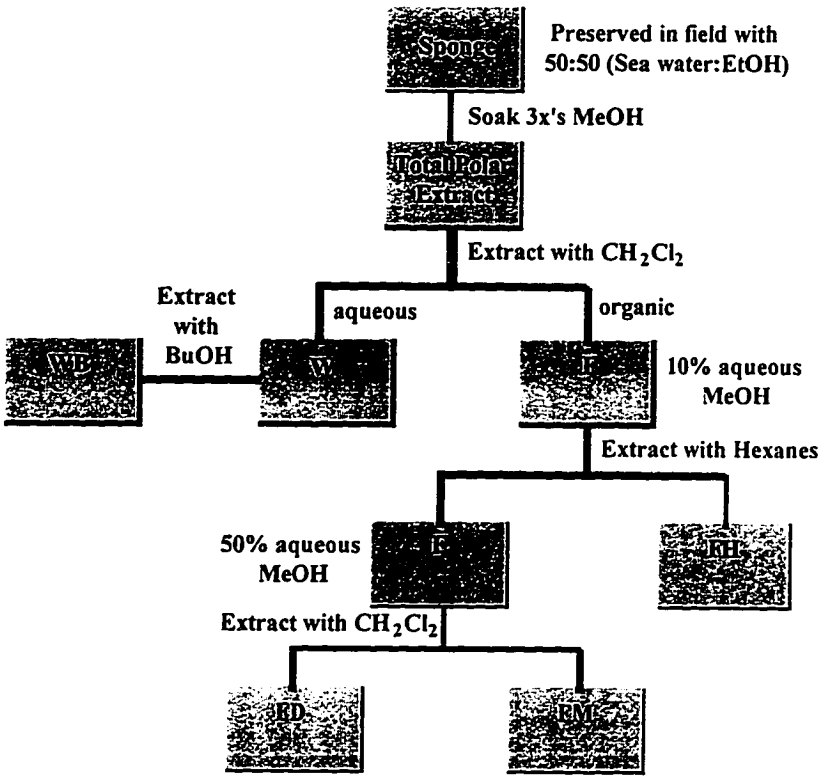
As for the future of marine natural products chemistry it is clear that this field serves an important role in the drug discovery process. The marine environment is a unique source of novel and interesting secondary metabolites which are not isolated from

terrestrial sources. With the advent of higher-field NMR instruments, more advanced bioassay screens and the increasing explosion of biotechnology, the chemistry of many marine organisms deserves to be reexamined. The potential for new discoveries and new applications in marine natural products chemistry is just beginning.

Appendix: Methods

Appendix A

General Extraction Procedure for Marine Sponges. The sponge is preserved in the field with 50:50 (Ethanol:Sea Water), soaked for 24 hours and discarded the following day. Once transported to the lab, the sponge is soaked in MeOH three times (minimum 24 hours for each pour-off) with the solvent being removed from each pour-off by rotary evaporation. The crude MeOH extracts are combined and subjected to our liquid-liquid extraction scheme (Scheme A1). The MeOH extract is partitioned between H_2O and CH_2Cl_2. The aqueous phase is extracted with butanol to give the (WB) fraction. After rotary evaporation of the organic phase, the crude dichloromethane fraction is further partitioned between hexanes (FH) and 10% aqueous MeOH to remove fats. Water is added to the 10% aqueous MeOH fraction to give a 50% aqueous MeOH (FM) solvent partition fraction which is extracted with dichloromethane (FD).



Appendix B

HPLC mobile phase optimization using the Solvent Triangle. I successfully applied the Solvent Triangle in all my marine natural products isolations. Previously, these principles had not been applied in the separation of unknowns, but only in the analytical separations of already identified compounds. My goal was to find a better method to perform HPLC in my field. Therefore, I took these basic principles and developed a system whereby anyone, with little or no experience, can use it and achieve excellent results in their HPLC separations the first time. It is efficient in that the mobile phase for HPLC is developed using thin-layer chromatography (TLC) instead of HPLC. This uses only minute quantities of organic solvents. The use of ternary and quaternary mixtures of solvents in the mobile phase enhances separations and in most cases gives baseline resolution. The application of this system is illustrated by the following study I conducted on a mixture on known compounds.

Procedure for mobile phase optimization using TLC and Solvent Triangle.

Step 1. Calculate eluting power (EP) of a binary solvent mixture that gives a R_f range of 0.15-0.35 for the extract (Figure B.1). Use % composition (% Comp.) of the organic and its respective solvent strength (SS) to calculate (see Table B.1 for SS's and Mobile Phase Triangle (Figure B.3) for formula; SS of H_2O = 0 in reverse phase,RP and SS of hexanes = 0 in normal phase, NP).

Step 2. Calculate EP ratio(s) (#1-10; Figure B.3) of desired solvent mixture using the eluting power calculated for the binary mixture.

Step 3. Calculate % composition of desired ternary or quaternary solvent mixture using appropriate solvents (miscible solvents in RP or NP). For best results choose solvents from three widely separated groups (see Solvent Group Triangle Figure B.3). (Note: I use 3cc syringes to measure solvents of ternary or quaternary mixtures with a total volume of 3ml.). The results of the example study are illustrated in Table B.2 and Figures B.3, B.4.

Table B.1. Updated Version of Snyder's Classification of Organic Solvents‡

Solvent	Strength (SS)	Solvent	Strength (SS)
		Solvent Group I	
triethyl amine	1.9	*i*-propyl ether	2.4
ethyl ether	2.8	butyl ether	2.1
		Solvent Group II	
n-octanol	3.4	*n*-butanol	3.9
t-butanol	4.1	*n*-propanol	4.0
i-propanol	4.2	ethanol	3.6
i-pentanol	3.7	methanol	3.0
		Solvent Group III	
tetrahydrofuran	4.4	methoxy ethanol	5.5
diethylene glycol	5.2	triethylene glycol	5.6
		Solvent Group IV	
acetic acid	6.0	ethylene glycol	6.9
benzyl alcohol	5.7		
		Solvent Group V	
methylene chloride	3.1	ethylene chloride	3.5
		Solvent Group VI	
ethyl acetate	4.4	acetonitrile	3.1
acetone	3.4	cyclohexanone	4.7
methyl ethyl ketone	4.7		
		Solvent Group VII	
toulene	2.4	phenyl ether	3.4
ethoxybenzene	3.3	anisole	3.8
		Solvent Group VIII	
chloroform	4.1	*m*-cresol	7.4

‡Snyder, L. R., *J. Chrom. Science* **1978**, *16*, 223-234.

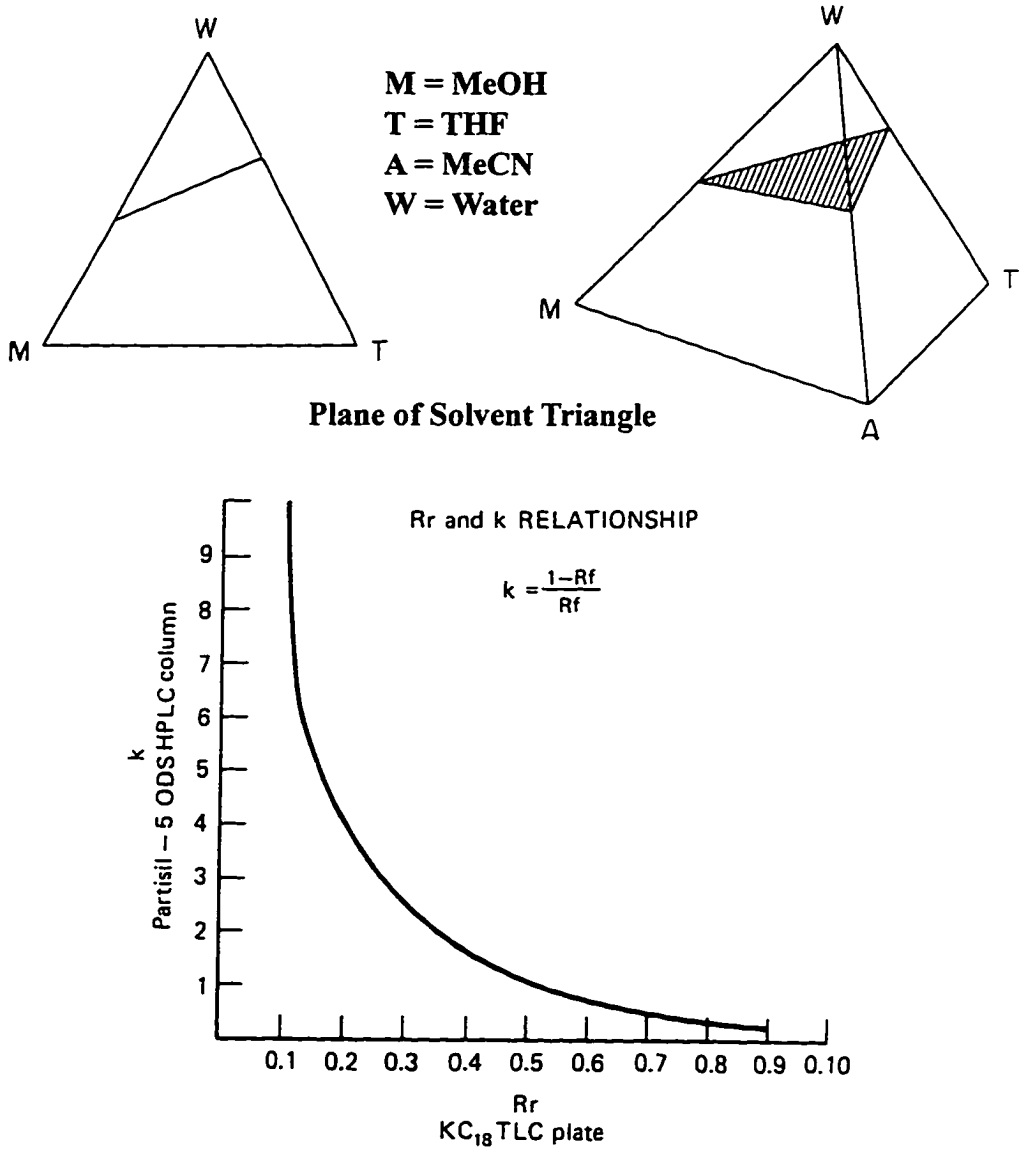


Figure B.1. Correlation of R_f in TLC to HPLC. Optimum Range = 0.15-0.35.

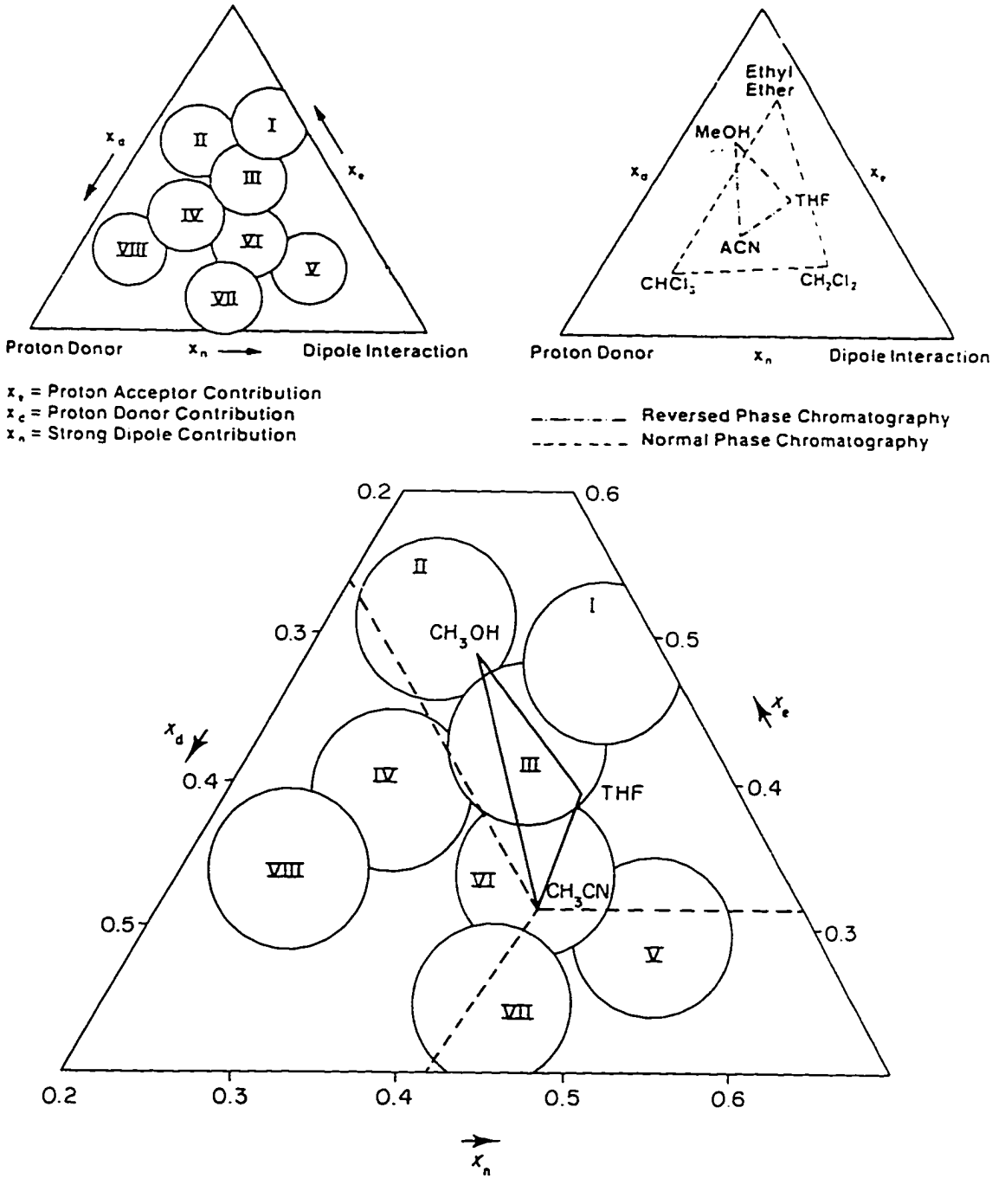
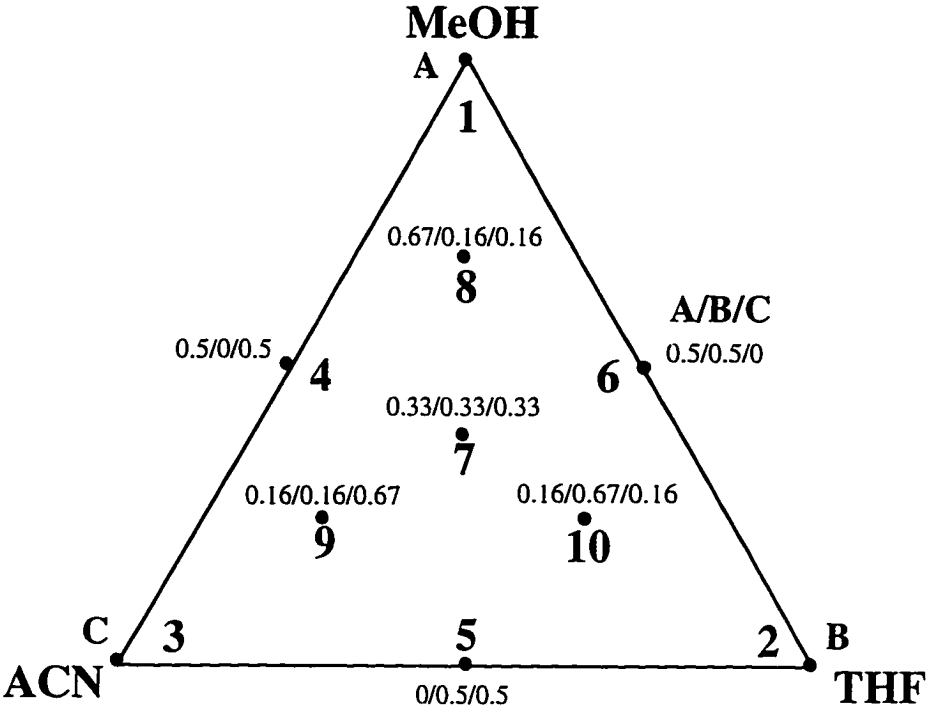


Figure B.2. Solvent Group Triangle.



EP = (% Comp.) (SS) + (% Comp.) (SS)

1. **MeOH:H_2O (60:40)** ⟶ **[1.8 = (.60) (3.0) + (.40) (0)]**
2. **THF:H_2O (41:59)**
3. **ACN:H_2O (58:42)**
4. **MeOH:ACN:H_2O (30:29:41)** ⟶ **[.9 = (% Comp) (3.0) MeOH; .9 = (% Comp) (3.1) ACN]**
5. **THF:ACN:H_2O (20:29:51)**
6. **MeOH:THF:H_2O (30:20:50)**
7. **MeOH:THF:ACN:H_2O (20:13:19:48)** ⟶ **[.59 = (% Comp) (3.0) MeOH; .59 = (% Comp) (4.4) THF; .59 = (%Comp) (3.1) ACN]**
8. **MeOH:THF:ACN:H_2O (41:7:9:43)**
9. **MeOH:THF:ACN:H_2O (10:7:39:44)**
10. **MeOH:THF:ACN:H_2O (10:28:9:53)**

Figure B.3. Calculated Mobile Phase Compositions for Test Mixture Using Mobile Phase Triangle (R_f = 0.11-0.40 for mobile phase #1-60:40 (MeOH:H_2O).

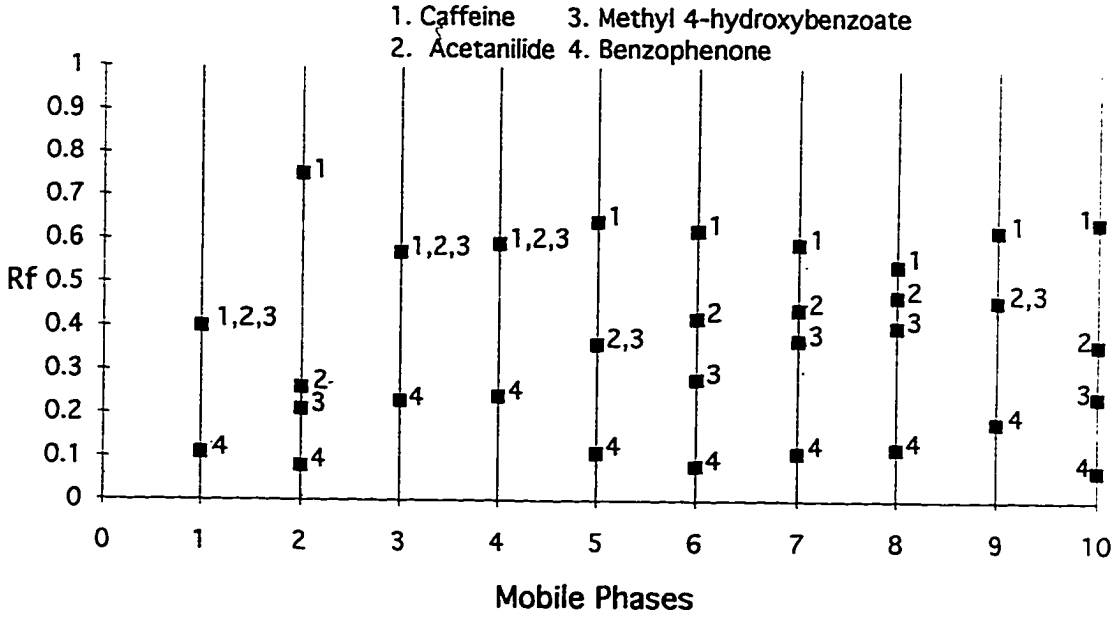


Table B.2. TLC Results of Test Mixture Using Calculated Mobile Phases.

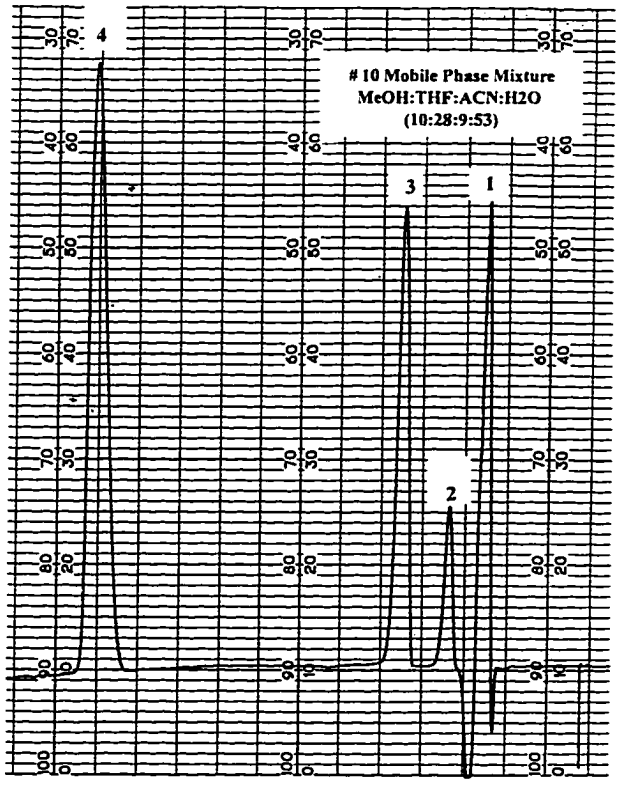


Figure B.4. HPLC Separation of Test Mixture Using Mobile Phase #10.

Appendix C

General procedure for brine shrimp assay.

Step 1. Make up a saturated solution of brine to grow up brine shrimp with sea salt and deionized water. It will take up to 48 hours to grow them up. They will be viable for 48-72 hours. Oxygenate brine solution with a slow air stream.

Step 2. Make up a 20 mg/ml solution of the crude extract to be tested in ethanol.

Step 3. Take 100 µl of above solution and add to 10 ml of brine solution to give a 200 µg/ml concentration (A).

Step 4. Take 100 µl of (A) and add to 10 ml of brine solution to give a 20 µg/ml concentration (B).

Step 5. Take 100 µl of (B) and add to 10 ml of brine solution to give a 2 µg/ml concentration (C).

Step 6. Take up 10-15 brine shrimp in the tip of a pipet to minimize volume change in concentration and add to vial (A). Repeat for vials (B) and (C).

Step 7. Measure mortality rate after 24 hours.