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Investigations of Natural Products from Sponges and Sponge Associated Marine Fungi

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UNIVERSITY OF CALIFORNIA

SANTA CRUZ

INVESTIGATIONS OF NATURAL PRODUCTS FROM SPONGES AND SPONGE ASSOCIATED MARINE FUNGI

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

DOCTOR OF PHILOSOPHY

in

CHEMISTRY

by

Mustafa Varoglu

June 1996

The dissertation of Mustafa Varoglu is approved:

Dr. Phil Crews, Research Advisor

Dr. Todd W. Wipke, Committee Chair

Dr. Rebecca Braslau, Committee Member

Dean of Graduate Studies and Research

ABSTRACT

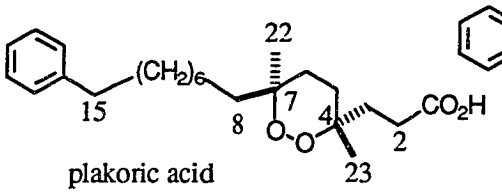
Two areas of marine natural products were explored in this work. The young but established area of sponge natural products was investigated as a source of anticancer secondary metabolites. In addition, the newly emerging field of marine fungal natural products was studied to provide insight on the capacity of marine fungi to produce novel natural products. The possibility that marine fungi are a microbial source of sponge derived natural products was examined through chemical investigations of fungi cultured from sponges.

The chemical investigation of both the sponges and fungi was carried out with the modern techniques and methods of natural products chemistry. Sophisticated chromatographic and spectroscopic methods were employed for the isolation and characterization of all isolated natural products

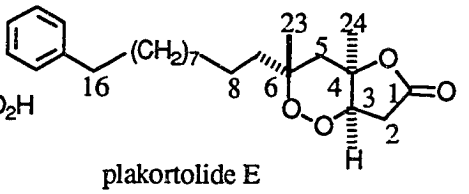
The four projects described in this thesis are presented in two parts. Chapters two and three describe the structures of plakortolide E and plakoric acid from a *Plakortis* species sponge and the structure of hipposulfate from a *Hippospongia* species sponge. The work on the *Plakortis* sp. sponge, chapter two, was initiated by whole cell cytotoxicity assays of slow growing melanoma tumor lines. The work on hipposulfate, chapter three, was initiated by a positive result in a matrix metalloproteinase enzyme assay. This enzymatic screening is a complimentary method to the whole cell assay method employed for the cytotoxic *Plakortis* sp. extracts.

Chapters four and five provide the details of the work on sponge derived marine fungal cultures. Chapter four discusses the young field of marine fungal natural products and provides a review of the sources and novelty of the compounds found to date in this new area. Chapter four also describes the first fungal project in this laboratory which yielded three new natural products, chloriolins A-C. Chapter five describes the structural elucidation of two novel compounds,

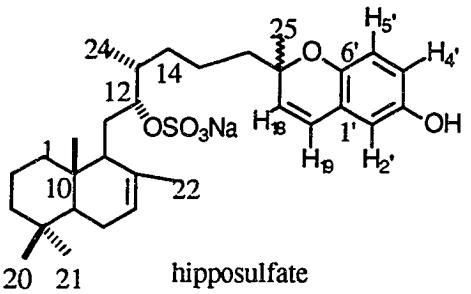
asperizine and asperic acid, and the dereplication of three known secondary metabolites, malformin C, pyrophen, and hexylitaconic acid, from a sponge derived *Aspergillus* c.f. *niger* fungus.



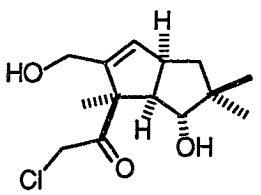
plakoric acid



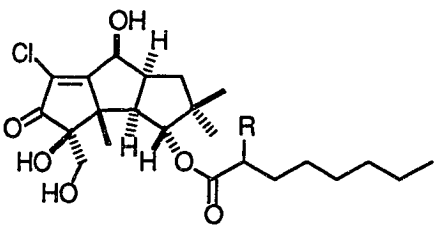
plakortolide E



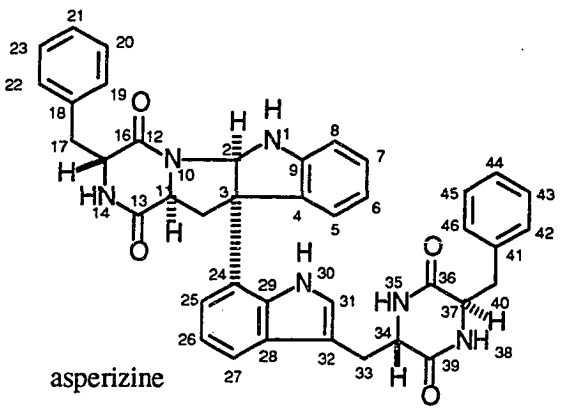
hipposulfate



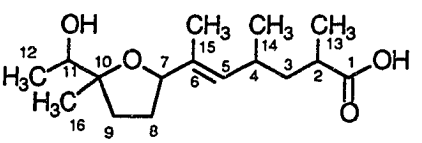
chloriolin A



chloriolin B R = OH
chloriolin C R = H



asperizine



asperic acid

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ACKNOWLEDGMENTS

The help and support of many people have made this endeavor possible. The work presented in this thesis would not have been possible without the guidance and encouragement of Professor Phil Crews. His help has extended beyond the valuable technical advice on the projects presented. Phil Crews' personal demonstration of the power of optimism and perseverance in the face of unending challenges has been inspiring. The integration of these traits with the research skills I have learned at U.C. Santa Cruz has made my stay here much more rewarding. I am also grateful to Phil Crews for the phone call he made to Vancouver, B.C. in 1990. That one phone call led to a graduate career that has been much more than I had expected.

During his time at Santa Cruz, Jaime Rodríguez provided my laboratory training. The majority of the techniques and skills I have learned were taught to me by Jaime in the years we shared Thimann 309 and 310. Aside from teaching me valuable laboratory lab skills Jaime demonstrated the pace that all research should proceed. Beyond his contributions to the chemistry of the Crews group Jaime brought Rosa Nieto with him to Santa Cruz. I am grateful to have experienced Rosa's kindness and generosity.

I thank Marcel Jaspars for his help in the lab and for dragging me up, down and sometimes on the mountains around Santa Cruz. I am sure somewhere on Empire, Felton, and Alba Grades I must have built character. I owe Sally Jaspars for the meals and company after chasing Marcel on his bike or trying to avoid him on his surfboard.

While Marcel unwittingly put me in danger in the ocean I thank Sam Sperry and Blaine Harrison for knowingly taking me into large waves breaking near rocks with no predictable pattern. I am also grateful to have learned the definitions of pearling, caught inside and over the falls first hand.

I have enjoyed the friendship and support of Jarrett J. Farias and Dale P. Clark since we all entered this program together.

I have appreciated Leif Abrell's advice and outlook since I met him in the Research Diving Class in 1991. I also appreciate the parties at his house and the continued opportunity to sample alternatives to B.C.'s finest.

Cristina Diaz and Lisa Hunter made diving abroad much more pleasant. I thank the two of them for teaching me the fundamentals of sponge taxonomy and how to take the difficulties of expeditions in stride. I am especially grateful to Cristina for her friendship, in general, her support in Amsterdam, and for introducing me to Larry Manes. Manatees now play a much larger role in my life. Miranda Sanders has continued the tradition of personal growth during expeditions, especially during our group therapy sessions in Puerto Rico.

With her compassion and friendship Andrea Michels helped me through the many large and small difficulties encountered on the way to this thesis. I thank Andrea for acting as a big sister; I will always value her frank advice. For the future graduate students to come I hope Andrea will remain here as a crucial part of the Crews group.

I am indebted to Amy Marie Yockel (AMY) for her love and support at home. In addition, to helping me enjoy my time outside of the Thimann I am grateful to Amy for her painstaking attention to the details of labeling, arranging, and laying out the graphics for this thesis. Her help has made writing this thesis on time possible. One of these years I promise to understand the concept of time.

I am grateful to Jim Loo for his help with all of the NMR machines that were used to acquire data for this thesis. I thank Jim not only for the times he has helped me during the week, but also for his willingness to come in on the weekends to change a probe or deal with real or imaginary emergencies.

Mr. Nakashima, my grade 11 chemistry teacher, fueled my earliest interest in chemistry through his encouragement and support. With his ability to teach and explain difficult concepts he laid the foundation of my chemistry education.

I thank my parents and sister for the models and values they have provided for me and for their continued support and encouragement in all aspects of my life.

CHAPTER ONE

Background, Significance and Techniques for the Study of Natural Products

Background

Natural products chemistry deals with the compounds produced by living organisms. All living entities have a primary metabolism for the generation of energy and macromolecules for growth and reproduction.[1] The organic compounds of primary metabolism are largely polymeric or oligomeric and are composed almost exclusively of a small number of building blocks such as carbohydrates, amino acids, lipids and nucleotides. The compounds and processes of primary metabolism are essential for the continued life of all organisms.

In contrast, some plants, animals and microorganisms possess other biological pathways which produce much more complex organic molecules that are not critical for the life of the producing organism.[2] These pathways are termed secondary metabolism and the compounds they produce are secondary metabolites. Primary metabolism end products, or intermediates, often act as the initial substrate for the production of secondary metabolism.[2,3] Examples of this include the diversion of methylmalonate from lipid biosynthesis for the production of polyketides and the use of amino acids to produce alkaloids. When organic chemists speak of natural products chemistry they are generally referring to the chemistry of secondary metabolites.

There has been much speculation on the role of secondary metabolites. While the amount of secondary metabolites produced by an organism is small compared to the amount of primary metabolites, highly specialized enzyme systems are devoted to the manufacture of these complex compounds.[4] One hypothesis is that these compounds are used to give advantage to organisms over enemies.[5] This may be the case for antifungal or antibacterial compounds. Other compounds are thought to be for the protection from predators[6] and it has been hypothesized that some compounds

may be used as metal chelators.[7] There is even speculation that secondary metabolites may have been used to modulate or catalyze primitive macromolecular reactions.[8]

Natural products chemistry grew out of the oral history of terrestrial medicinal herbs and plants. The use of willow bark (aspirin) and poppy seed pods (opium) for pain relief and the bark of the cinchona tree (quinine) to cure malaria are a few of the examples of traditional medicinal natural products. These plant-derived concoctions were originally used without knowledge of the active chemical species. At the turn of the 20th century many advances were made in the isolation and identification of these compounds and plant natural products are still extensively studied today.

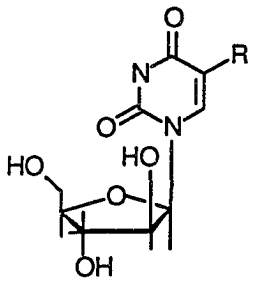
The 1930s saw the advent of microbial natural products chemistry with the discovery of the antibacterial penicillin. This marked the discovery of a new source of natural products.[9] The development of new sources of secondary metabolites lead to new compound classes. The search for novel natural products has benefited from the exploration of new biological sources. While the terrestrial environment has been well-explored, the oceans have been traditionally ignored as a source of new secondary metabolites. It is expected that marine organisms will have the ability to produce new structural types.

In the 1950s SCUBA (Self Contained Underwater Breathing Apparatus) allowed marine natural products to move past the intertidal zone. SCUBA provides access to deep water organisms with greater specificity. The marine environment contains completely different phyla, i.e., sponges, dinoflagellates, and tunicates, not observed on land. For the phyla that are found both on land and in the ocean, such as fungi and bacteria, the marine habitat may provide different species compared to the terrestrial counterparts. Since the 1950s many new structure classes have been isolated from marine organisms.[10]

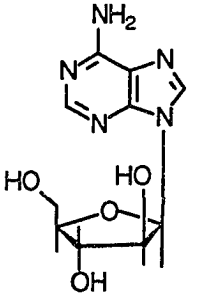
Significance

Terrestrial natural products have displayed a wide range of biological activity including cytotoxicity to cancer cells *in vitro* and *in vivo*. Approximately half of the cancer chemotherapeutics are either natural products or are derived from natural products,[11] including the recently FDA approved yew-tree derived compound taxol. One of the goals in natural products chemistry is to identify novel chemical skeletons as drug leads. A compound isolated from nature is rarely the most effective compound in terms of biological activity and toxicity however natural products provide important new ideas for the structures of potent therapeutics. A strategy for the discovery of new natural products is to examine new organisms from unexplored ecological sources.

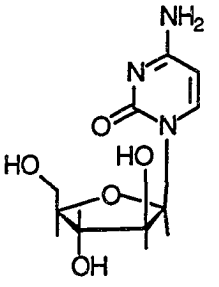
While many marine natural products have been discovered with selective activity against a variety of enzymes and cell types only a few have advanced to clinical trials or developed as drugs. Werner Bergmann was responsible for the discovery of the cytotoxic lead compounds spongouridine (**1**) and spongothymidine (**2**).[12] These arabinose nucleosides led to the development of the anticancer drugs ara-A (**3**) and ara-C (**4**) as well as antiviral agents which have culminated in acyclovir (**5**). Other marine natural products in clinical trials are didemnin B (**6**),[13] from the tunicate *Tridemnum solidum*, bryostatin 1 (**7**),[14] from the bryozoan *Bugula neretina*, and giradazole (**8**),[15] a simple yet cytotoxic compound from the sponge *Pseudaxinyssa cantharella*.



1 spongouridine R = H
2 spongothymidine R = CH_3



3
ara - A



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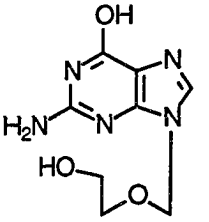
ara - C



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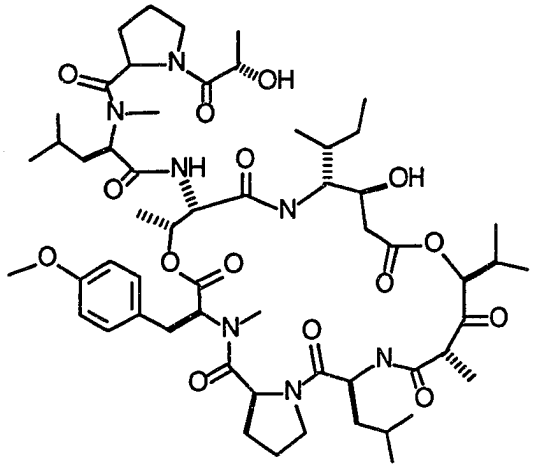
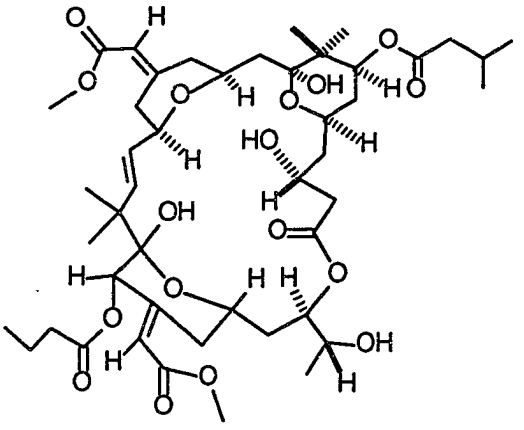
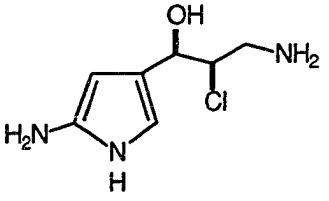
acyclovir



6
didemnin B



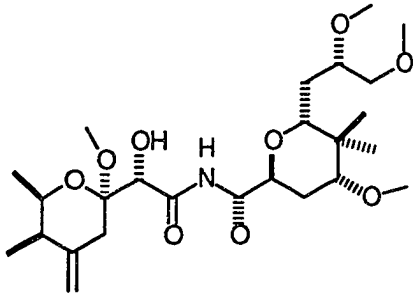
7
bryostatin 1



8
girodazole

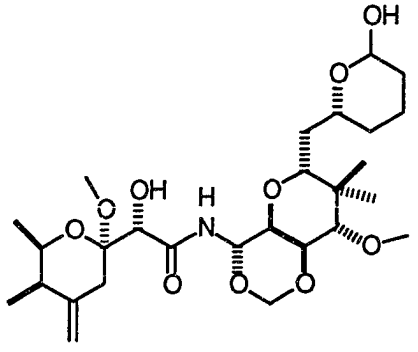
Generally, marine natural products are structurally divergent from terrestrial secondary metabolites. However, there are exceptions in which very similar compounds have been isolated from very different terrestrial and marine organisms. Pederin (**9**), a compound isolated from the blister beetle *Paederus fuscipes*,[16] is similar to the theopederins (**10**),[17] from the sponge *Theonella* sp., and the mycalamides (**11**)[18] isolated the sponge *Mycale* sp. Mimosamycin (**12**) was first isolated from the terrestrial bacterium *Streptomyces lavendulae*.[19] Mimosamycin and a related compound reinerone (**13**) were subsequently isolated from a *Reniera* sp. sponge[20] and these compounds were also found in *Cribochalina sp.*[21] and *Xestospongia* sp.[22] sponges. These

observations have fueled a debate on the true source of some marine natural products. In other cases when extremely divergent natural products are isolated from the same sponge there has also been the question of the true source of the marine natural products. This is the case for the marine sponge *Dysidea herbacea* in which brominated phenols, terpenes, and chlorinated peptides have been isolated.[23,24] *Dysidea herbacea* is often found associated with the cyanobacteria *Oscillatoria spongeliae*. It has been conclusively shown the chlorinated peptides and the brominated phenols represented by dysidin (**14**) and 2(2'-4'-dibromo-phenyl)-4,6-dibromophenol (**15**), are produced by the cynaobacteria *Oscillatoria spongeliae* while the terpene compounds herbadysinolide (**16**) and spyrodysin (**17**) are synthesized by the sponge.



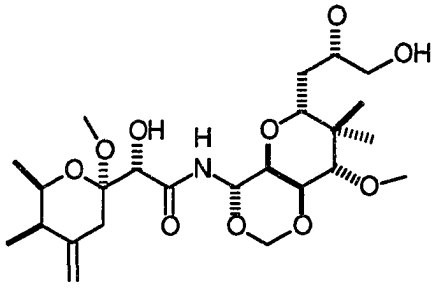
9

pederin



10

theopederin



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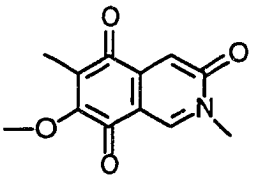
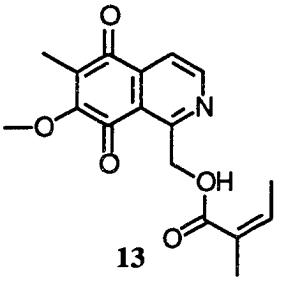
mycalamide



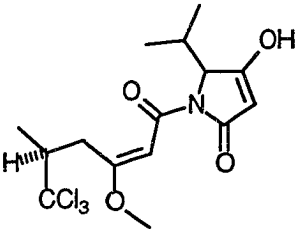
12

mimosamycin



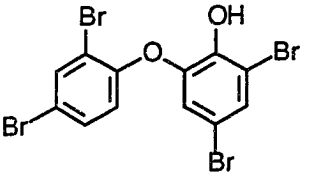
13

reinerone



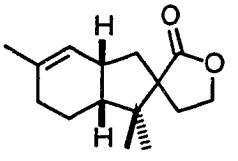
14

dysidin



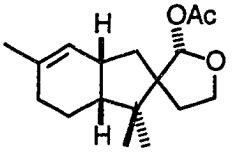
15

2(2'-4'-dibromophenyl)-4,6-dibromophenol



16

herbadysinolide

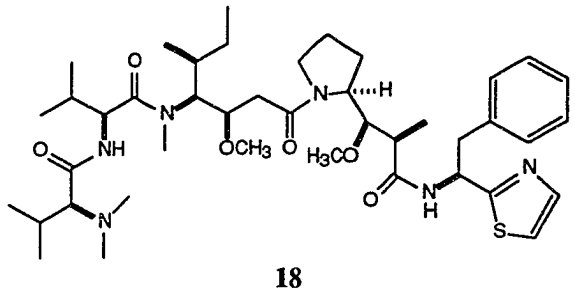


17

spirodysin

Generation of a sufficient amount of biologically active compounds for human testing is a key difficulty in the development of marine natural products. The dolastatins, from the sea hare *Dolabella auracula*, exemplify the difficulty of generating supply. Approximately 1000 kg of sea hare was collected to yield 28.7 mg of the most active dolastatin, dolastatin 10 (**18**).[25] The finite supply of marine macroorganisms such as sponges, tunicates, and sea hares requires a search for alternative sources of biologically active natural products. The exploration of sponge and other marine macroorganism associated microorganisms, such as cyanobacteria, bacteria, actinomycetes, and fungi, will allow the large scale culture and production of natural products. While the source

of the *Dysidea herbacea* peptides and phenols has been determined as the cyanobacteria *Oscillatoria spongeliae* there have been no reports of the culture of these cyanobacteria.



18

dolastatin 10

There is well documented research on the natural products of marine bacteria and cyanobacteria.[26-28] The main culture source of bacteria is sediment and the surfaces of marine materials.[28] The culture of marine cyanobacteria is usually from collection of wild specimens.[27] Marine fungi have been examined sporadically since the late 1970s and several reviews have documented the emerging natural products of marine fungi.[29-32]

When confronted with the biodiversity of the ocean the first question is which organism to investigate for new secondary metabolites. In plant terrestrial natural products there is an ethnopharmacological basis for the collection of specific plant species. For marine natural products research there is rarely such a history. Instead, the initial approach was one of random collection of a wide variety of phyla. Over the last forty years certain patterns have emerged when examining the biosynthetic range of the different phyla. Sponges are able to produce the widest variety of compounds from a number of different biosynthetic pathways.[33] Many of the compounds isolated from sponges are cytotoxic or otherwise biologically active.[34,35] In contrast the area of marine fungal natural products is so new that trends in structure types and biological activity are just now starting to emerge.

The research presented in this thesis explores both established and emerging aspects of marine natural products. The ability of sponges to continue to be a source of new cytotoxic compounds is examined. In a complementary fashion empirical evidence on the ability of marine fungi to biosynthesize novel natural products is investigated.

Techniques - collection of samples

Before the chemistry of a marine sponge or fungi can be examined the organism must be collected from an often remote field site and returned to the laboratory. The collection technique will vary depending on whether sponges or fungi are sampled. Both sponges and fungi are collected by SCUBA to depths of 40 m. If samples from deeper water are desired this can be accomplished by trawling or submersibles. SCUBA provides the ability to collect more selectively and with more flexibility than either submersibles or trawlers. With appropriate preservation techniques, collection by SCUBA can be accomplished wherever there is a commercial dive operation. This reduces the need for home ports for trawlers or large support vessels for submersibles.

Many methods exist to collect fungal cultures.[36,37] Core samples of sediment may be acquired and dissolved in sterile water. This solution and its serial dilutions may be used to inoculate plates containing appropriate antibacterial agents to encourage the growth of fungi. Marine substrates such as sponges, mangrove roots, driftwood, or parts of alga or sea animals may also be used to start cultures. Sometimes these samples are surface sterilized to ensure that the cultures that emerge from these samples are truly endogenous to the samples.

If the sponge samples are not extracted in the field then they must be preserved for transport to the laboratory. Preservation may be by either freezing or chemically fixing the sponge. The Crews laboratory preserves sponge samples with a solution of 50:50 water: alcohol (methanol or ethanol) in which the sample is soaked for at least 24 hours. The preserving solution is then discarded and the sponge remains fixed and will not rot before return to the laboratory. This allows

for collection of samples in areas where freezer facilities are not available. Sponge samples that are collected for the culture of fungi are surface sterilized and cut into small pieces before being placed on the culture plates. Fungal cultures which are observed to originate from the sponge are transported back to the laboratory.

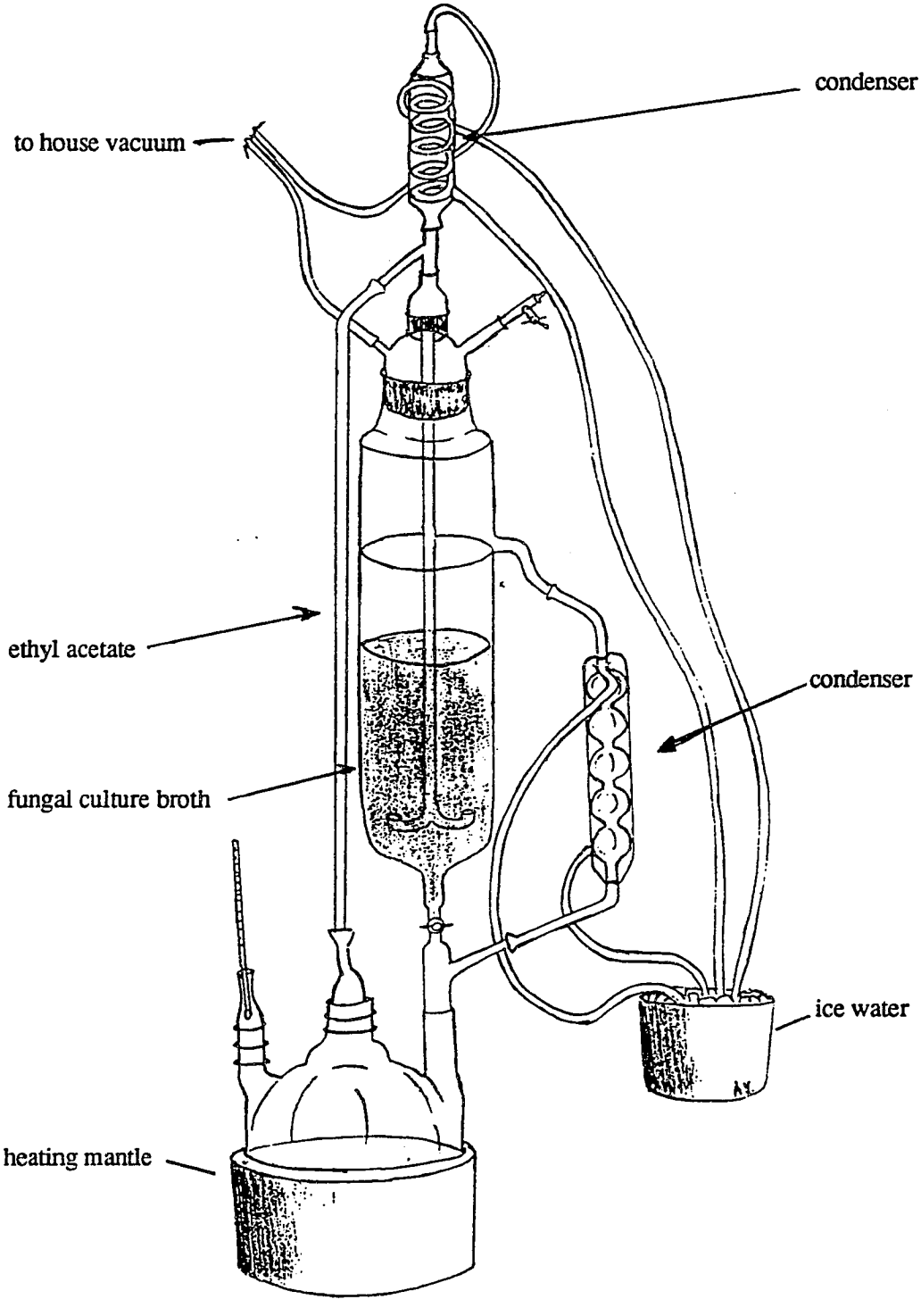
Techniques - extraction, assay and purification of compounds

To prioritize sponges or fungal cultures for natural products investigation the samples must first be extracted. Both sponges and fungal mycelia are extracted in the same manner with methanol. The culture broth that the fungi were grown in is extracted with ethyl acetate. This extraction may be by either separatory funnel or by continuous liquid-liquid extraction using a specially designed large scale extractor. A large scale extractor for the continuous extraction of fungal broths was designed in this laboratory and is shown in Figure 1.1.

The crude extracts are partitioned by a series of solvent-solvent partitions before priority can be assigned. A modification of the partition scheme developed by Kupchan[38] is used to group the compounds with the same polarity together in a fraction, Figure 1.2.

A wide variety of biological screens exist to assay crude extracts and fractions. The earliest screens measured antifungal, antibacterial, and brine shrimp toxicity.[39] Sea urchin egg division assays to indicate potential neoplastic activity and the use of cancer cell lines have been developed more recently.[40] The use of purified enzymes to search for specific inhibitors is also a commonly used screening method to discover novel therapeutics for cancer, heart disease, and inflammatory disease.[41,42] Non-medical assays that search for biofouling inhibitors are also used in marine natural products.[43] Once biological activity is noted in an extract, isolation and identification of the active chemical species is the next step.

Figure 1.1. Drawing of large scale (8L) continuous liquid-liquid extractor for the extraction of fungal broths.



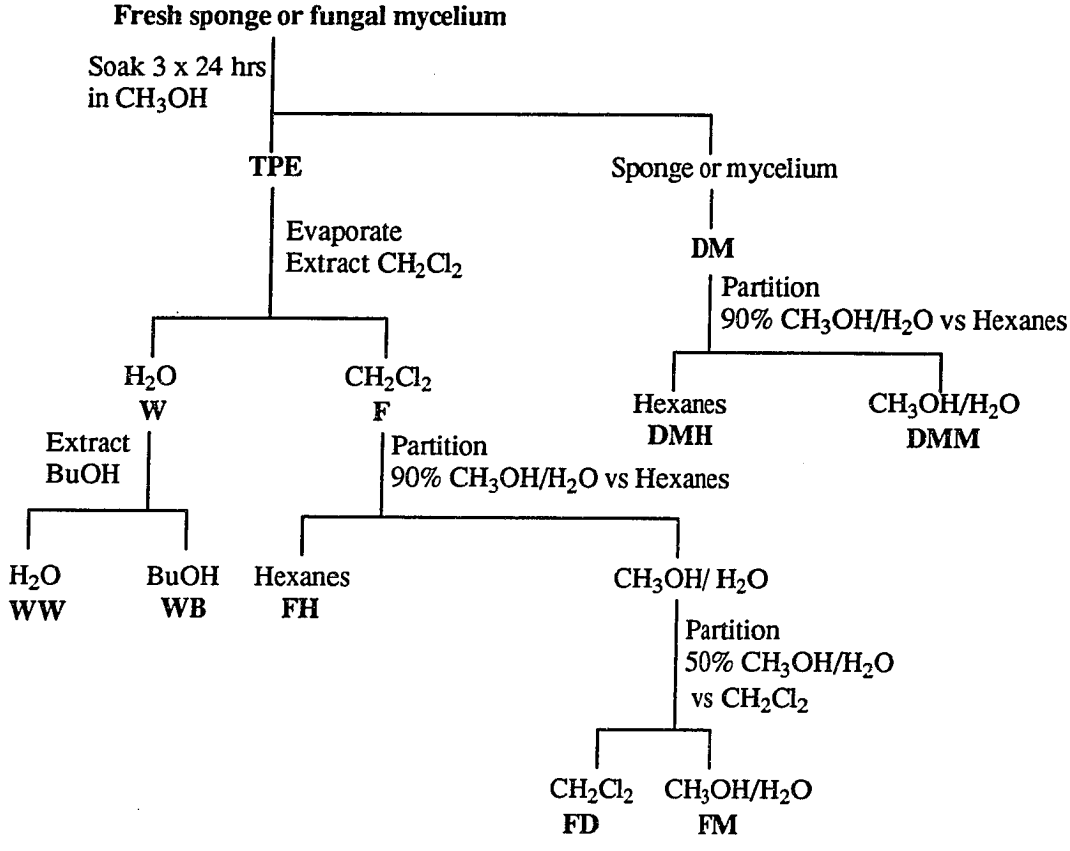


Figure 1.2. Solvent-solvent partition scheme for partitioning crude extracts.

Many different types of chromatography have been developed for the separation of biologically active compounds.[44] In general, if there is a large amount of material present in the active crude fraction a size exclusion column will be used for separation. Since compounds often come in families with similar skeletons but differing functional groups, size exclusion chromatography will tend to group all of the compounds with the same skeleton together. In bioassay-guided extraction these size exclusion chromatography fractions will be sent for bioassay, however, often from examination of the ^{1}H and ^{13}C NMR spectra of these compounds the "good" fractions can be differentiated from the "bad" fractions. Further separations may be performed

using adsorbent chromatographic methods such as flash columns or HPLC. This usually leads to the isolation of a pure compound. Other avenues for separation include countercurrent chromatography, a method that uses solvent partition coefficients to separate compounds, or preparative thin layer chromatography.

Techniques - structure elucidation

It is usually necessary to have a pure compound in hand for structure elucidation to begin. If one is fortunate enough to obtain a good quality crystal then the structure elucidation can be performed by X-ray crystallography. If a noncrystalline pure compound is isolated a standard set of data consisting of ^{1}H and ^{13}C NMR and mass spectra is acquired before any thoughts can be given to structure elucidation. To confirm that the compound isolated is truly novel, the molecular weight from mass spectroscopy and the number of carbon atoms from the ^{13}C NMR spectrum of the isolated compound can be used to derive a tentative molecular formula.

Determination of whether the compound known is usually accomplished by searching the known features of the molecule in some form of a database. This process is termed dereplication. The database used may be a review or a book on natural products, or it may be computer searchable database. There are several computer databases available for natural products chemists, such as a proprietary database of sponge compounds as in this laboratory, or MarinLit (University of Canterbury, New Zealand), which contains all the published literature on marine natural products. Other larger specialized databases exist for microbial natural products, i.e., Antibase (Chemical Concepts, GmbH), as well as an all inclusive database such as the Chemical Abstracts Registry. As the number of isolated natural products grows the use of databases for the management of molecular structure information is critical.

If the compound isolated is believed to be new, then structure elucidation can begin in earnest. If possible, the molecular weight of the compound is determined by mass spectrometry.

New advances in mass spectrometry have led to the development of techniques to consistently generate the molecular ion. One of the first of these "soft ionization" techniques was fast atom bombardment mass spectrometry (FABMS).[45] While this technique usually provides molecular ions it must be run in the presence of a matrix which add extraneous peaks to the spectrum. A newer technique for the generation of molecular ions is electrospray ionization mass spectrometry (ESIMS).[45] This method does not use a matrix and usually the molecular ion is observed clearly. Once the molecular formula is established the connectivities within the molecule are then determined.

Generally, a guess of the functional groups present is made from the values of the ^{1}H and ^{13}C NMR shifts. As summarized in Figure 1.3, first the proton spectra is reconciled with the carbon spectra by use of a one bond proton carbon *J* coupled correlation spectroscopy experiment. This can be done by a homonuclear multiple quantum correlation, (HMQC) experiment on an inverse probe.[46,47] The previous experiment to correlate protons to carbons by one bond couplings was the heteronuclear correlation ^{1}H-^{13}C COSY experiment with the $J_{C\text{-}H}$= 140 Hz.[48-51] The logical path of structure elucidation from this point usually involves the generation of substructures using H-H homonuclear *J* coupled spectroscopy, ^{1}H-^{1}H COSY.[52] This will allow determination of independent spin systems that are terminated with a quaternary carbon or hetero-atom. In order to link the disparate spin systems discerned from the ^{1}H-^{1}H COSY experiment a long-range carbon hydrogen correlation experiment is used. Previously, this was a ^{1}H-^{13}H COSY experiment with $J_{C\text{-}H}$ = 9 Hz.[53] On an inverse probe the experiment for two and three bond carbon hydrogen connectivity is the heteronuclear multiple bond correlation, (HMBC) experiment.[47] Assuming that there are no overlapped signals or other ambiguities this should produce the two dimensional structure of the compound.

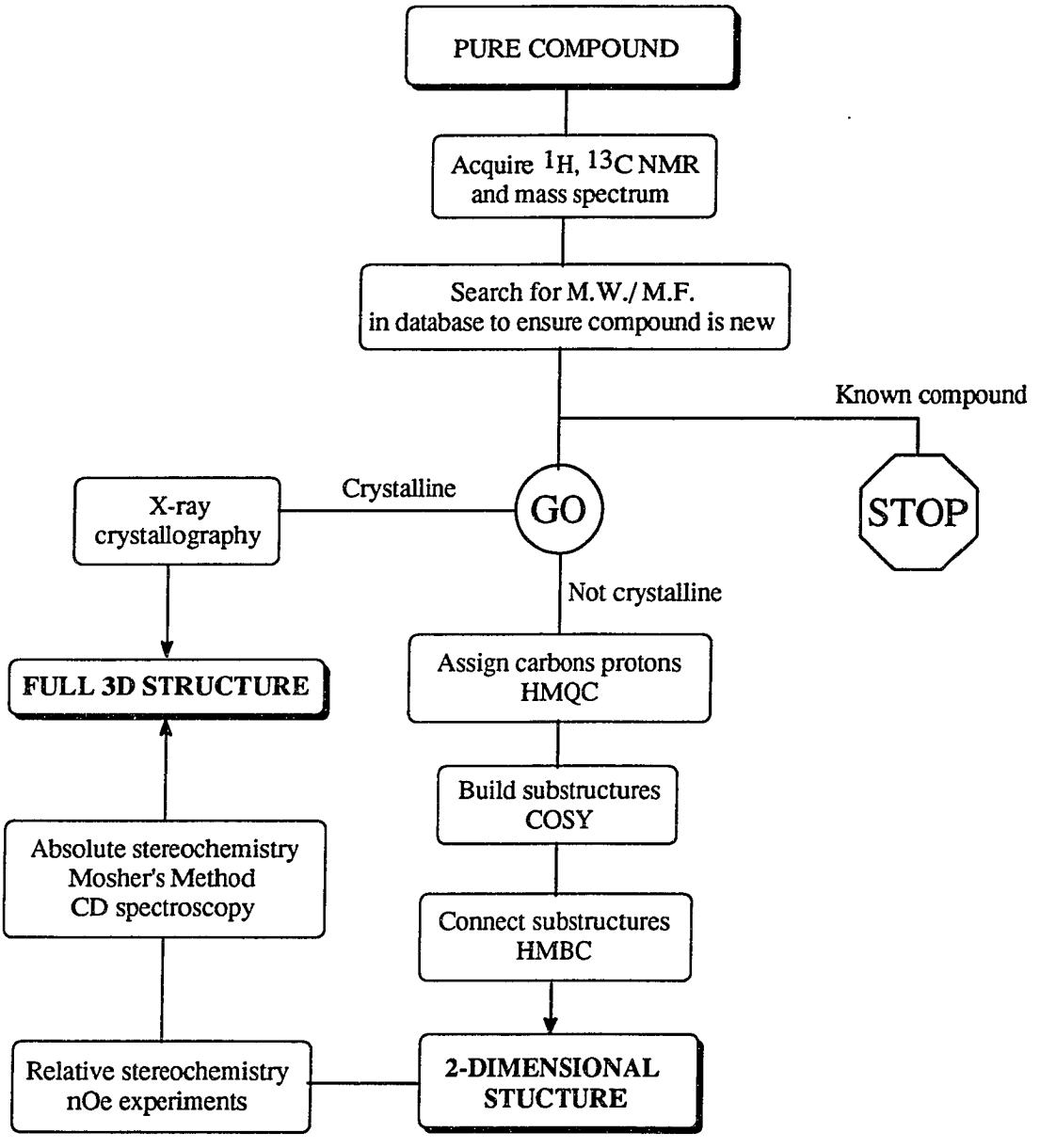


Figure 1.3. Sequence of events for structure elucidation.

Once the two-dimensional structure is elucidated the three-dimensional structure can be solved. The relative stereochemistry of the compound is often solved by the use of the nuclear Overhauser effect which when applied in a one-(1D difference nOe) or two-dimensional experiment (NOESY)[54,55] allows determination of nuclei that are near each other in space. This interaction

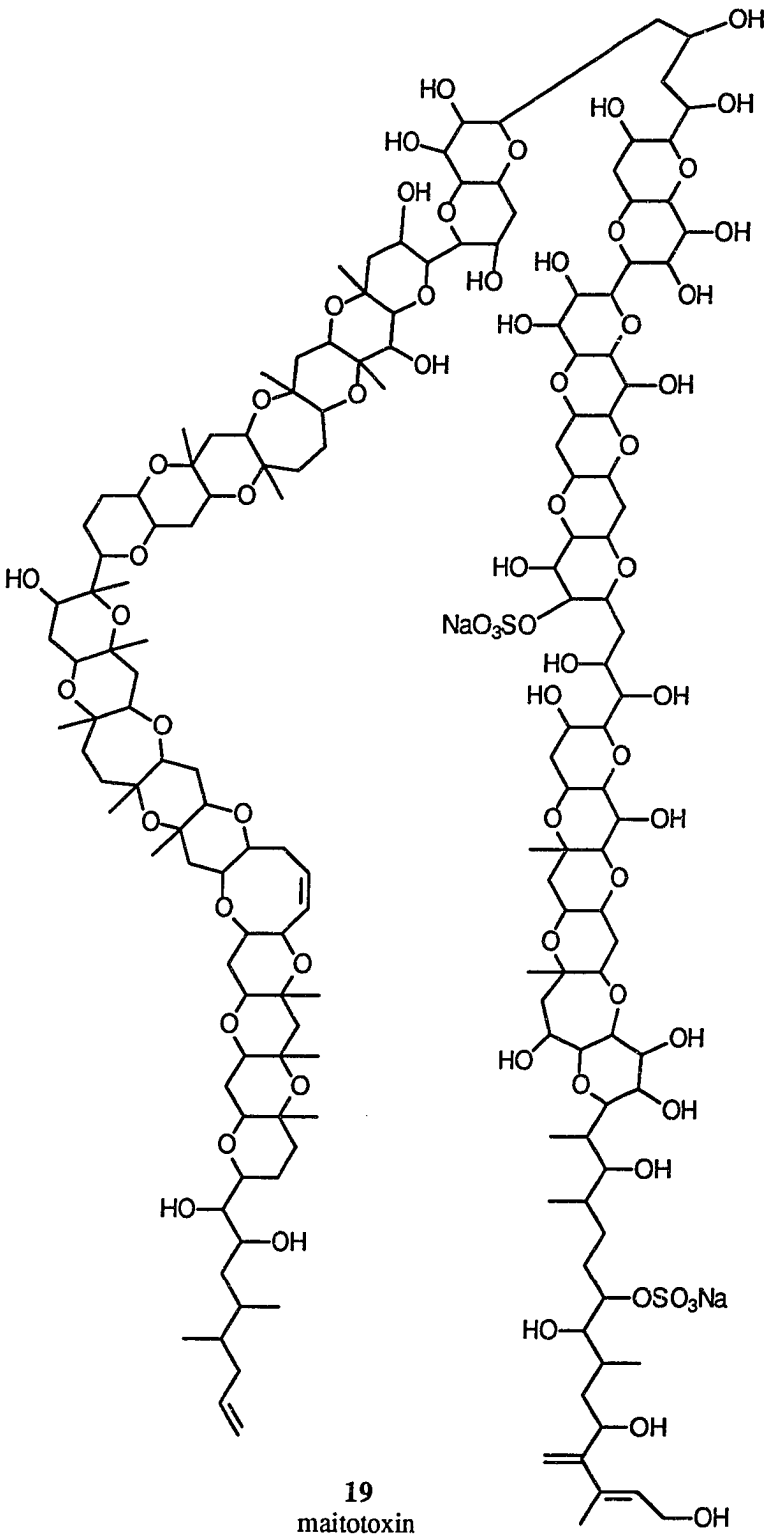
occurs through a dipole-dipole coupling of the nuclei which is strongly distant dependent. Other methods of determining relative stereochemistry include the use of coupling constant analysis for the determination of axial or equatorial groups on cyclohexane rings by using the Karplus equation. This equation correlates the diaxial angle between two coupled nuclei as a function of the coupling constant.

Many methods have been developed for the determination of absolute stereochemistry. Originally, the absolute stereochemistry of a new compound was determined either by total synthesis of the compound or by degrading the new compound to form a compound whose stereochemistry has been determined. It is not necessary to determine the absolute stereochemistry of every stereogenic center since the knowledge of the absolute stereochemistry of one center with a known relative stereochemical relationship to the other centers is enough to determine the absolute stereochemistry of the whole molecule.

Beyond total synthesis and degradation of the new compound, spectroscopic methods have been developed for the elucidation of absolute stereochemistry. Circular dichroism may be used when there is a UV/vis absorbing unit near a chiral center.[56] Improvements in NMR technology and field strength have allowed the use of novel NMR methods for the determination of absolute stereochemistry. Mosher developed this area in the 1970s by using the ^{19}F NMR resonances of triflouro esters of secondary alcohol derivatives on the substrate in question.[57] With an enantiomeric pair of acids, a pair of diastereomeric esters are produced and the flourine shifts provide information on the environment about the stereogenic secondary alcohol being examined. More recently, the use of the modified Mosher's method has employed various carboxylic acids containing a phenyl group and an *O*-methoxy group.[58,59] The shielding or deshielding effect of the phenyl group on the proton near the stereogenic alcohol is diagnostic, due to the conformational rigidity of the resulting ester. Further developments of this type have recently provided a method to determine the absolute stereochemistry of an asymmetric center adjacent to a carboxylic acid on a

novel natural product.[60] While these methods require the presence of a secondary alcohol or carboxylic acid, chemical transformation can be used to generate these functional groups in the novel compound.

The techniques outlined above have recently been used to solve the structure of maitotoxin (**19**). With a molecular weight of 3422 Da maitotoxin is the largest non-biopolymer found to date. Maitotoxin has drawn interest, not only for its large size, but also because of its acute toxicity and occurrence in edible coral reef food fish. The structure elucidation of maitotoxin is a testament to the strength of the current techniques of marine natural products.



19
maitotoxin

OVERVIEW OF WORK PRESENTED

The four projects described in this thesis are presented in two parts. Chapters Two and Three describe the structures of plakortolide E and plakoric acid from a *Plakortis* species sponge and the structure of hipposulfate from a *Hippospongia* species sponge. The work on the *Plakortis* sponge, Chapter Two, was initiated by whole cell cytotoxicity assays of slow-growing melanoma tumor lines. The work on hipposulfate, Chapter Three, was initiated by a positive result in a matrix metalloproteinase enzyme assay. This enzymatic screening is a complementary method to search for anticancer active extracts compared to the whole cell assay method used for the *Plakortis* sp. extracts.

Chapters Four and Five provide the details of the work on sponge-derived marine fungal cultures. Chapter Four describes the first fungal project in this laboratory which yielded three new natural products, chloriolins A-C. Chapter Four also discusses the young field of marine fungal natural products and provides a review of the sources and novelty of the compounds found to date in this new area. Chapter Five describes the structural elucidation of two novel compounds, asperizine and asperic acid, and the dereplication of three known secondary metabolites, malformin C, pyrophen and hexylitaconic acid, from a sponge-derived *Aspergillus* c.f. *niger* fungus.

REFERENCES

(1) Mathews, C. K.; van Holde, K. E. *Biochemistry*; Banjamin /Cummings Publishing Co.: Menlo Park, CA, 1990.

(2) Luckner, M. *Secondary Metabolism in Microorganisms, Plants, and Animals*; 3 ed.; Springer-Verlag: New York, 1990.

(3) Herbert, R. B. *The Biosynthesis of Secondary Metabolites*; 2nd ed.; Chapman Hall: New York, 1989.

(4) Luckner, M. *Secondary Metabolism in Microorganisms, Plants, and Animals*; 3rd ed.; Springer-Verlag: New York, 1990.

(5) Faulkner, D. J. *Chem. Rev.* **1993**, *93*, 1671-1672.

(6) Pawlik, J. R. *Chem. Rev.* **1993**, *93*, 1911-1922.

(7) Michael, J. P.; Pattenden, G. *Angew. Chem. Int. Ed. Eng.* **1993**, *32*, 7-23.

(8) Davies, J. *Mol. Microbio.* **1990**, *4*, 1227-1232.

(9) Stanier, R. Y.; Ingraham, J. L.; Wheelis, M. L.; Painter, P. R. *The Microbial World*; 5th ed.; Prentice-Hall: Eaglewood Cliffs, 1986.

(10) Faulkner, D. J. *Nat. Prod. Rep.* **1995**, *12*, 223-269.

(11) Crews, P.; Slate, D. L.; Gerwick, W. H.; Schmitz, F. J.; Schatzman, R.; Struvlovici, B.; Cannon, P.; Hunter, L. M. In *Anticancer Drug Discovery Natural Products and New Molecular Models.*; Valeriote, F. A., Corbett, T. H., Baker, L. H., Eds.; Kluwer Academic Publishers: Boston, 1994, p 365.

(12) Bergmann, W.; Feeney, R. J. *J. Org. Chem.* **1951**, *16*, 981-987.

(13) Rinehart, K. L. J.; Gloer, J. C.; Cook, J. C. J.; Mizsak, S. A.; Scahill, T. A. *J. Am. Chem. Soc.* **1981**, *103*, 1857-1859.

(14) Pettit, G. R. In *Progress in the Chemistry of Organic Natural Products*; Herz, W., Grisebach, H., Kirby, G. W., Tamm, C., Eds.; Springer Verlag: New York, 1991, pp. 153-195.

(15) Lavelle, R.; Zerial, A.; Fizames, C.; Rabault, B.; Caraudeau, A. *Investig. New. Drugs.* **1991**, *9*, 233-244.

(16) Matsumoto, T.; Yangiya, M.; Maeno, S.; Yasuda, S. *Tetrahedron Lett.* **1968**, 6297.

(17) Matsunaga, S.; Fusetani, N.; Nakao, Y. *Tetrahedron* **1992**, *48*, 8369.

(18) Perry, N. B.; Blunt, J. W.; Munro, M. H. G.; Thompson, A. M. *J. Org. Chem.* **1990**, *55*, 223.

(19) Arai, T.; Yazawa, K.; Mikami, Y. *J. Antibiot.* **1976**, *29*, 398.

(20) He, H.; Faulkner, D. J. *J. Org. Chem.* **1989**, *54*, 5822-5824.

(21) Pettit, G. R.; Collins, J. C.; Herald, D. L.; Doubek, D. L.; Boyd, M. R.; Schmidt, J. M.; Hooper, J. N. A.; Tackett, L. P. *Can. J. Bot.* **1992**, *70*, 1170-1175.

(22) McKee, T. C.; Ireland, C. M. *J. Nat. Prod.* **1987**, *50*, 754.

(23) Unson, M. D.; Faulkner, D. J. *Experientia* **1993**, *49*, 349-353.

(24) Unson, M. D.; Holland, N. D.; Faulkner, D. J. *J. Mar. Biol.* **1994**, *119*, 1-11.

(25) Pettit, G. R.; Kamano, Y.; Herald, C. L.; Tuimann, A. A.; Boettner, F. R.; Kizu, H.; Tomer, K. B.; Bontens, R. J. *J. Am. Chem. Soc.* **1987**, *109*, 6883-6885.

(26) Valeriote, F.; Moore, R. E.; Patterson, G. L. M.; Paul, V. J.; Scheuer, P. J.; Corbett, T. In *Anticancer Drug Discovery and Development : Natural Products and New Molecular Models.*; Valeriote, F. A., Corbett, T. H., Baker, L. H., Eds.; Kluwer Academic Publishers: Boston, 1994, pp 1-20.

(27) Gerwick, W. H.; Bernart, M. W. In *Marine Biotechnology*; Attaway, D. H., Zaborsky, O. R., Eds.; Plenum Press: New York, 1993; Vol. 1 Pharmaceutical and Bioactive Natural Products, p 101-152.

(28) Fenical, W. *Chem. Rev.* **1993**, *93*, 1673-1684.

(29) Liberra, K.; Lindequist, U. *Pharmazie* **1995**, *50*, 583.

(30) Kobayashi, J.; Ishibashi, M. *Chem. Rev* **1993**, *93*, 1753-1770.

(31) Fenical, W.; Jensen, P. R. In *Marine Biotechnology, Volume 1 Pharmaceutical and Bioactive Natural Products*; 1st ed.; Attaway, D. H., Zaborsky, O. R., Eds.; Plenum Press: New York, 1993; Vol. 1, pp. 419-474.

(32) Davidson, B. S. *Env. Biotech.* **1995**, *6*, 284.

(33) Ireland, C. M.; Roll, D. M.; Molinski, T. F.; McKee, T. C.; Zabrieski, T. M.; Swersey, J. C. In *Biomedical Importance of Marine Organisms*; Fautin, D. G., Ed.; California Academy of Science: San Francisco, 1988, pp. 41-57.

(34) Munro, M. H. G.; Blunt, J. W.; Lake, R. J.; Litaudon, M.; Battershill, C. N.; Page, M. J. In *Sponges in Time and Space, Biology, Chemistry, and Paleontology*; van Soest, R. W. M., van Kempen, T. M. G., Braekman, J.-C., Eds.; A. A. Balkena: Rotterdam, 1994, pp. 473-484.

(35) Schmitz, F. J. In *Sponges in Time and Space, Biology, Chemistry, and Paleontology*; van Soest, R. W. M., van Kempen, T. M. G., Braekman, J.-C., Eds.; A. A. Balkema: Rotterdam, 1994, pp. 485-496.

(36) Hyde, K. D.; Jones, E. B. G. *J. Linn. Soc.* **1990**, *100*, 237.

(37) Seifert, K. A. In *Isolation of Biotechnological Organisms from Nature*; Labeda, D. L., Ed.; McGraw Hill: San Francisco, 1990, pp. 21-52.

(38) Kupchan, M. S.; Britton, R. W.; Ziegler, M. F.; Sigel, C. W. *J. Org. Chem.* **1973**, *38*, 178-179.

(39) Sam, T. W. In *Bioactive Natural Products : detection, isolation, and structural elucidation.*; Colegate, S. M., Molyneux, R. J., Eds.; CRC Press: Boca Raton, 1993, pp. 442-456.

(40) Boyd, M. R. *Principles and Practice of Oncology Updates* **1989**, *3*, 1-12.

(41) Swaffar, D. J.; Ireland, C. M.; Barrows, L. R. *Anti-Cancer Drugs* **1994**, *5*, 15.

(42) Johnson, R. K.; Hertzberg, R. P. *Ann. Rep. Med. Chem.* **1990**, *25*, 129.

(43) Wang, G. Y. S.; Kuramoto, M.; Uemura, D.; Yamada, A. *Tetrahedron Lett.* **1996**, *37*, 1813-1816.

(44) Daloze, D.; Braekman, J. C. In *Sponges in Time and Space Biology, Chemistry, Paleontology*; van Soest, R. W. M., van Kempen, T. M. G., Braekman, J.-C., Eds.; A. A. Blakena: Rotterdam, 1994, pp. 441-452.

(45) Dass, C. In *Mass Spectrometry, Clinical and Biomedical Applications*; Desiderio, D. M., Ed.; Plenum Press: New York, 1992; Vol. 2, pp. 1-52.

(46) Bax, A.; Subramanian, S. *J. Magn. Res.* **1986**, *67*, 565.

(47) Bax, A.; Sparks, S. W.; Torchin, D. A. *J.Am. Chem. Soc.* **1988**, *110*, 7926.

(48) Bodenhausen, G.; Freeman, R. *J. Magn. Reson.* **1977**, *28*, 471.

(49) Bodenhausen, G.; Freeman, R. *J. Am. Chem. Soc.* **1978**, *100*, 320.

(50) Maudsley, A. A.; Kumar, A.; Ernst, R. R. *J. Magn. Reson.* **1977**, *28*, 303.

(51) Maudsley, A. A.; Ernst, R. R. *Chem. Phys. Lett.* **1977**, *50*, 368.

(52) Aue, W. P.; Bartholdi, E.; Ernst, R. R. *J. Chem. Phys.* **1976**, *64*, 2229.

(53) Kessler, H.; Griesinger, C.; Zarbock, J.; Loosli, H. R. *J. Magn. Reson.* **1984**, *57*, 331.

(54) Macura, S.; Ernst, R. R. *Mol. Phys.* **1980**, *41*, 95.

(55) Jeener, J.; Meier, B. H.; Bachmann, P.; Ernst, R. R. *J. Am. Chem. Soc.* **1980**, *101*.

(56) Lightner, D. A. In *Circular Dichroism - Principles and Applications*; Nakanishi, K., Berova, N., Woody, R. W., Eds.; VCH Publishers: New York, 1994.

(57) Dale, J. A.; Mosher, H. S. *J. Am. Chem. Soc.* **1973**, *95*, 512.

(58) Trost, B. M.; Curran, D. P. *Tetrahedron Lett.* **1981**, *49*, 4929.

(59) Trost, B. M.; Belletire, J. L.; Godleski, S.; McDougal, P. G.; Balkovec, J. M.; Baldwin, J. J.; Christy, M. E.; Ponticello, G. S.; Varga, S. L.; Springer, J. P. *J. Org. Chem.* **1986**, *51*, 2370.

(60) Nagai, Y.; Kusumi, T. *Tetrahedron Lett.* **1995**, *36*, 1853-1856.

CHAPTER TWO

Structures, Stereochemistry and Cytotoxicity of Cyclic Peroxides from a *Plakortis* sp. Sponge

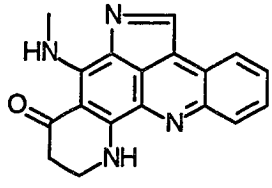
INTRODUCTION

Screening for anticancer cytotoxicity by model systems is an effective means for discovering cancer chemotherapeutics.[1] The screens may be either *in vivo*, or *in vitro*. Ethical considerations and expense prevent the *in vivo* screening of animals until activity in an *in vitro* model has shown promise for a compound or extract. There are essentially two types of *in vitro* screens available, one is mechanism blind and involves cultured cancer cells,[2] either human or murine (mouse), while the other screen is mechanism specific and involves targeting specific enzymes thought to be important in cancer cells.[3,4]

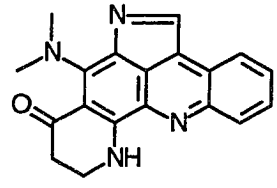
Whole cancer cell assays have several advantages over mechanism based models including the identification of compounds with the ability to kill or inhibit the growth of cancer cells by new mechanisms of action. The original cell lines used for screening for anticancer drugs have been the murine L1210 and P388 leukemia cell lines.[5] Both of these cell lines are rapidly dividing cancer lines. The L1210 murine cancer cell line was the primary screening line from the 1950s to the 1970s, however, in the 1970s the murine P388 leukemia cell line replaced the L1210 cell line.[5] Using L1210 and P388 the cancer chemotherapeutics ara-C, daunorubicin, vincristine and others were developed.[5] While these drugs are effective against rapidly dividing cells, chemotherapeutic agents for slow growing tumors are scarce. Slow growth tumor *in vitro* assays have been developed using melanoma and other cancer cell lines to identify potential cytotoxic agents. It is believed the mechanism blind aspect of these assays will bring forward anticancer agents that act by a variety of mechanisms.[6]

An *in vitro* melanoma cancer assay administered by Abbot Laboratories identified cytotoxic activity in the nonpolar partition of a Fijian *Plakortis* species. Cytotoxic compounds have been isolated from *Plakortis* sponges in this laboratory and by other investigators of marine natural products. The *Plakortis* sponges (order Homosclerophorida, family Plakinidae) are known to produce both planar alkaloids and oxygenated polyketides.

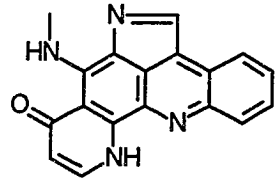
The few planar alkaloids isolated are a rare exception to the polyketides generally found in *Plakortis* species. Two independent reports of the plakinidines (**1-3**) have occurred.[7,8] The identification of the *Plakortis* species producing plakinidines was performed by two different taxonomists.



1
plakinidine A



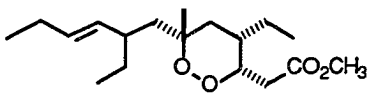
2
plakinidine B



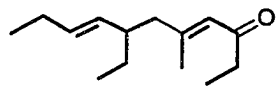
3
plakinidine C

Overwhelmingly the secondary metabolite chemistry of *Plakortis* species (and of the family Plakinidae) are oxygenated polyketides, usually containing cyclic peroxides. The first example of a cyclic peroxide from a *Plakortis* sponge was plakortin (**4**) from *Plakortis halichondriodes* in 1978.[9] Also found with plakortin (**4**) were the structurally related ketone (**5**) and further aromatic polyketide peroxides with varying levels of oxygenation from carboxylic acids and lactones to hydrocarbons. *Plakortis halichondriodes* was a rich source of peroxides and many peroxide structural types that would be isolated later were first discovered in this sponge. Along with the cyclic peroxides, lactone peroxides containing a butenolide ring fused to the peroxide ring (**6**) were also isolated.[10] A carboxylic acid peroxide (**7**) with the same carbon skeleton as **6** was also isolated from the same sample of sponge.[10] After more than a decade a second butenolide peroxide, plakortolide (**8**),[11] was identified and shortly thereafter three more peroxide lactones, plakortolides

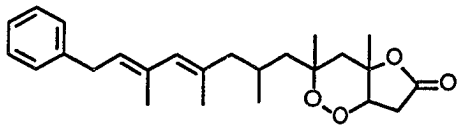
B-D (**9-11**),[12] were isolated from a *Plakinestrella* sponge (family Plakinidae). Other structural types of polyketide peroxides have been identified. These include 3,5 epidioxy cyclic peroxides (**12**)[13] as opposed to the more commonly encountered 2,5 epidioxy peroxides. A polyketide compound containing a bis peroxide (**13**) has been recently identified.[14]



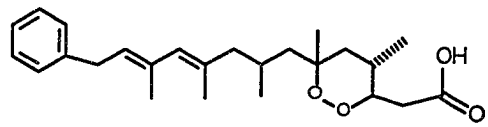
4
plakortin



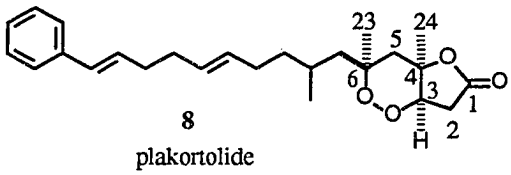
5
unnamed ketone



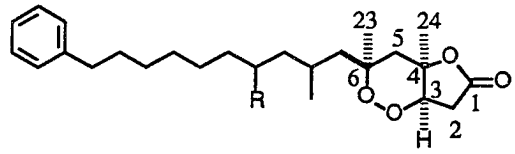
6
unnamed butenolide



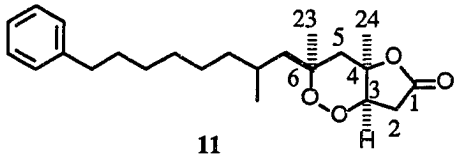
7
butenolide related acid



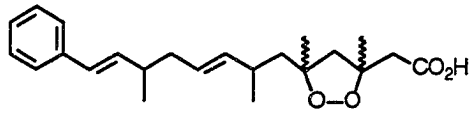
8
plakortolide



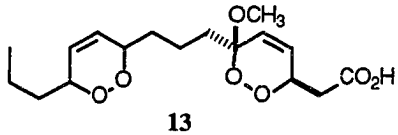
9 plakortolide B, R = H
10 plakortolide D, R = CH_3



11
plakortolide C

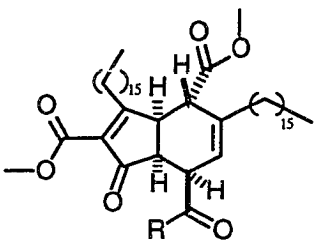


12
plakinic acid

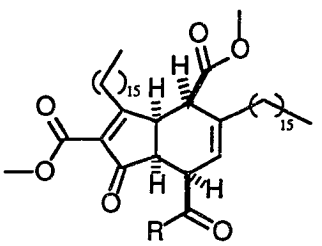


13
bis peroxide

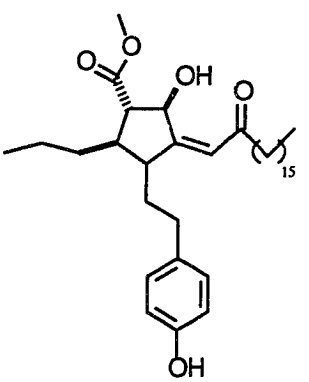
Another group of polyketides isolated from *Plakortis* sponges are the fused fatty acids that make up the manzamenones A-F (**14-19**).[15,16] In addition to the manzamenones the tyramine substituted plakoridine A (**20**)[17] is a further cyclised fatty acid natural product. The fatty acid untenone-A (**21**), a putative precursor of the manzamenones has also been isolated.



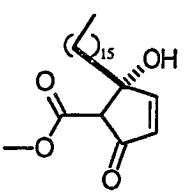
14 manzamenone A, R = OH
16 manzamenone C, R = OEt
17 manzamenone D, R= NH_2
18 manzamenone E, R = valine
19 manzamenone F, R = O-n-butoxide



15
manzamenone B, R = OH



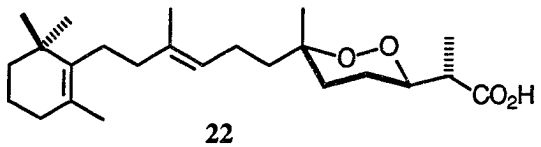
20
plakoridine A



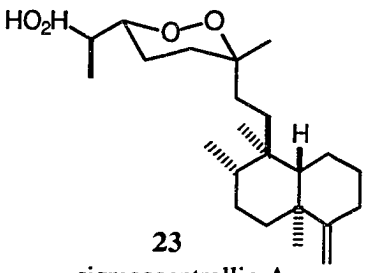
21
untenone A

While polyketide peroxides are a hallmark of *Plakortis* sponges, terpene cyclic peroxides are known from several other sponges. Norsesterpene peroxides have been isolated from the genera *Prianos*, *Sigmosceptrella*, *Latrunculia*, and *Mycale*. Though *Sigmosceptrella*, and *Latrunculia* are in the same family (family Latrunclidae, order Hadromerida) they differ taxonomically at the

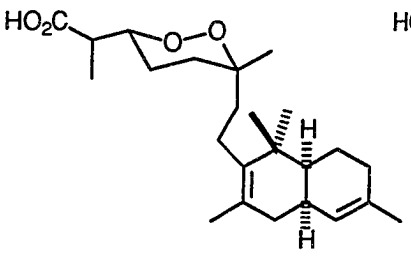
order level from both *Prianos* (order Halichondriidae) and *Mycale* (order Poescilosclerida). The compounds isolated from all four genera are very similar to each other. Muquibillin (**22**)[18] was isolated from a *Prianos* sponge and sigmosceptrellin (**23**)[19] and trunculin A (**24**)[20] were isolated from *Sigmosceptrella laevis* and *Latrunculia brevis* respectively. Along with labdane norditerpenes, antipodal sigmosceptrellin (**25**)[21] was isolated from the sponge *Mycale arcorina.* The norditerpene methyl nuapapuanoate (**26**) was isolated from a Tongan *Prianos* sponge.[22]



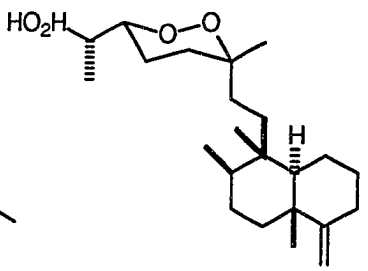
22
muquibillin



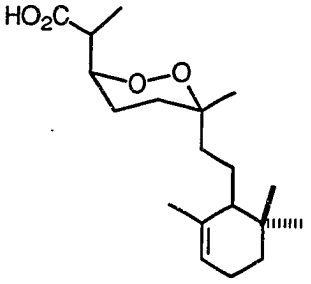
23
sigmosceptrellin-A



24
trunculin A



25
ent-sigmosceptrellin-A



26
methyl nuapapuanoate

The availability of a wide variety of cyclic peroxides led to the development of several methods for determining the stereochemistry of this structure class. The ^{13}C NMR shift values of the methyl groups on the peroxide rings have been used to assign relative axial or equatorial stereochemistry. The axial methyl groups resonate at approximately 20 ppm while the shift value of the equatorial methyl groups are at 24 ppm or lower field.[23] Both one and two dimensional nOe experiments have been used to assign the relative stereochemistry of 1,3 diaxial methyl groups on the cyclic peroxide rings.[11] The absolute sterochemistry of the terpene cyclic peroxides has been

determined by X-ray crystallography, CD spectral analysis, and differential asymmetric esterification of the peroxide-derived diols.[23,24] While the stereochemistry of the terpene peroxides is well understood, the absolute stereochemistry of a polyketide peroxide has not been evaluated.

There has been a continuing interest in sponge derived cyclic peroxides due to the cytotoxicity that many of these compounds exhibit. The polyketide derived cyclic peroxides, with and without fused lactone rings, as well as the terpene derived cyclic peroxides have all displayed biological activity in a variety of anticancer screens. These anticancer screens include inhibition of sea urchin egg division assays,[22] the cell lines P 388[25] and the NCI 60 cell line disease oriented screen.[26] The unidentified *Plakortis* species studied in this project continues the established trend of chemistry and biological activity of other *Plakortis* species.

RESULTS

The sponge examined in this work has only been found on the Fijian habitat known as "the great white wall" of Taveuni. An initial collection of this sponge (collection # 89134), Plate 2.1, was extracted and cytotoxic activity was observed in the crude extract. Barbara Peters, a former postdoctoral fellow, isolated very small amounts (1-2 mg) of two pure peroxides. One of the compounds was tentatively assigned a butenolide peroxide structure while the other was believed to be a peroxide carboxylic acid. When Barbara Peters left UCSC there was uncertainty about the structure of the peroxides because the mass spectrum did not match the predicted molecular weight from NMR spectroscopy. There was also an insufficient amount of material to perform two and three bond proton-carbon correlation NMR experiments to confirm some aspects of the structures.

Isolation of plakortolide (27) and plakoric acid (34)

In this thesis work, a subsequent extraction and isolation of a second sample of sponge was carried out and this led to the confirmation of the proposed structures as well as the assignment of absolute stereochemistry of the butenolide peroxide. The same *Plakortis* sp. as collection # 89134 was recollected by SCUBA and given the collection number 92123, Plate 2.2. This second sample was preserved in the field by overnight soaking in 1:1 ethanol:water. The ethanol water solution was discarded and the damp sponge was transported at ambient temperatures to U.C. Santa Cruz where it was soaked in methanol (3 L, three times for 72 h). The combined methanol extracts were concentrated and the resultant oil was partitioned between 1:1 CH_2Cl_2:H_2O. A series of solvent-solvent partitionings took place to yield a pale yellow oil of 0.99 g from the carbon tetrachloride fractions, Figure 2.1.

Plate 2.1. Underwater photograph of collection # 89134, *Plakortis* sp.

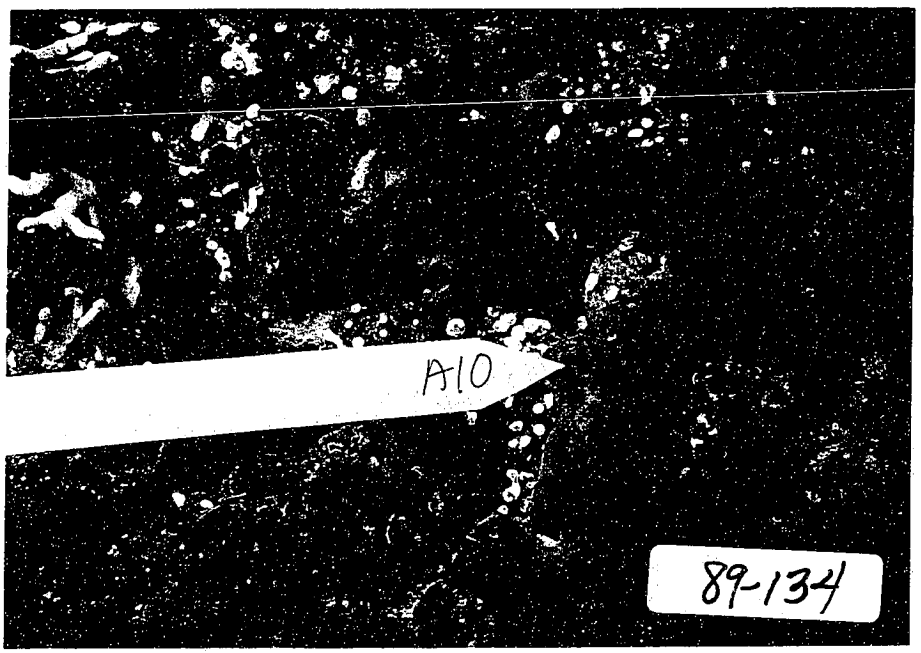
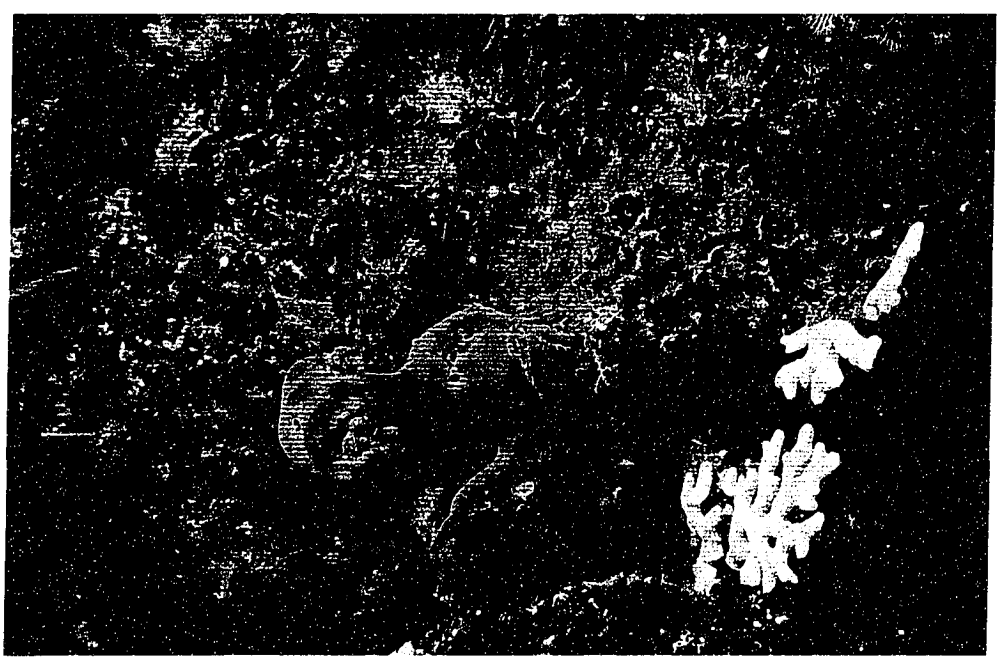


Plate 2.2. Underwater photograph of collection # 92123, *Plakortis* sp.



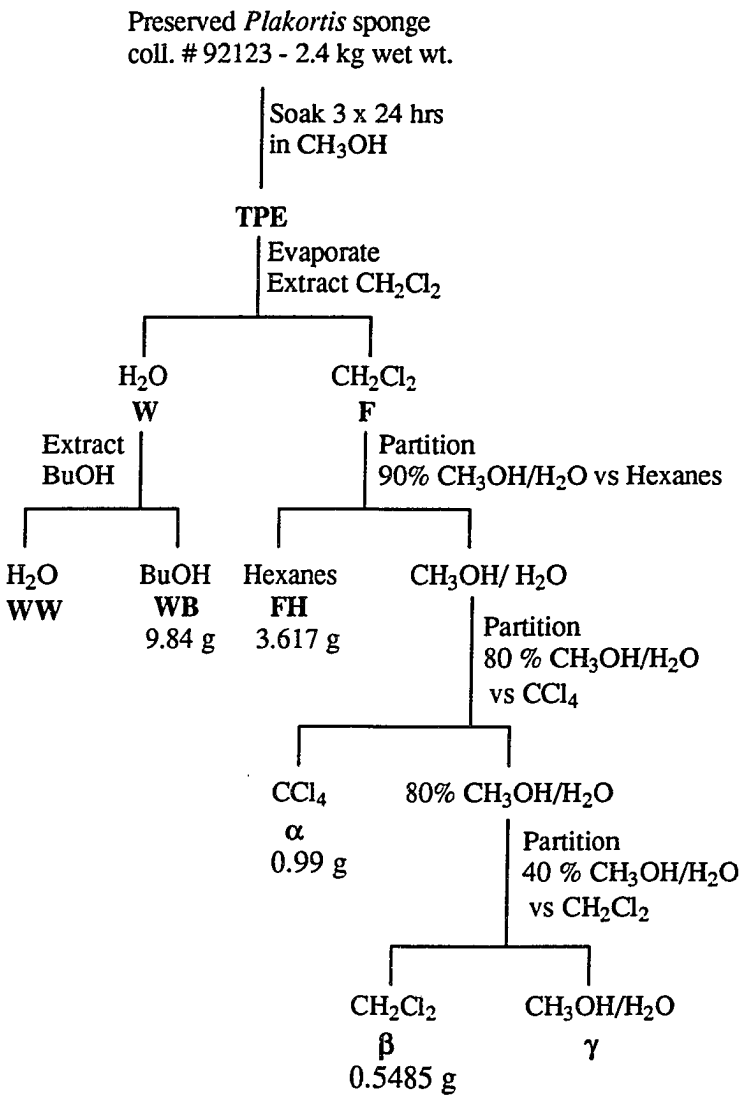


Figure 2.1. Solvent-solvent partition of crude extract of 92123, *Plakortis* sp.

Due to the known instability of peroxides isolated from *Plakortis* sponges, separations were performed with either size exclusion chromatography or reverse phase octadecyl bonded silica. The CCl_4 soluble fraction was applied to a Sephadex LH-20 column (MeOH). Select fractions were further purified using a C_{18} silica flash column (eluted with 15% aqueous MeOH,

100% MeOH, then CH_3CN), followed by C_{18} silica HPLC (eluted with 25% aqueous CH_3CN) to yield plakortolide E (**27**) (76.5 mg) and plakoric acid (**34**) (19.3 mg).

An examination of the ^1H NMR spectra of the compounds Barbara Peters isolated and the newly isolated material indicated that they were the same compounds. A comparison of the ^1H NMR of plakortolide E (**27**) from collection # 89134, Figure 2. 2, with the ^1H NMR of plakortolide E (**27**) from collection # 92123, Figure 2.3 confirms this. The ^1H NMR of plakoric acid from collection # 89134, Figure 2.4 shows the same compound as the ^1H NMR spectra of plakoric acid from collection #92123, Figure 2.5.

Structure elucidation of plakortolide (27)

The major structural features of plakortolide E (**27**) were defined from the ^{13}C NMR resonances (Table 2.1, entry 1) and Figure 2.6. The primary functionality consisted of a monosubstituted benzene ring, a peroxide (δ 73.8 d (C3), 72.9 s (C6)), and an ester (δ 175.2 s (C1), 90.1 s (C4)) Figure 2.7. From the outset, the highest hreims spectral peak at *m/z* 372.2664, assigned as $C_{24}H_{36}O_3$ (Δ 0.1 mmu of calcd), was suspected to be from an M^+-O fragmentation, Figure 2.8.[27] The benzene ring atoms (C_6H_5) plus the additional ^{13}C APT count of $C_{18}H_{31}$, indicated that no hetero-atom protons were present, Figure 2.9. A bicyclic skeleton like that in previously discovered plakortolides accounted for the remaining elements of unsaturation. The bicyclic ring system was consistent with the ^1H NMR AMX multiplet pattern for H3 (δ 4.19), H2 (δ 2.93) and H2' (δ 2.55) ^3J = 1.8, 6.7 Hz and ^2J = 18.3 Hz; the AB pattern for H5 (δ 2.15) and H5' (δ 2.09); (Table 2.1, entry 7)and Figure 2.3, and long range ^1H-^{13}C COSY correlations from H5/5' to C23 and C24, Figure 2.10. A combination of ^1H-^1H COSY, and ^1H-^{13}C COSY (J = 140 Hz and J = 9 Hz) correlations justified the assignment of all ^1H and ^{13}C resonances, except those which were overlapping for atoms 9 to 14.

Figure 2.2. ^{1}H NMR of plakortolide E (**27**) from coll. # 89134 isolated by Barbara Peters, 250 MHz, $CDCl_3$.

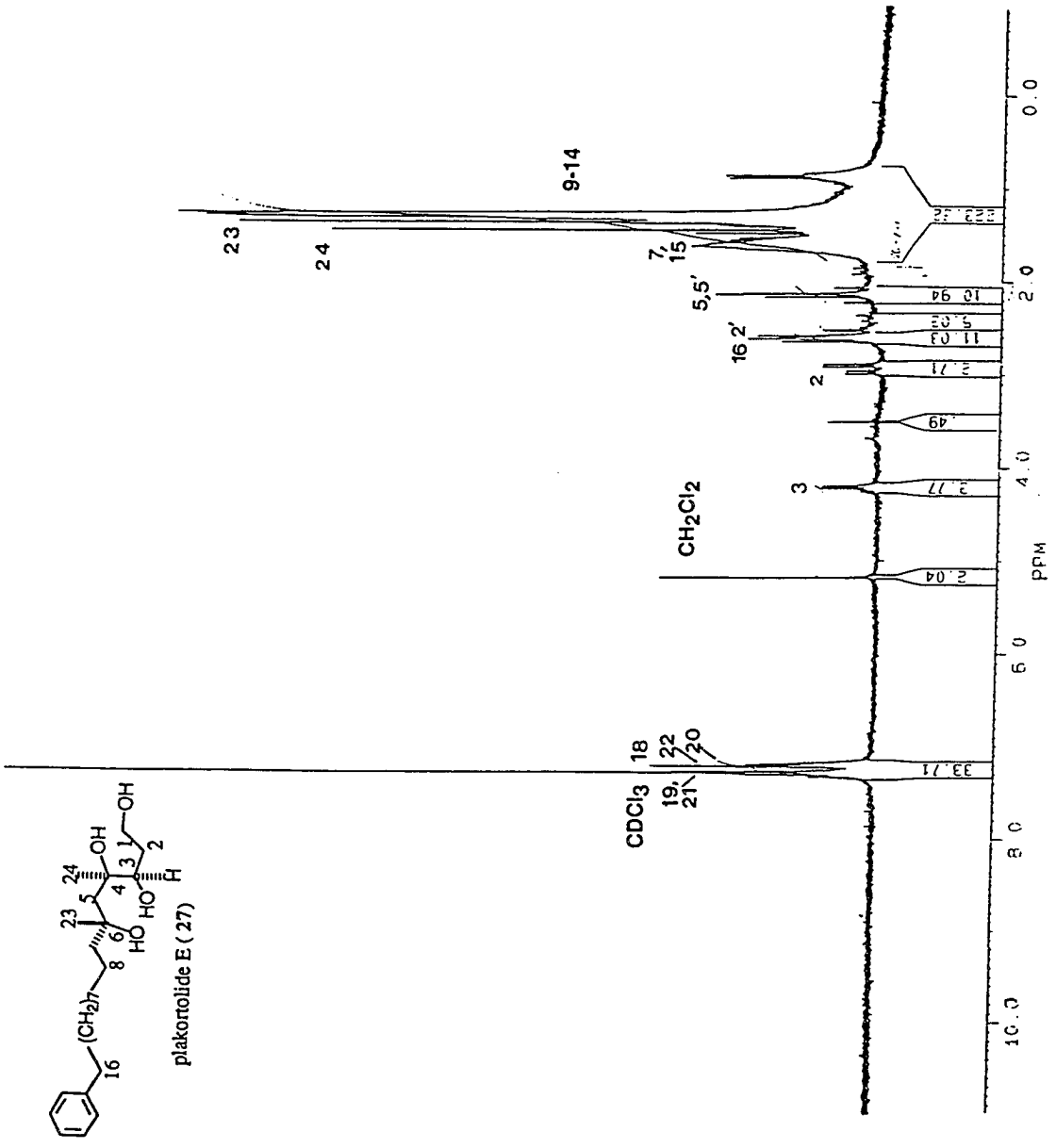


Figure 2.3. ^1H NMR of plakortolide E (**27**) from coll. # 92123, 250 MHz, $CDCl_3$.

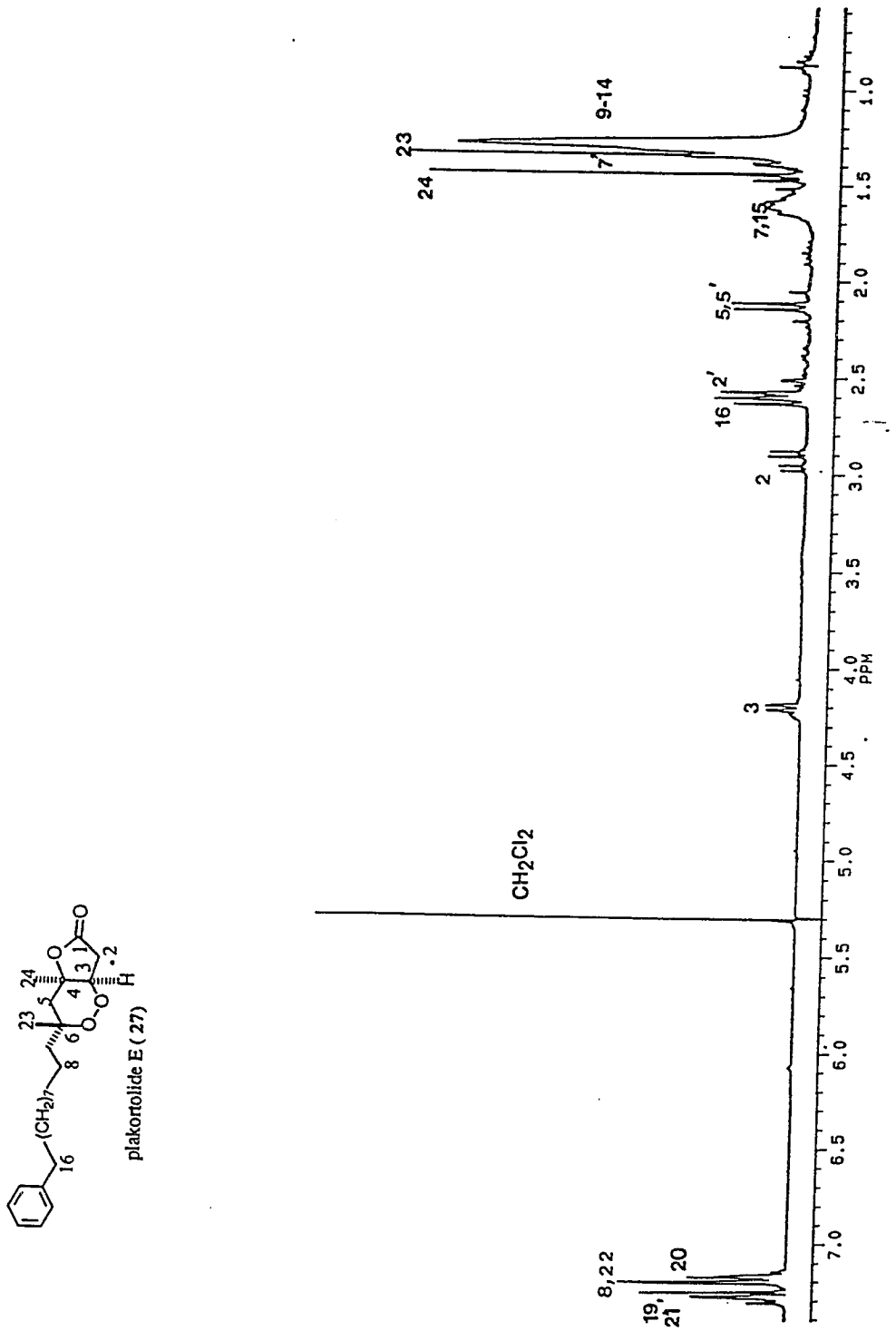


Figure 2.4. ^1H NMR of plakoric acid (**34**) from coll. # 89134 isolated by Barbara Peters, 250 MHz, $CDCl_3$.

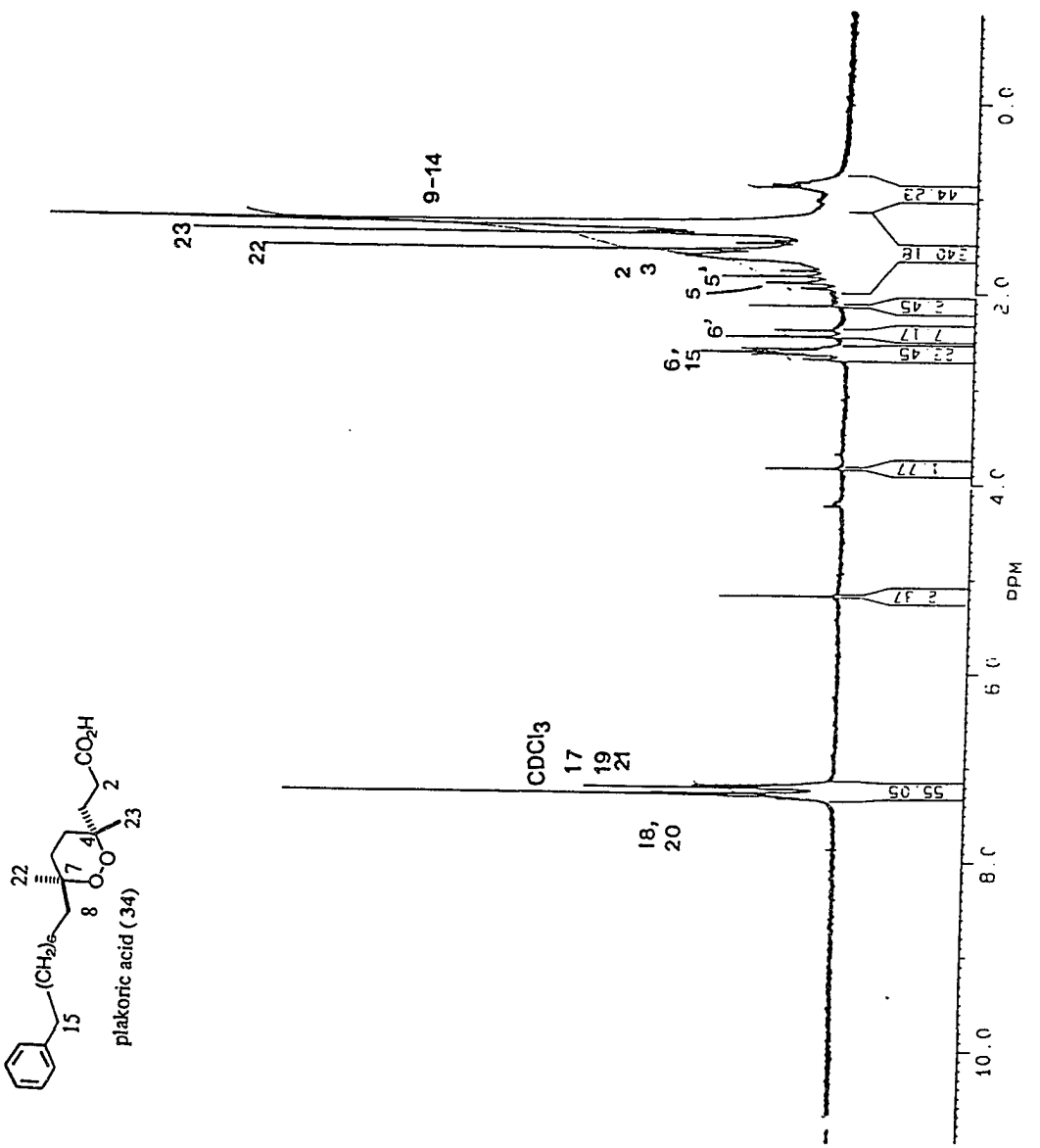


Figure 2.5. ^{1}H NMR of plakoric acid (**34**) from coll. # 92123, 250 MHz, $CDCl_3$.

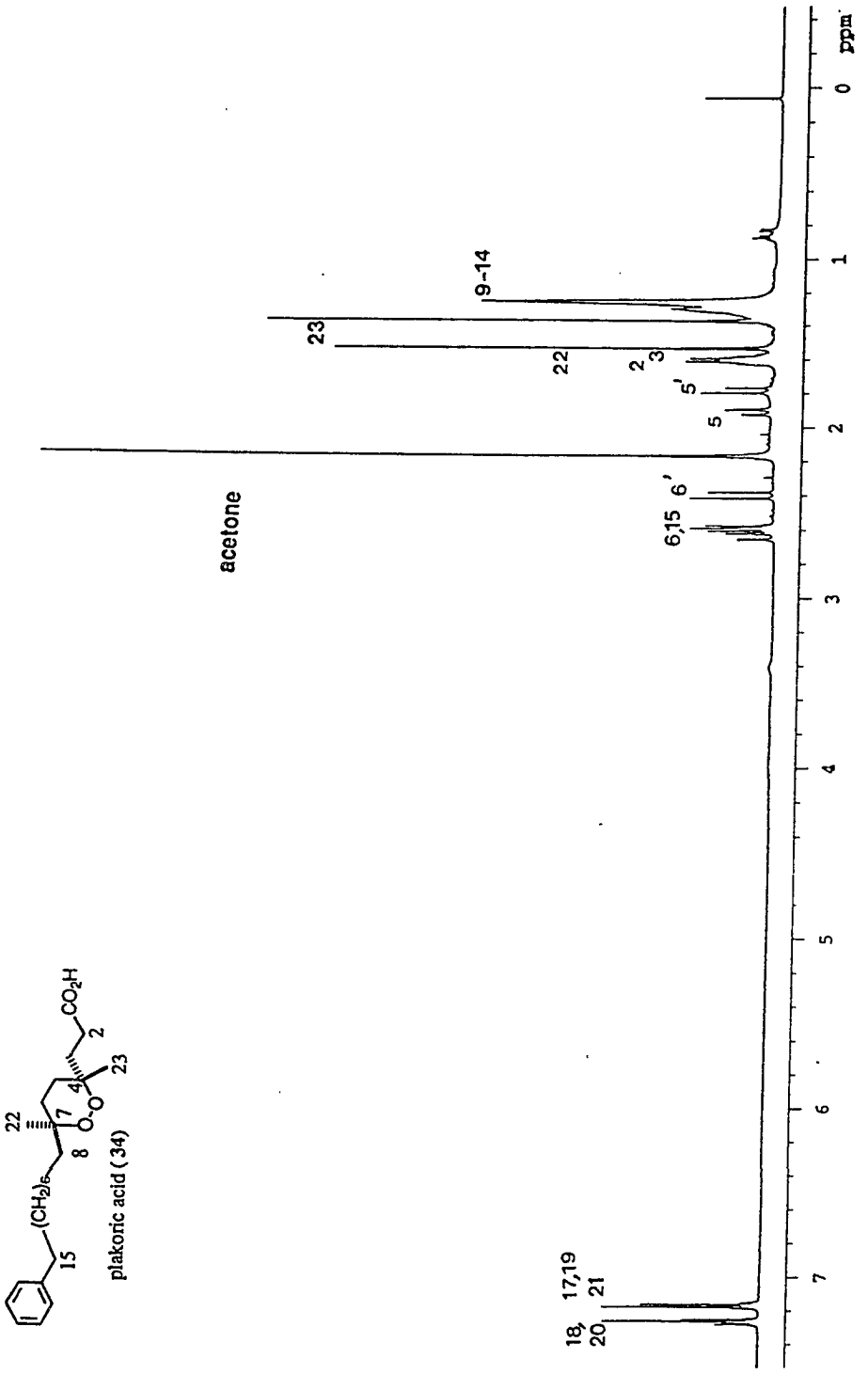
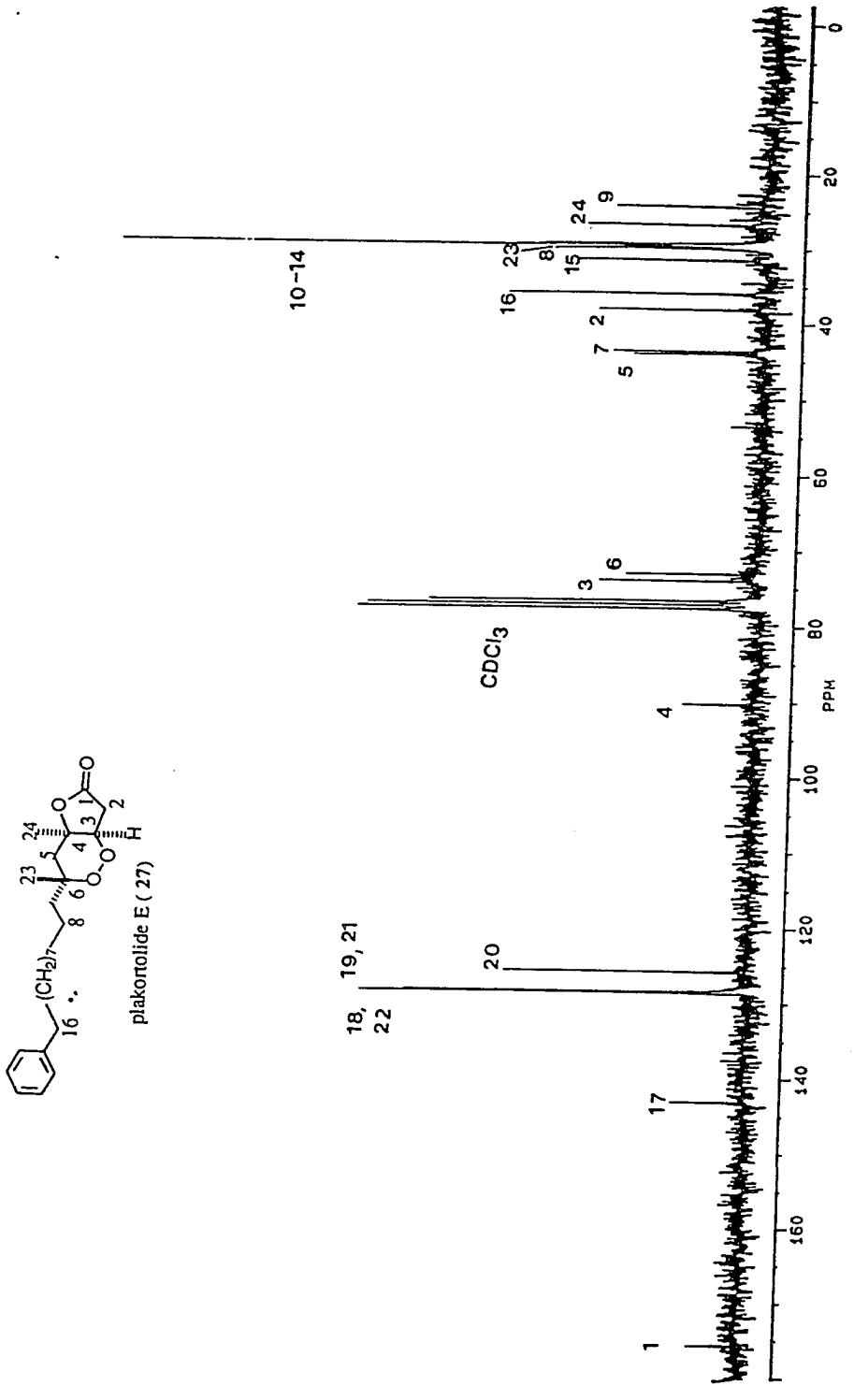


Figure 2.6. ^{13}C NMR of plakortolide E (**27**), 62.9 MHz, $CDCl_3$.



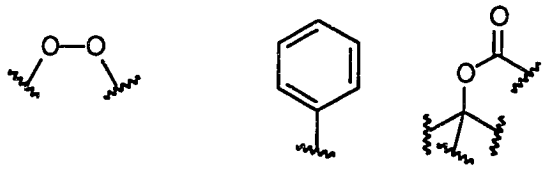


Figure 2.7. Substructures of plakortolide E (**27**).

Table 2.1. NMR shifts of selected peaks of **27**, **32**, **8**, **9**, and **6** in $CDCl_3$ and C_6D_6.

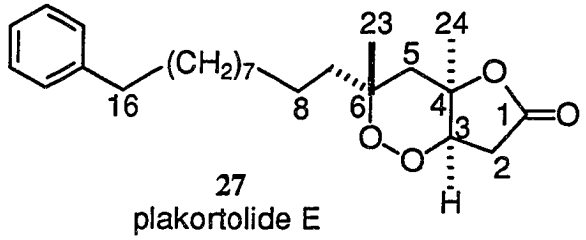
A. ^{13}C shifts of selected peaks in $CDCl_3$ and C_6D_6.

Entry	Compound	Solvent	C2	C3	C4	C5	C6	Me23	Me24	C16	Ref.
1	Plakortolide E (**27**)	$CDCl_3$	38.1	73.8	90.1	43.9	72.9	29.9	26.9	35.9	a
2	Plakortolide ether (**32**)	$CDCl_3$	36.5	81.0	94.5	49.1	84.8	23.6	26.1	35.9	a
3	Plakortolide ether (**32**)	C_6D_6	36.4	81.1	93.1	49.3	84.2	23.1	25.9	36.3	a
4	Plakortolide (**8**)	$CDCl_3$	34.2	80.9	82.8	42.0	80.7	24.9	25.9	N/A	9
5	Plakortolide B (**9**)	$CDCl_3$	34.2	81.6	82.8	42.1	80.7	24.9	25.9	N/A	10
6	**6**	$CDCl_3$		81.3	82.5		80.4			N/A	8

B. ^{1}H shifts of selected peaks in $CDCl_3$ and C_6D_6.

Entry	Compound	Solvent	H2	H2'	H3	H5	H5'	Me23	Me24	H16	Ref.
7	Plakortolide E (**27**)	$CDCl_3$	2.93	2.55	4.19	2.15	2.09	1.35	1.44	2.6	a
8	Plakortolide ether (**32**)	$CDCl_3$	2.78	2.65	4.35	2.40	1.87	1.24	1.51	2.6	a
9	Plakortolide (**8**)	$CDCl_3$	2.89	2.58	4.44	2.19	1.67	1.22	1.35	N/A	9
10	Plakortolide B (**9**)	$CDCl_3$	2.90	2.60	4.45	2.12	1.70	1.25	1.37	N/A	10
11	**6**	$CDCl_3$	2.90	2.58	4.45	2.21	1.70	1.25	1.36	N/A	8

[a]This work.



27
plakortolide E

Figure 2.8. High and low resolution EIMS of plakortolide E (**27**).

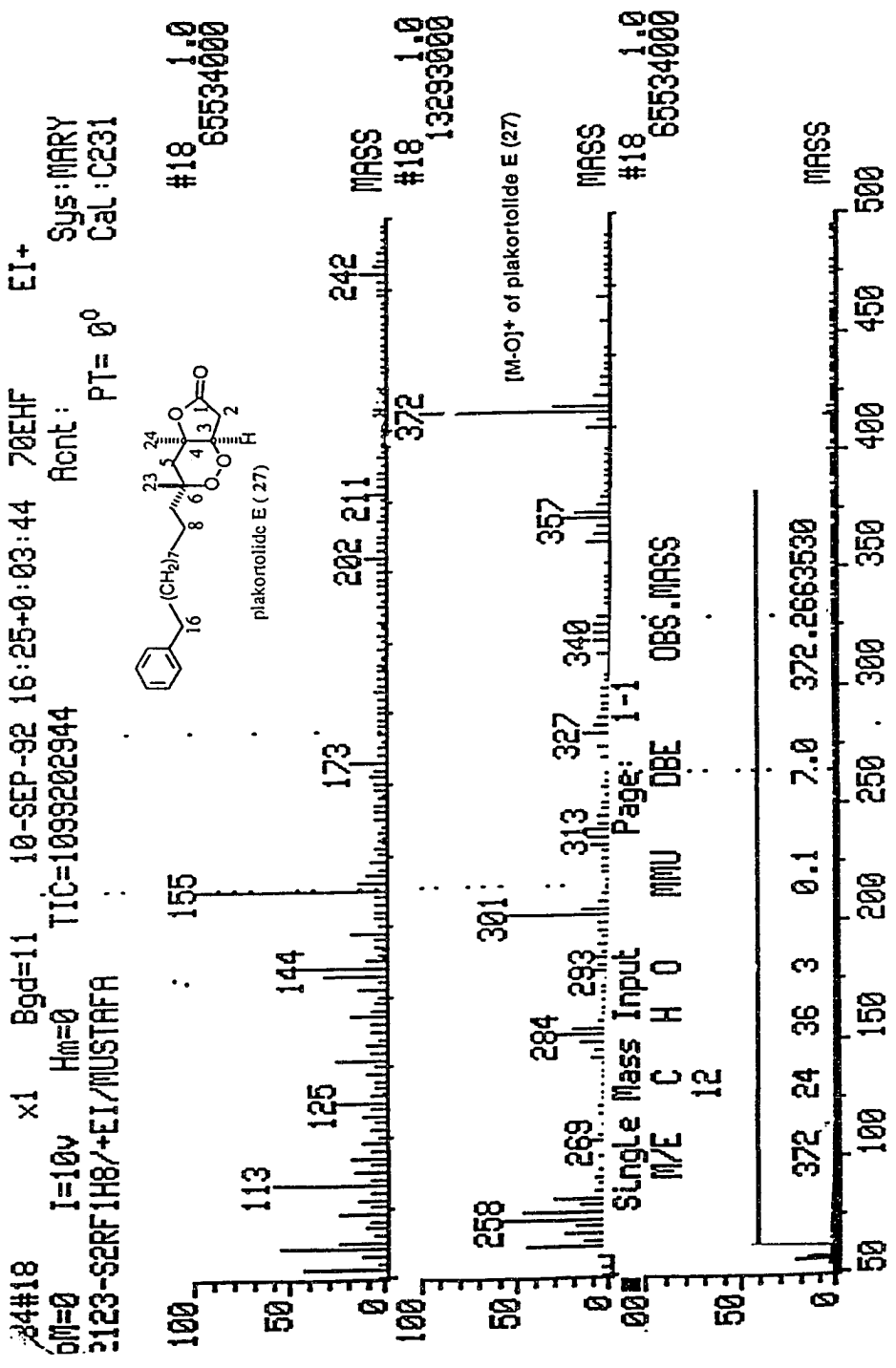


Figure 2.9. APT spectrum of plakortolide E (**27**), 62.9 MHz, $CDCl_3$.

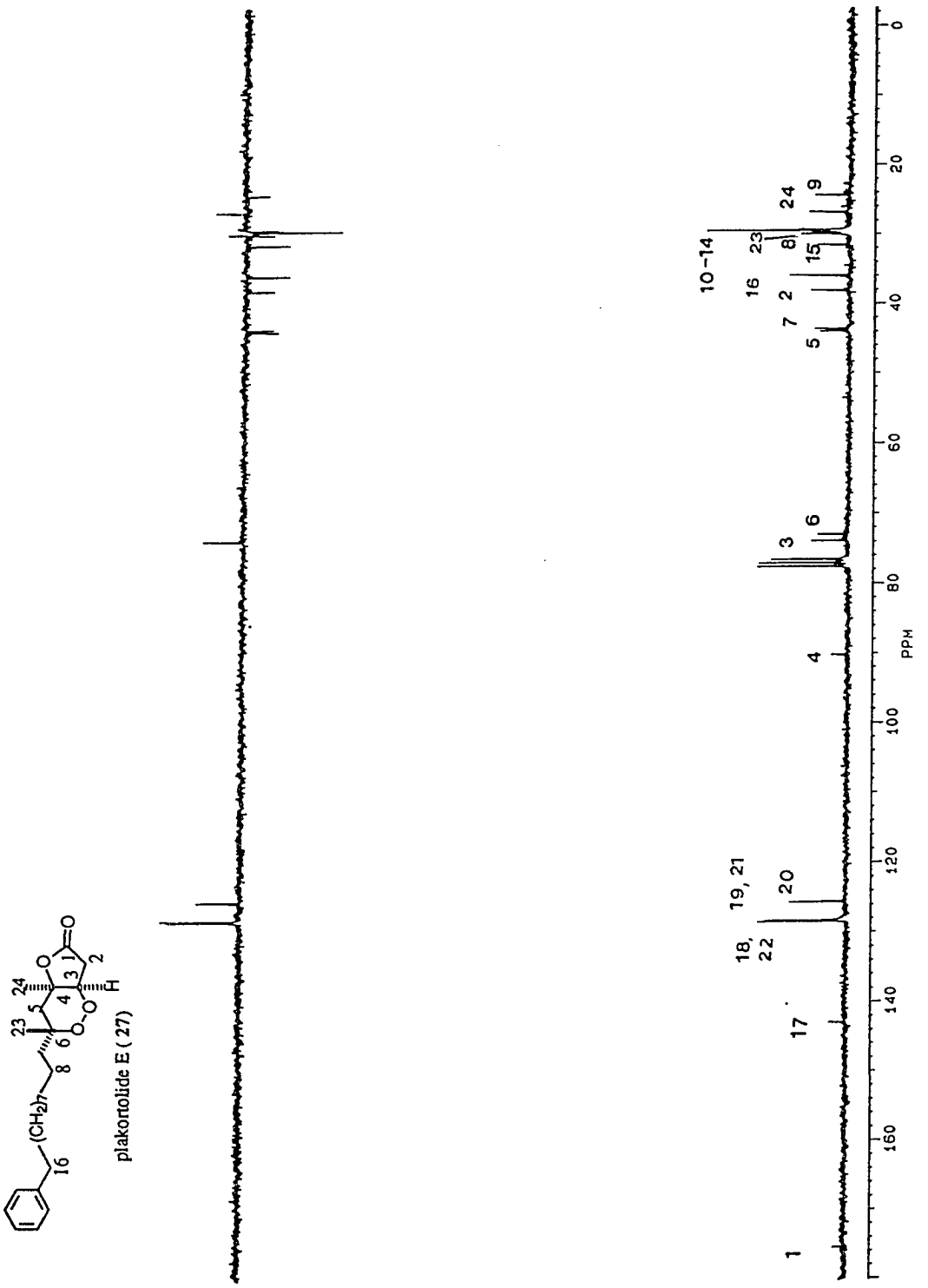
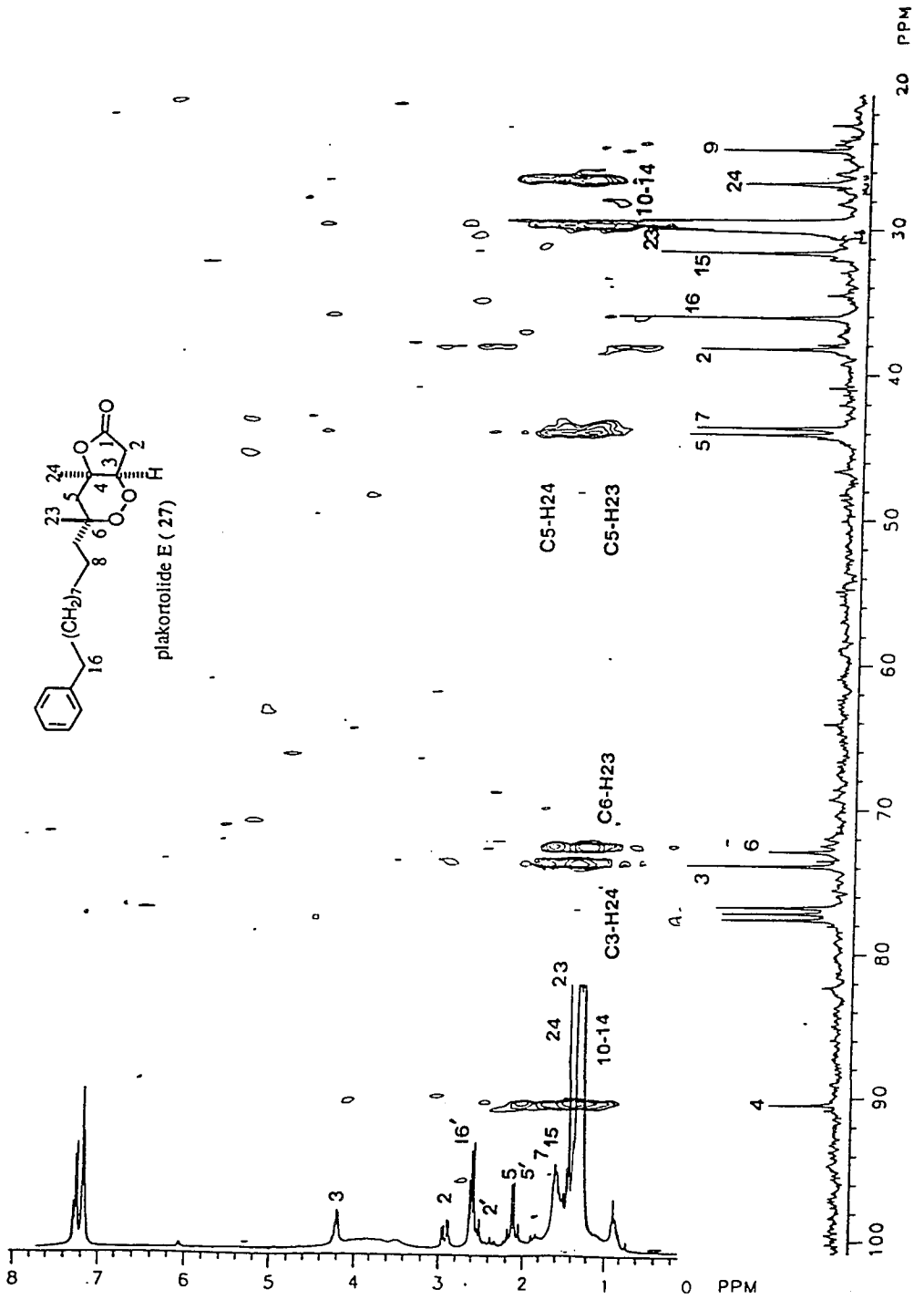
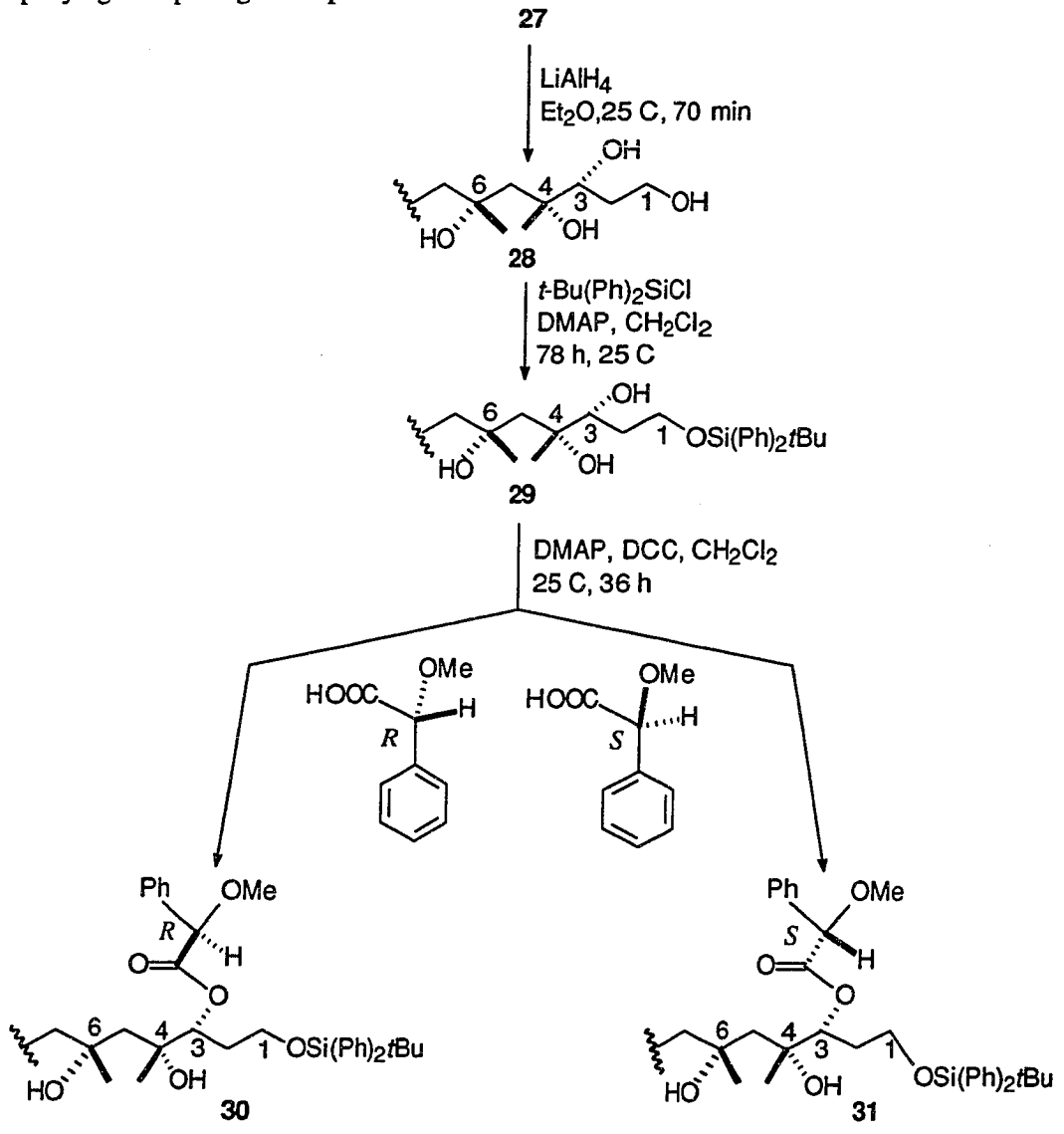


Figure 2.10. ^{13}C-^{1}H COSY (J_{C-H} = 9 Hz) of plakortolide E (**27**) - low field ^{13}C region, 300 MHz, $CDCl_3$.



To confirm the assumption of a peroxide moiety, plakortolide E was converted to the tetraol **28** by $LiAlH_4$ reduction, Scheme 2.1. The assignment of four oxygens in **28** was justified by the lrfabms peak at 395 ($[M+1]^+$ $C_{24}H_{42}O_4$), Figure 2.11, and four characteristic ^{13}C NMR signals (δ 78.5 (d), 75.3 (s), 74.1 (s), 61.4 (t)), Figure 2.12. The upfield shift of C4 from δ 90.1 in **27** to δ 75.3 or 74.1 in **28** was consistent with the loss of a β-substituent increment additivity effect accompanying the opening of the peroxide.



Scheme 2.1. Preparation of *O* - methyl mandelate esters **30** and **31**.

Figure 2.11. Low resolution FABMS of **28**, NBA matrix.

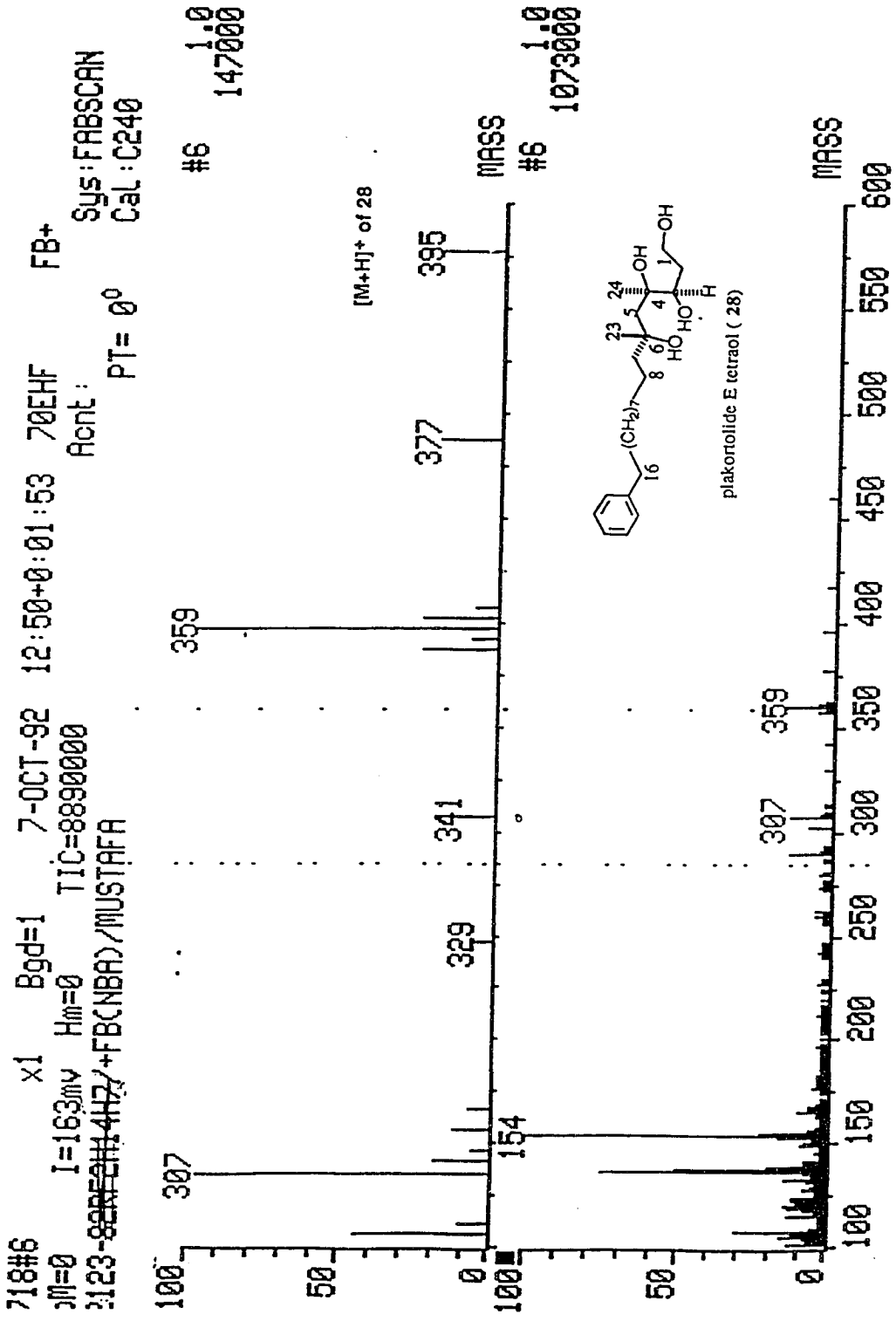
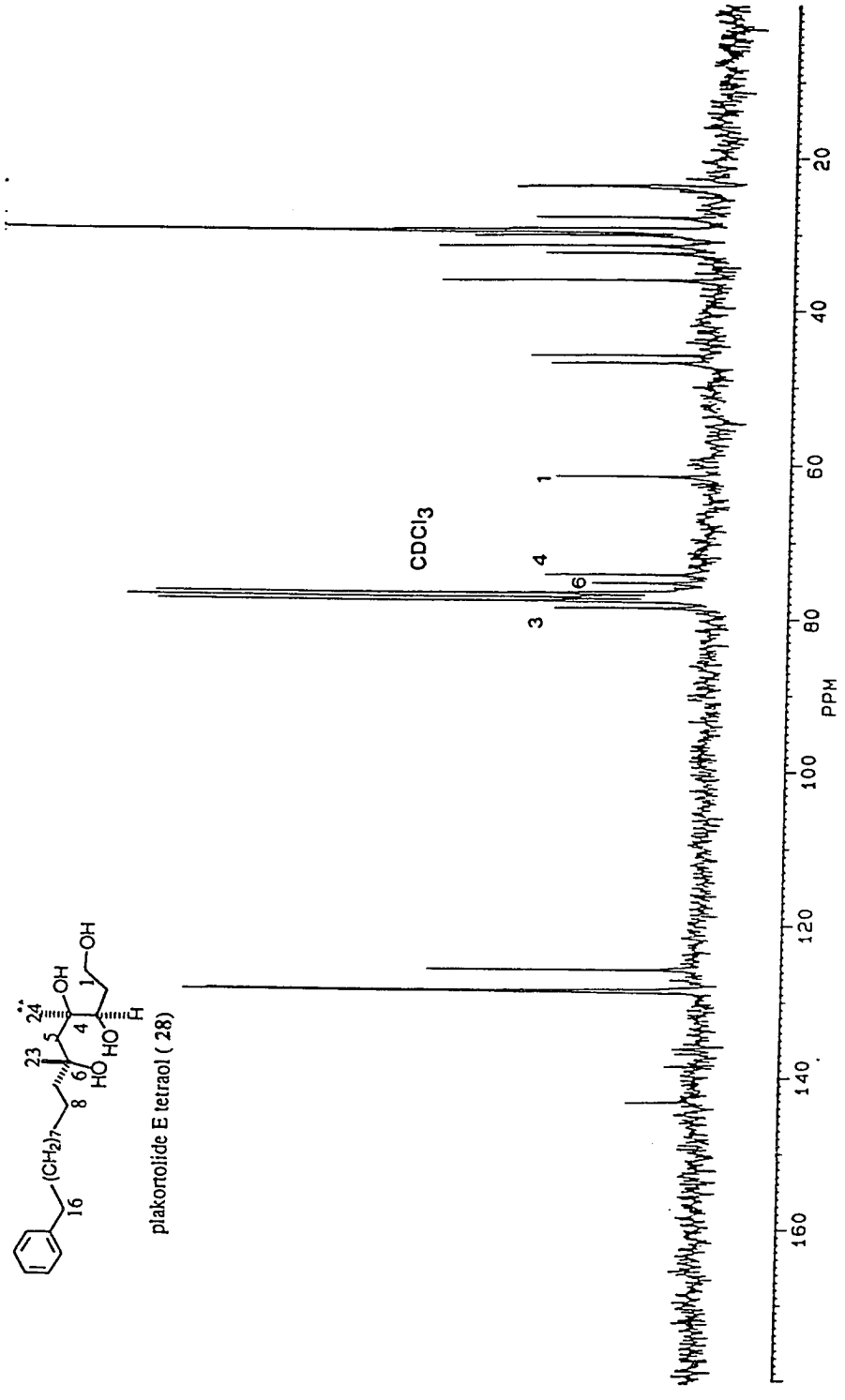


Figure 2.12. ^{13}C NMR of **28**, 62.9 MHz, $CDCl_3$.

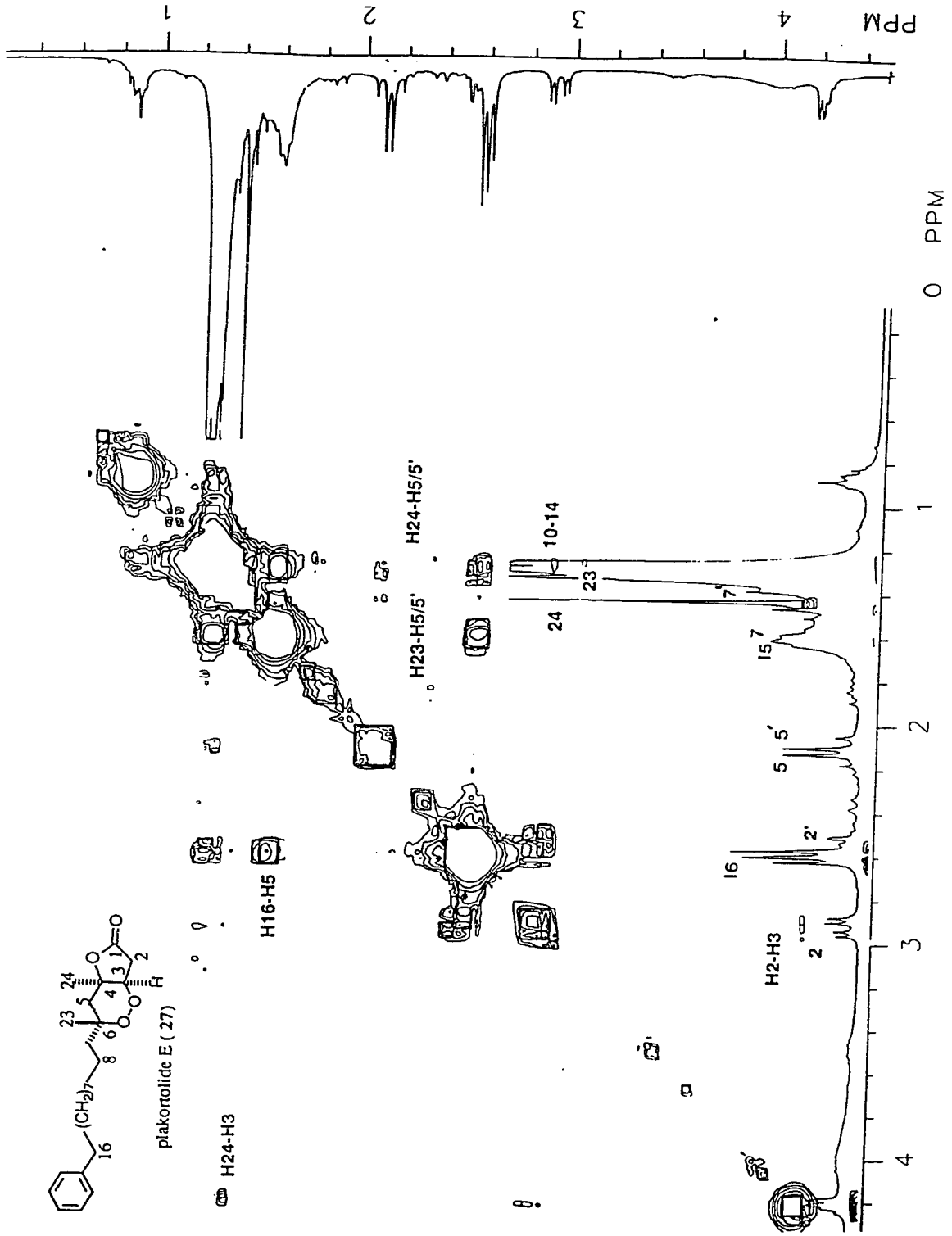


The relative stereochemistry of **27** was determined by nOe and NOESY data and comparisons to the literature data of the other plakortolides.[10-12] The syn junction of the ring system was determined by the strong NOESY cross peaks between H3 and Me24 in either $CDCl_3$ or C_6D_6, Figure 2.13. A comparison of the ^{13}C NMR shifts of C6, C4, C3, Me23 and Me24 of compounds **6**, **8** and **9** (Table 2.1, entries 4 - 6) against the ^{13}C NMR shifts of the same nuclei of plakortolide E (**27**) (Table 2.1, entry 1) indicated that the bicyclic ring of **27** must have a diastereomeric configuration relative to that of **6**, **8** and **9**. Furthermore the downfield shift of Me23 from δ 24.9 for **8** and **9** to δ 29.9 for **27** indicated that this group had moved from an axial to an equatorial position,[28] and justified the relative stereochemistry shown for plakortolide E (**27**).

The absolute stereochemistry of **27** was determined by the modified Mosher's empirical shift correlation method using *O*-methyl mandelate derivatives.[29-31] The progression of the synthetic work, shown in Scheme 2.1, afforded secondary esters of the (*R*) and (*S*) *O*-methyl mandelic acids as compounds **30** and **31**. In order to eliminate the concern of more than one methoxy mandelate ester forming from tetraol **28** this compound was first protected as the *t*-butyldiphenylsilyl ether **29**. Further treatment of **29** with either the *R*(+) or *S*(-) enantiomers of *O*-methyl mandelic acids gave the diastereomers **30** and **31** respectively, Scheme 2.1.

The diagnostic resonances shown in Figure 2.14 include those of the (*R*) mandelate **30**, δ 3.63 ($H_2$1) and δ 1.75/1.61 (H2/2'); and the (*S*) mandelate **31**, δ 3.42 ($H_2$1) and δ 1.61/1.51 (H2/2'). The greater shielding of the H1 and H2 protons in the *S*(-) mandelate **31** indicates that the phenyl group of the mandelate is positioned over that side of the molecule which supports assignment of absolute stereochemistry of (*R*)-3 in both **30** and **31**. Employing the relative stereochemistry assigned above, the additional stereochemistry of (*R*)-4 and (*R*)-6 is indicated for **27** and **28 - 31**.

Figure 2.13. NOESY spectrum of **27**, 300 MHz, $CDCl_3$.



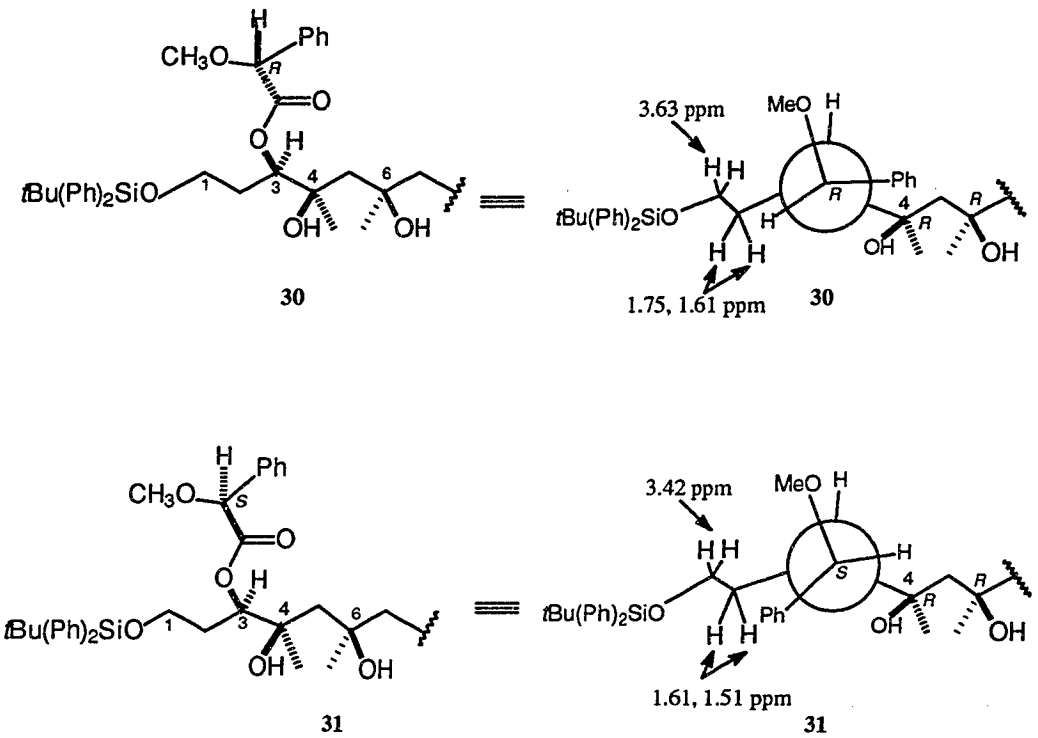
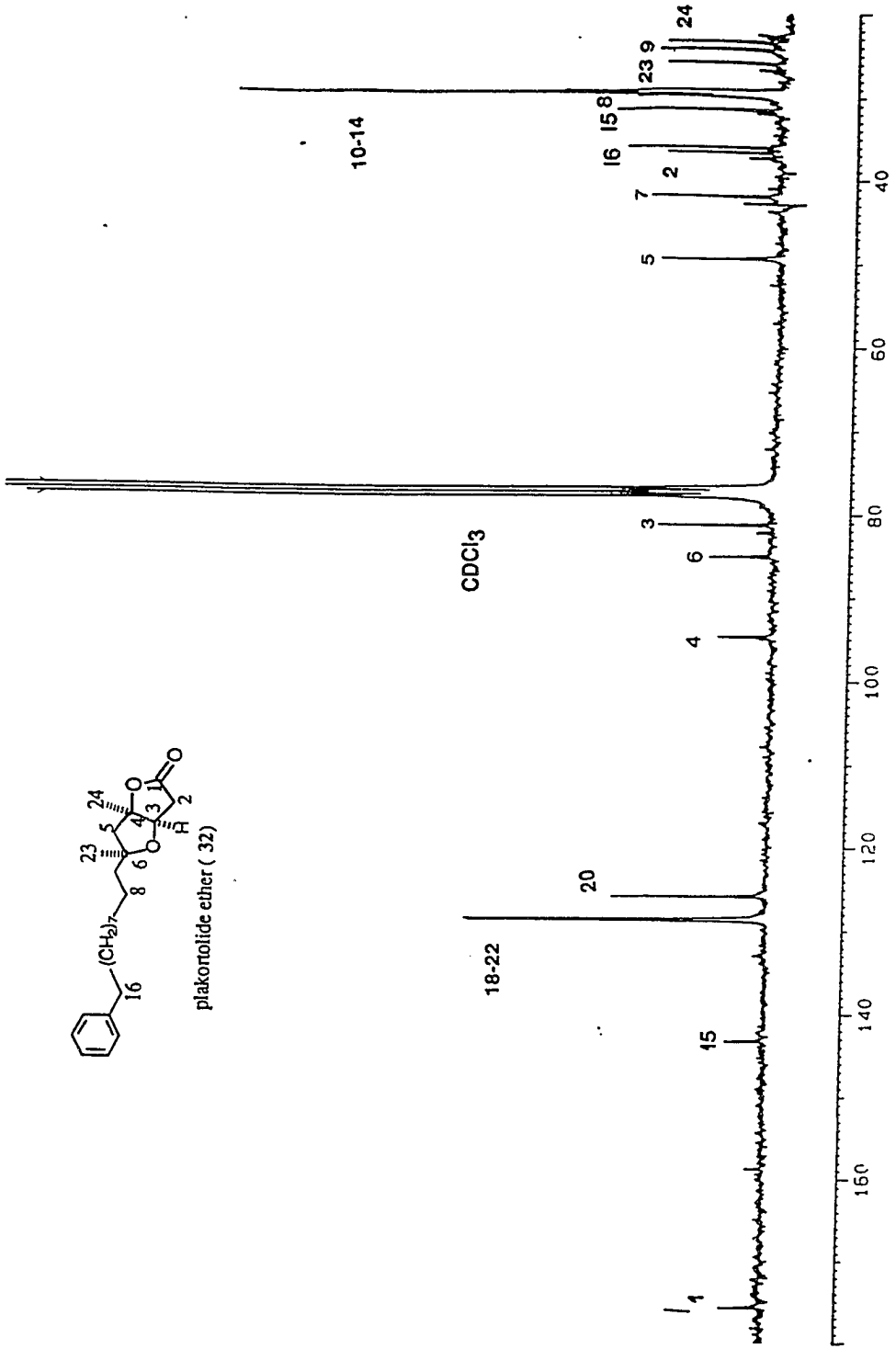


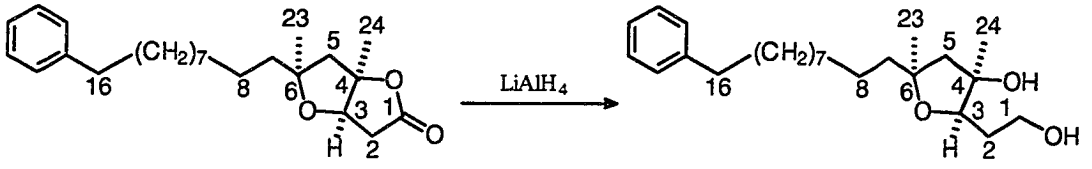
Figure 2.14. Results of modified Mosher's method with *O* methoxy mandelates to determine the absolute stereochemistry of plakortolide E (**27**).

After storage for approximately one year a change was observed in the plakortolide E (**27**) obtained from collection # 92123. The transformation in **27** was evident by comparing the ^{13}C NMR shifts of C6, C4, C3, Me23 and Me24, Figure 2.6, of the original sample (Table 2.1, entry 1) versus the aged sample (Table 2.1, entry 2), Figure 2.15. The molecular formula, $C_{24}H_{36}O_3$, of this new compound designated as plakortolide ether (**32**) was established by the hrfabms *m/z* = 373.2702 $[M+1]^+$ (Δ 4.1 mmu of calcd). Further chemical investigation of this new compound began with $LiAlH_4$ reduction to generate an ether diol (**33**) of formula $C_{24}H_{40}O_3$, also established by hrfabms *m/z* = 377.3047 $[M+1]^+$ (Δ 0.9 mmu of calcd), Scheme 2.2. Additional confirmation of this formula was provided by observing a mass shift by generating the sodium adduct of **33**,

Figure 2.15. ^{13}C NMR of **32**, 62.9 MHz, $CDCl_3$.



$C_{24}H_{40}O_3Na$, (hrfabms *m/z* = 399.2874 $[M+Na]^+$, Δ 0.1 mmu of calcd), Figure 2.16. The stereochemistry of **32** was determined by the correlations observed from H3 to both Me23 and Me24 in a ROESY NMR experiment, Figure 2.17. It appears that the most stable of the C6 epimers of **32** was formed in the transformation; the minimized calculated energies revealed that **32** with the C6 configuration as shown is 0.64 kcal lower in energy than the C6 epimer of **32**.



plakortolide ether (**32**) ether diol (**33**)

Scheme 2.2. Conversion of plakortolide ether (**32**) to ether diol (**33**).

Structure elucidation of plakoric acid (34)

The second metabolite, plakoric acid A (**34**), was isolated as an amorphous brown solid. Analogous to the situation in **27**, the highest hrfabms peak at *m/z* = 361.2733, which required the formula of $C_{23}H_{37}O_3$ (Δ 0.9 mmu of calcd), was assumed to represent the $[M+1-O]^+$ ion, Figure 2.18. The NMR spectra contained evidence for the three elements of functionality consisting of a monosubstituted benzene ring, a peroxide (δ 84.3 s (C7), 69.3 s (C4)), and an acid (δ 170.1 s (C1)), Figure 2.19. The two ^1H NMR singlet methyls, Figure 2.5, were assumed to be attached to the peroxide ring and this was confirmed by HMBC correlations, Figure 2.20, from each of these protons, H23 and H22, to the peroxide carbons C4 and C7. Additional support for the carboxylic acid group was provided by the sharp, intense IR absorption at 1713 cm^{-1} and a broad absorption from 3200-3400 cm^{-1}, Figure 2.21. The long methylene chain was characterized by broad and intense overlapping NMR signals observed in the proton and carbon spectra, Figures 2.5 and 2.19. Consistent with the C2-C3 and C5-C6 groups in **34** were the two isolated spin systems observed in the ^1H-^1H COSY spectrum, Figure 2.22. One spin system consisted of a double-doublet at δ 2.64

Figure 2.16. Low resolution FABMS of **33**, dithiothreotol/dithioerythrotol matrix.

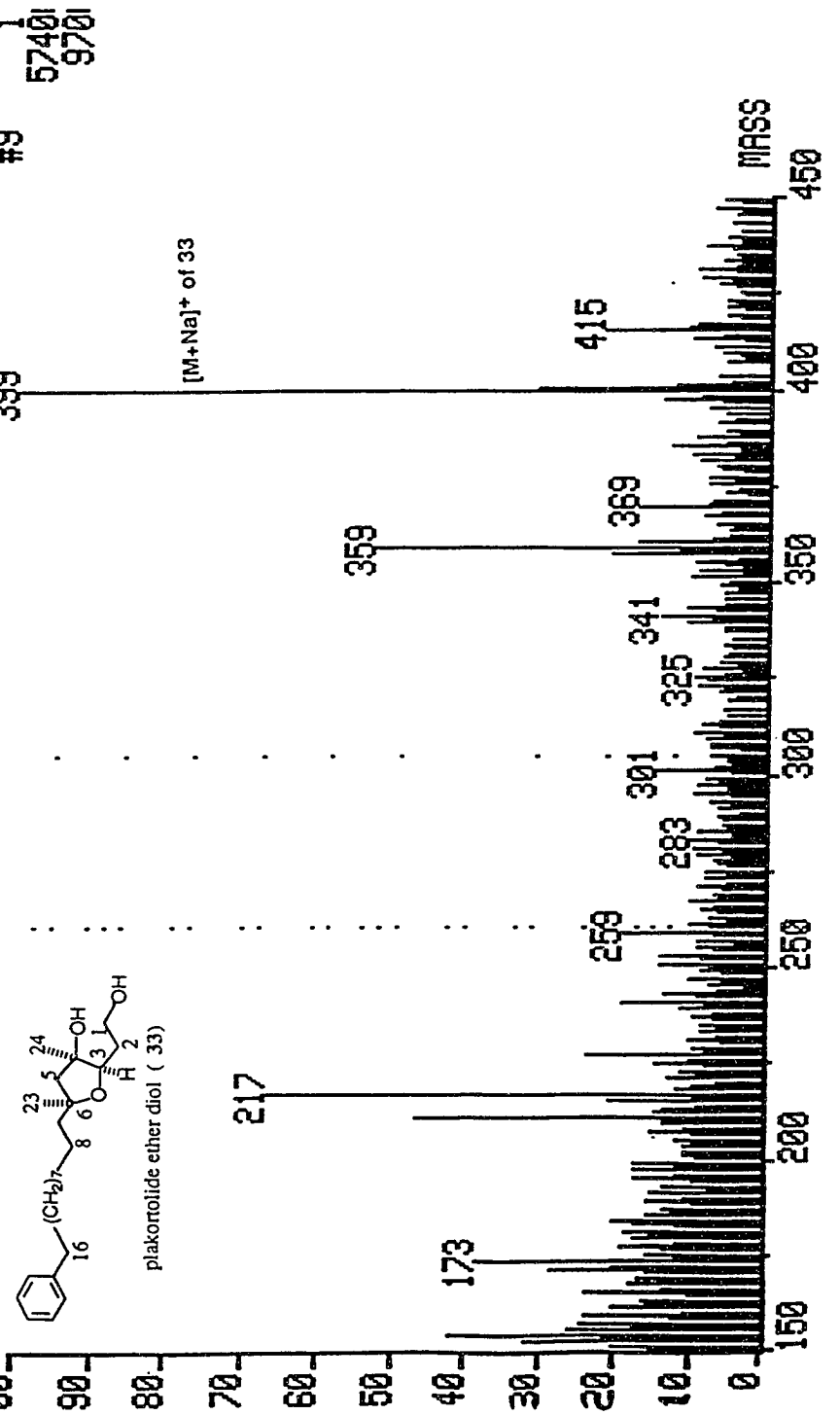


Figure 2.17. ROESY spectrum of **32**, 300 MHz, $CDCl_3$.

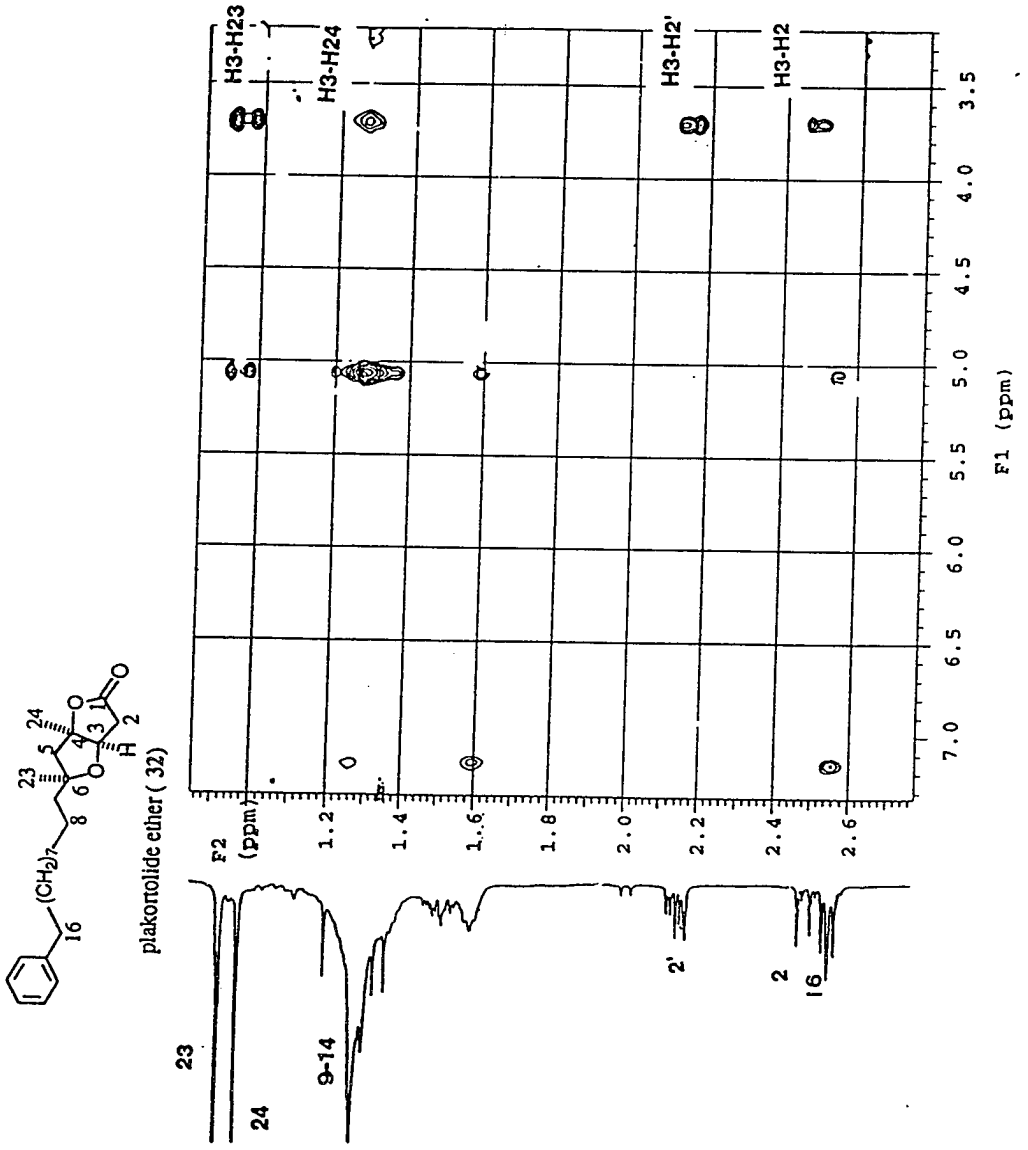


Figure 2.18. Low and high resolution FAB MS of plakoric acid (**34**), glycine matrix.

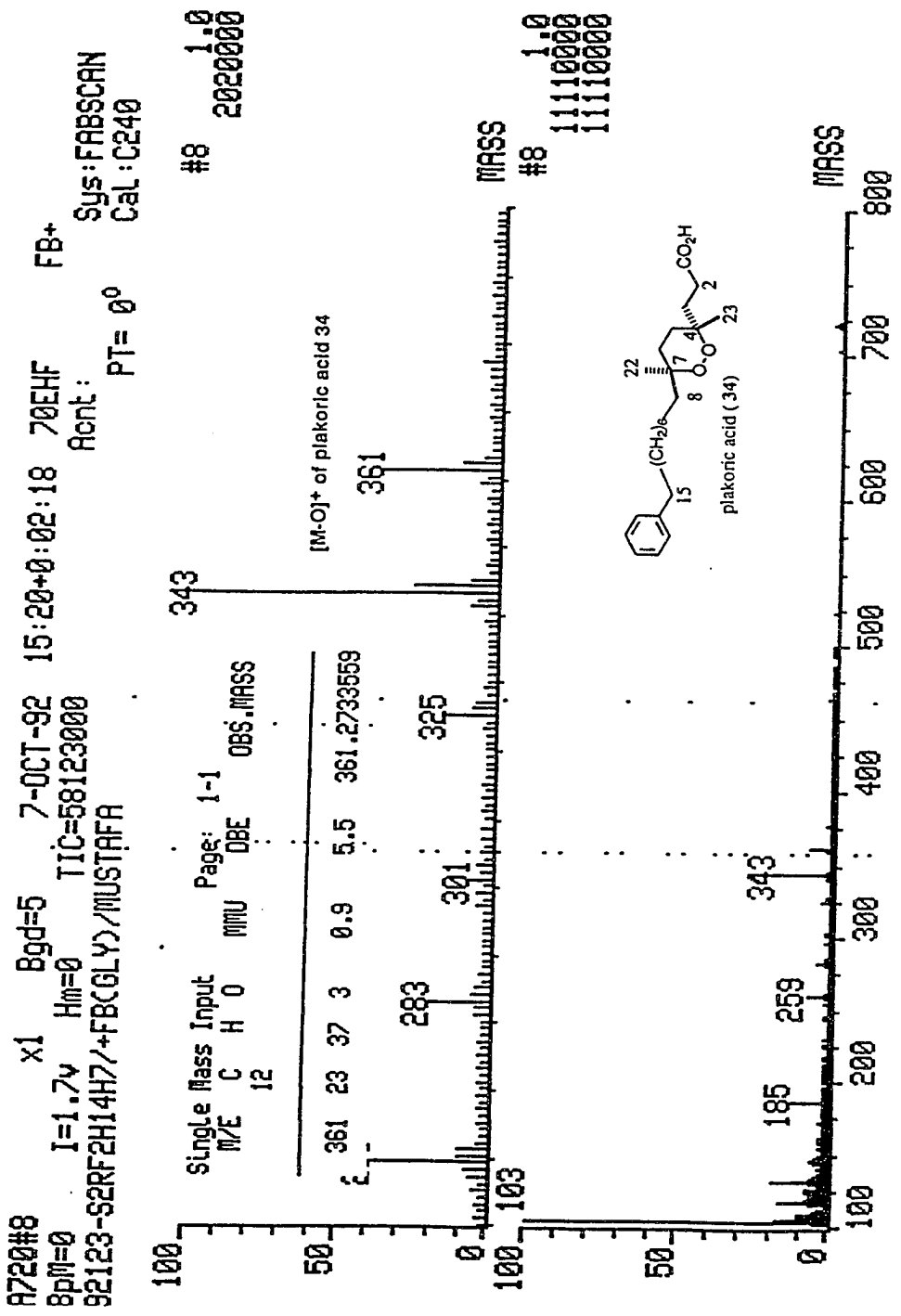


Figure 2.19. ^{13}C NMR of plakoric acid (**34**), 125 MHz, $CDCl_3$.

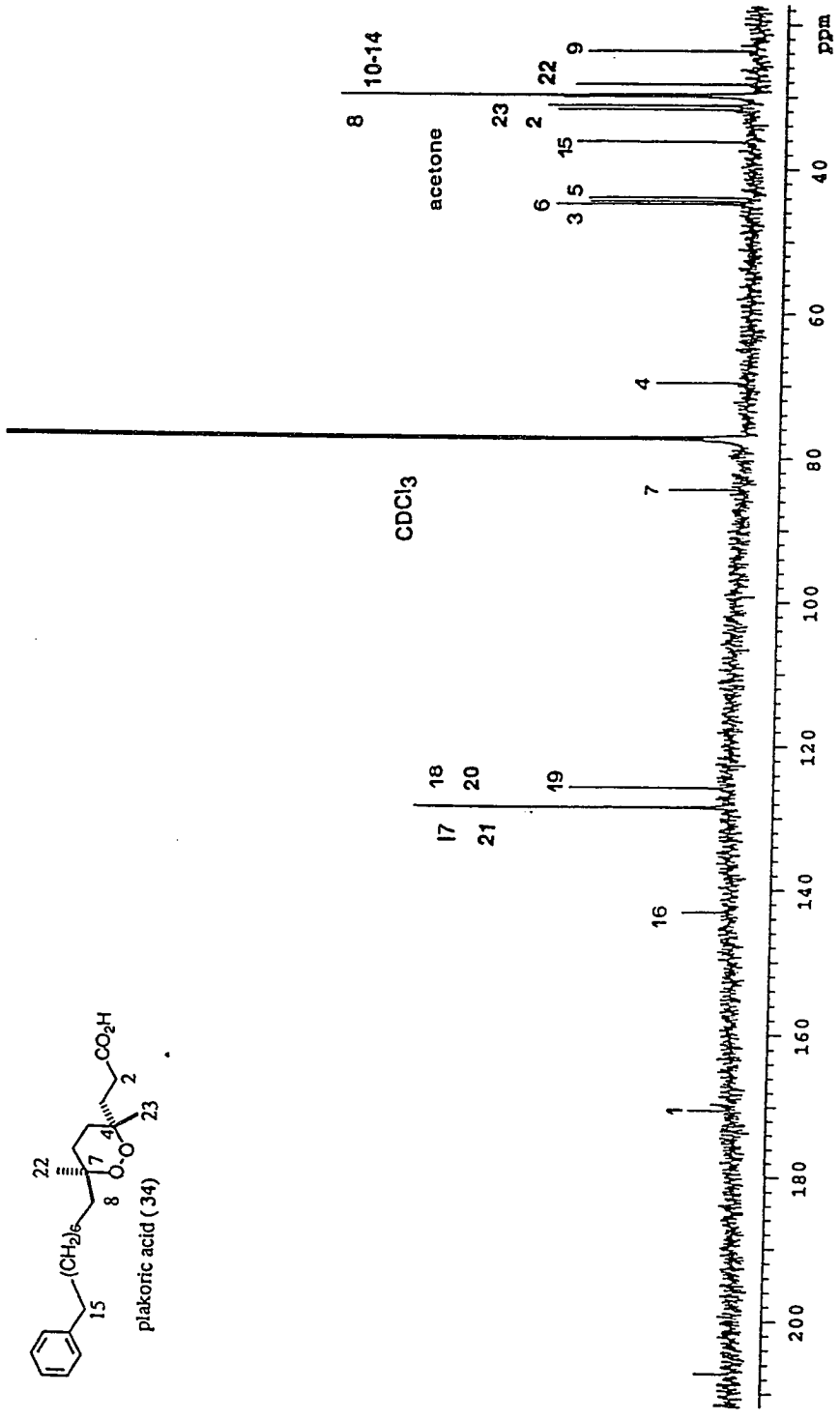


Figure 2.20. HMBC of plakoric acid (**34**), 500 MHz, $CDCl_3$.

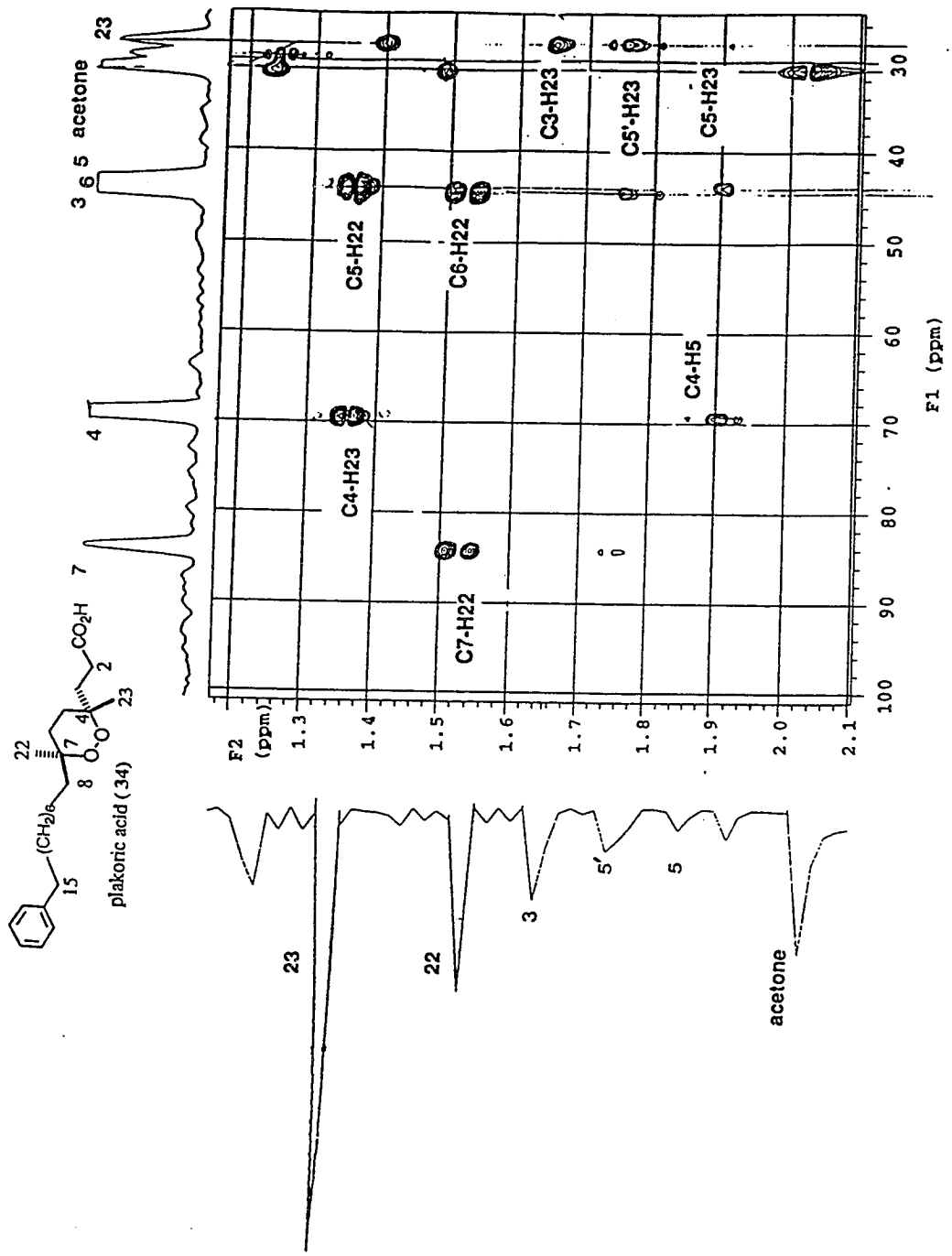


Figure 2.21. Infrared spectrum (film) of plakoric acid (**34**).

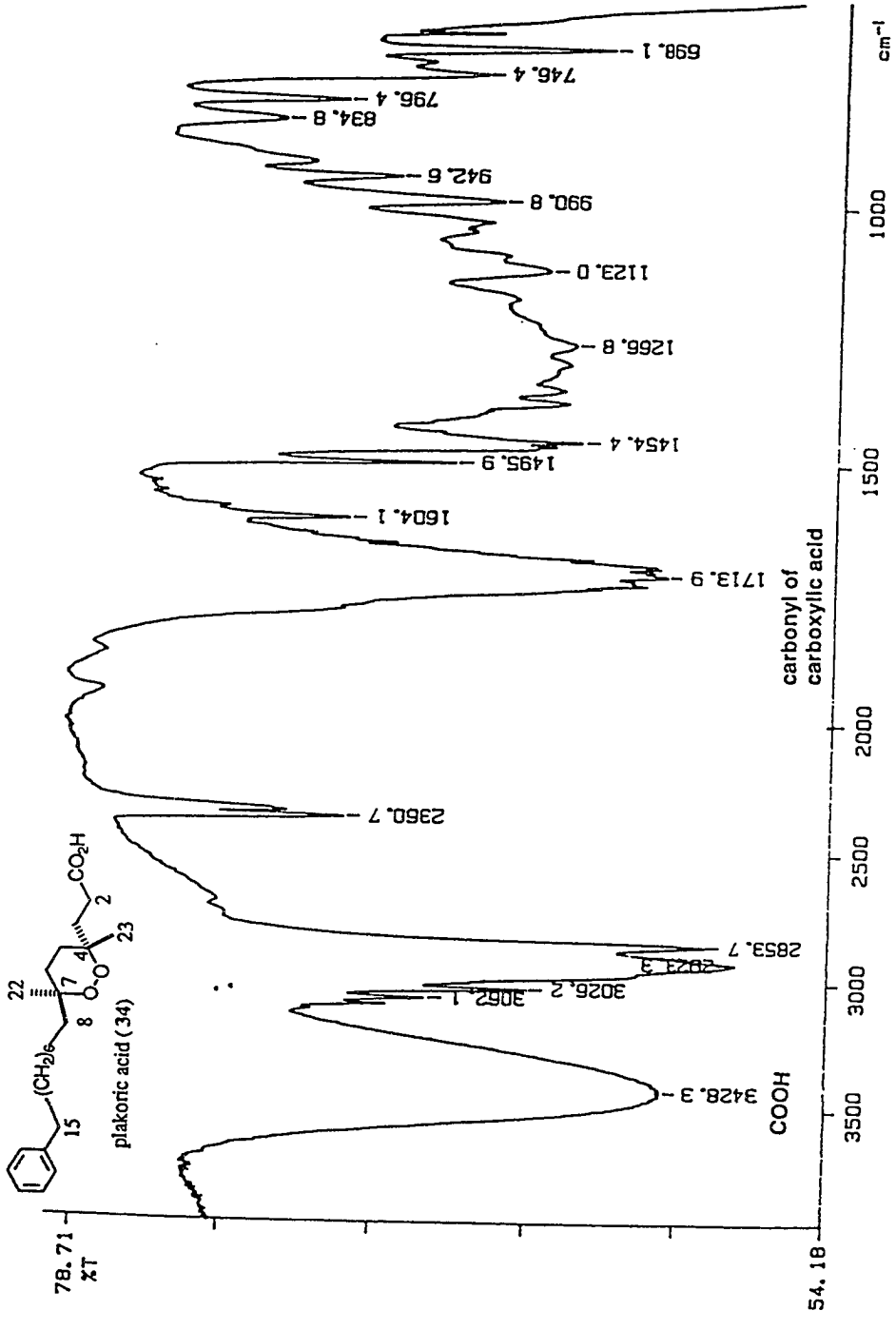
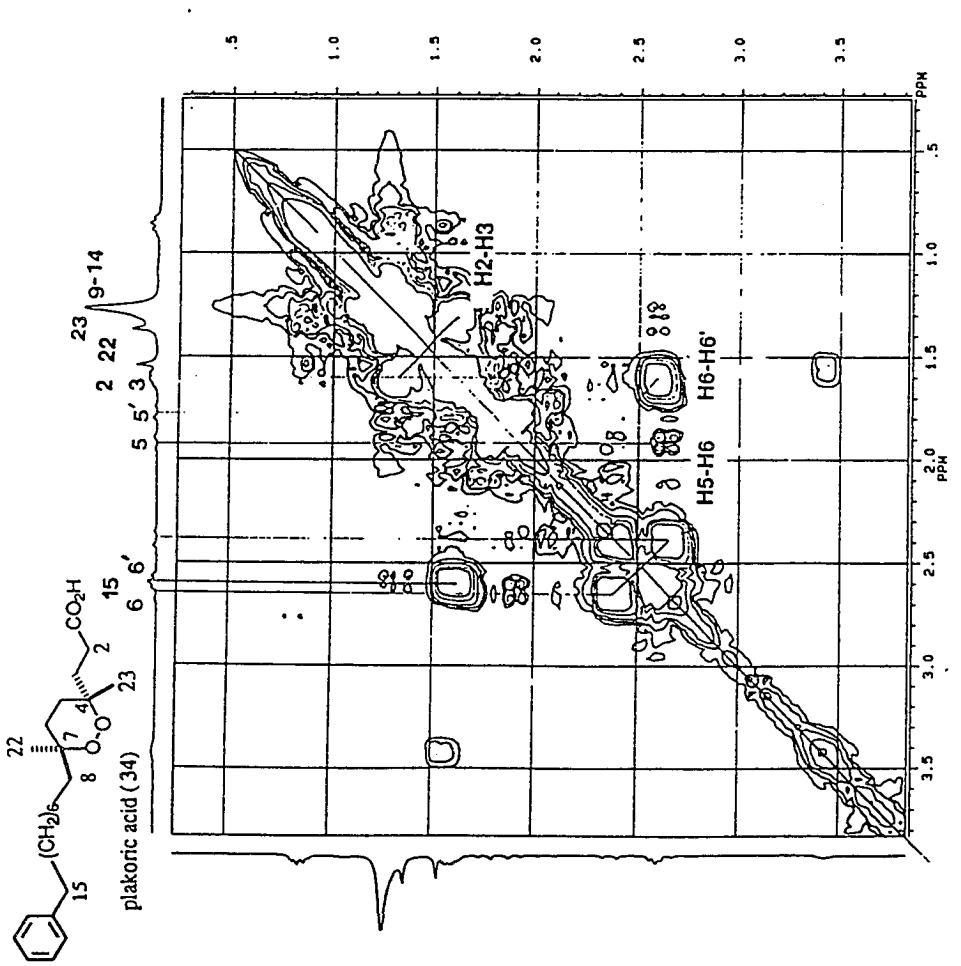


Figure 2.22. ^{1}H-^{1}H COSY spectrum of plakoric acid (**34**), 250MHz, $CDCl_3$.



(J = 2.5 and 17.5 Hz, H6) and a doublet at δ 2.40 (J = 17.5 Hz, H6') which were coupled to another double-doublet at δ 1.93 (J = 2.5 and 14.5 Hz, H5) and a doublet at δ 1.78 (J = 14.5 Hz, H5'). The other spin system appeared as overlapped multiplets centered at δ 1.59 ($H_2$3 and $H_2$2). These substructures are summarized in Figure 2.23.

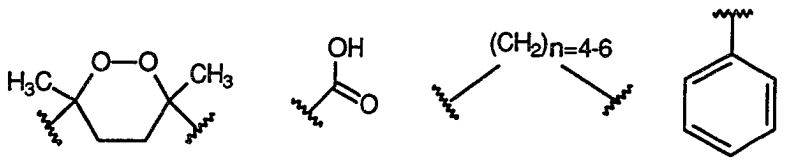
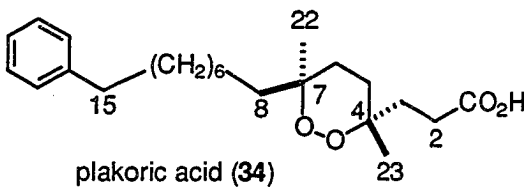
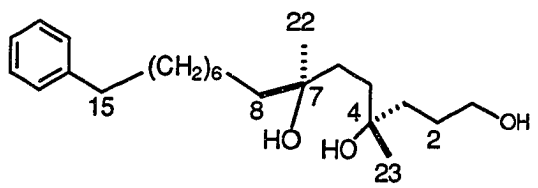


Figure 2.23. Substructures of plakoric acid (**34**).

The HMBC correlations from C6 and C7 to H22 indicated the position of Me22, Figure 2.20. Likewise HMBC correlations from C3, C4 and C5 to Me23 allowed the determination of a connection from Me23 to C4. Overall these and other HMBC correlations established the connectivities from C1 to C8. Reduction of **34** with $LiAlH_4$ afforded the expected triol (**35**), $C_{23}H_{40}O_3$ (hrfabms m/z = 365.3037 $[M+1]^+$, Δ 1.8 mmu of calcd). In addition, one of the quaternary carbons, C7, bearing oxygen shifted upfield in the conversion from **34** to **35** (to δ 75.0 s or 74.5 s), Figure 2.24. The lowfield ^{13}C NMR resonances of the methyls (δ 31.0 q, (C23) and 28.0 q, (C22)) of **34** suggests that both of these methyls are in the equatorial position.[24]

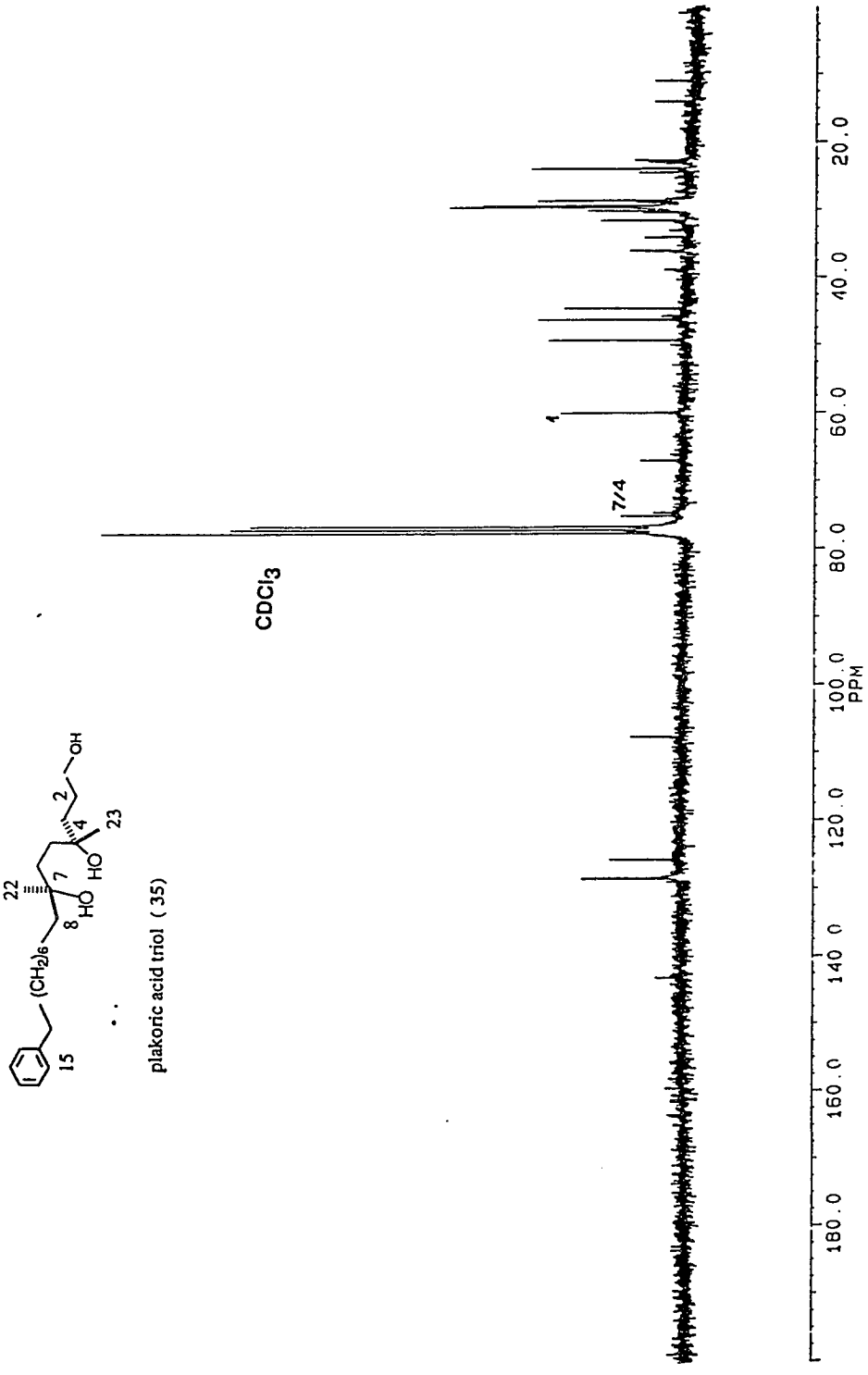


plakoric acid (**34**)



plakoric acid triol (**35**)

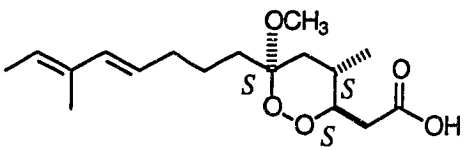
Figure 2.24. ^{13}C NMR spectrum of **35**, 62.9 MHz, $CDCl_3$.



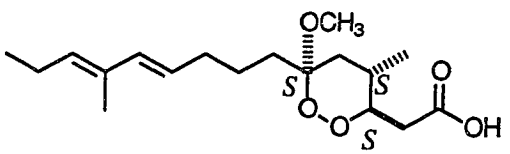
DISCUSSION

The isolation of the butenolide peroxide plakortolide E (**27**) and the carboxylic acid peroxide, plakoric acid (**34**) continues the trend observed for sponges of the genus *Plakortis*. The occurrence of two different cyclic peroxide ring structures has been observed before in *Plakortis halichondriodes*. The first butenolide peroxide and an accompanying acid peroxide (both unnamed) **6** and **7** were purified from the same sponge.[10] These compounds also contained the monosubstituted phenyl group that is present in plakortolide and plakoric acid.

To determine the absolute stereochemistry of plakortolide E (**27**) a novel application of the modified Mosher's method was employed. This led to the assignment of (*R*)-3, (*R*)-4, and (*R*)-6 for the absolute stereochemistry of **27-31**. Previously the absolute stereochemistry of cyclic peroxides has been elucidated by X-ray crystallography, CD spectroscopy using a exo methylene group or by asymmetric esterification of the diols derived from cleavage of the peroxides.[23] However only the absolute stereochemistry of terpene peroxides produced by non-*Plakortis* sponges has been elucidated. This work represents the first determination of the absolute stereochemistry of a polyketide peroxide. A second example of this method was recently applied to determine the stereochemistry of manadic acids A (**36**) and B (**37**), in which zinc and acetic acid were used for cleavage of the peroxide instead of $LiAlH_4$.[32]



36
manadic acid A



37
manadic acid B

Plakortolide E (**27**) also presents the first example of oxygen extrusion from a cyclic peroxide to form a cyclic ether in the absence of other reagents. The transformation of cyclic peroxides to hemiketals was observed in a specimen of *Plakortis halichondriodes* soaked in

ethanol for a prolonged period of time.[10] However, in the case of plakortolide E (**27**), there was no observation of a hemiketal, (no hemiketal carbon at approximately 100 ppm in the ^{13}C NMR). The small amount of plakortolide E (**27**) isolated from collection # 89134 did not undergo the same transformation as the newer plakortolide E (**27**) from collection # 92123. The conversion to plakortolide ether (**32**) occurred in a dry sample that was left in a vial for approximately one year. The mechanism for this reaction is not known.

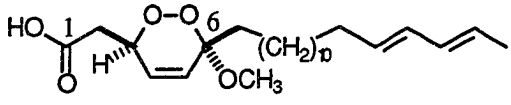
The observed bioactivity in a melanoma screen of the crude extract was the original motivation for this work. Plakortolide E (**27**) was identified as the active compound in the mixture while plakoric acid (**34**) was inactive. The butenolide peroxide skeleton has been shown to be cytotoxic since Davidson reported plakortolide (**8**) in 1991.[11] Unfortunately the butenolide peroxide (**6**) reported by Faulkner in 1980 was not assayed in an anticancer screen. Plakortolides B-D (**9-10**),[11,12] with the same general structural features as plakortolide (**8**) and plakortolide E (**27**) have also been found to be cytotoxic in anticancer assays.

The importance of the peroxide ring for the activity of plakortolide E (**27**) is demonstrated by the loss of activity upon formation of plakortolide ether. This suggests that there is a crucial dependence on the presence of the peroxide ring. Branching and cyclization of the chain do not appear to hamper the cytotoxic properties of these compounds as the norditerpene muquibillin (**22**) is found to be toxic as well.

Cyclic peroxides without a butenolide ring have also been found to be active in cancer assays. A comparison of the anticancer activity of the free acid versus the methyl ester of several cyclic peroxides indicates that the carboxylic acid containing compounds are more active.[25] However, in some cases, the ester containing cyclic peroxide compounds have been shown to be active.[26] It is possible the only active compounds are the cyclic peroxide free acids and both the ester peroxides and lactone (butenolide) peroxides operate as prodrugs that must be converted to the free acids to display cytotoxic effects. However the inactivity of plakoric acid (**34**) serves as a

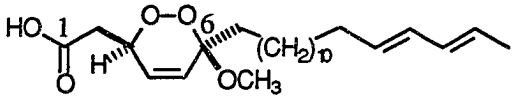
warning that there may be other mechanisms at work. There are no reports at this stage on the mechanism of action of cyclic peroxides so it is difficult to determine what is needed for activity. There are a number of drugs which kill cancer cells by a free radical mediated mechanism, including adriamycin, vincristine, and bleomycin.[33] It is possible free radicals from the peroxides are responsible for the observed cytotoxicity.

There are also cases where greater subtlety is displayed in structure activity relationships than the presence or absence of a carboxylic acid. Xestins A (**39**) and B (**40**),[26] a diastereomeric pair, have both been screened in the NCI 60 cell line anticancer screen. Only xestin A (**39**) is active in the NCI's cancer cell panel. Xestin B (**40**) differs from xestin A in the configuration of one stereogenic center, C6, in the orientation of the methoxy carbon and the fatty side chain. This small difference in configuration is enough to provide high selectivity or inactivity in the 60 cell line screen for these two compounds.



39

xestin A



40

xestin B

Even among cytotoxic cyclic peroxides, differences in selectivity toward different cancer types are observed. The NCI's 60 cell line disease screen is a good test of the cytotoxic potential of compounds against a variety of cancer types. While both plakortolide E (**27**) and xestin A (**39**) were active in this screen they affect different cancer cell lines, Figures 2.25 and 2.26. Plakortolide E (**27**) targets the melanoma and breast cancers while xestin A (**39**) is specifically active against equal numbers of colon and renal cancer cell lines. A terpene peroxide, methyl nuapapunoate B is also active in the NCI 60 cell line screen, however it predominantly affects the renal cancer cell lines and only affects one of the colon cell lines, Figure 2.27.[34] What accounts for this selectivity is unclear.

Figure 2.25. NCI 60 cell line GI_{50}, TGI, and LC_{50} for plakortolide E (**27**).

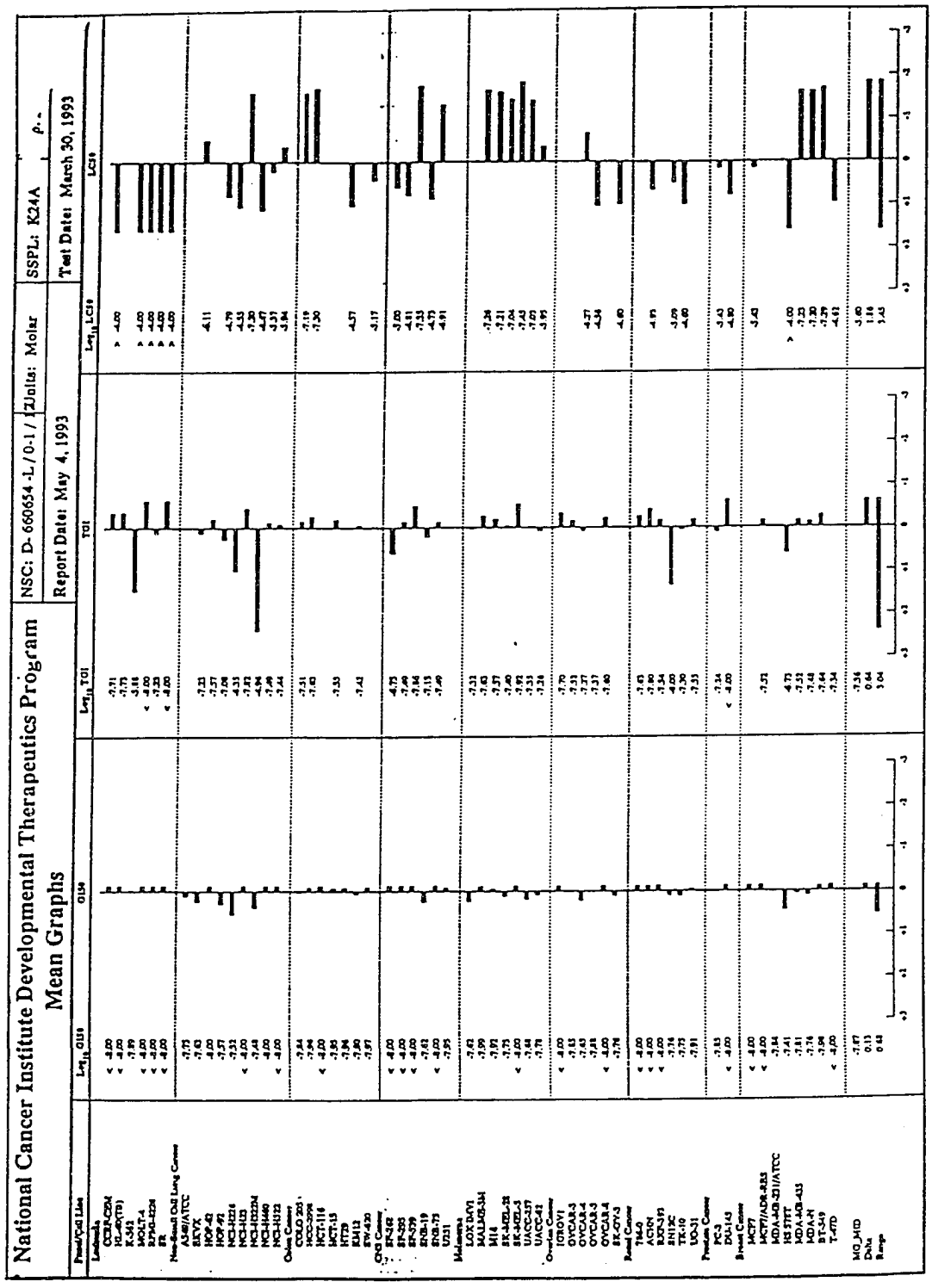


Figure 2.26. NCI 60 cell line GI_{50}, TGI, and LC_{50} for xestin A (**39**).

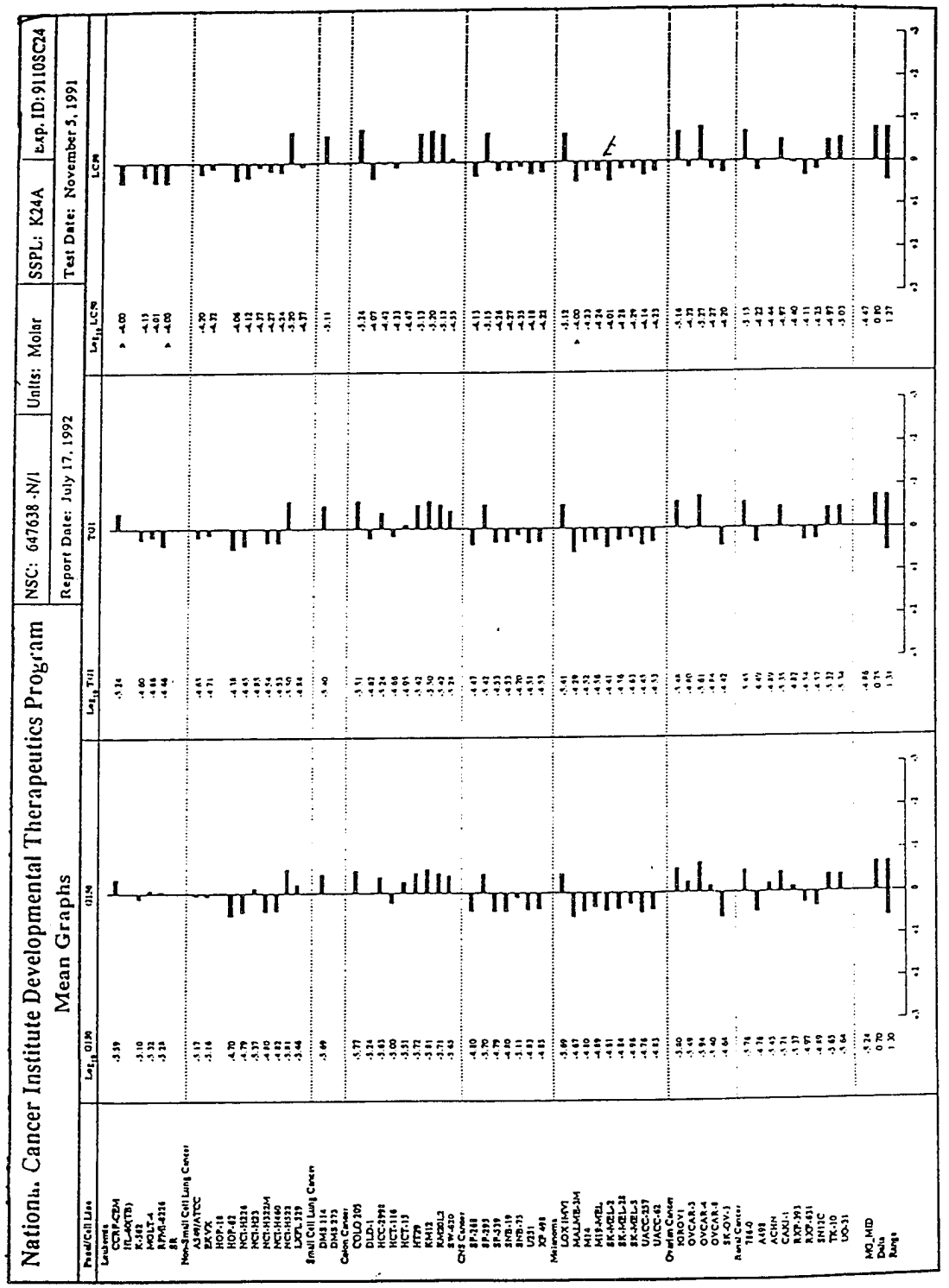
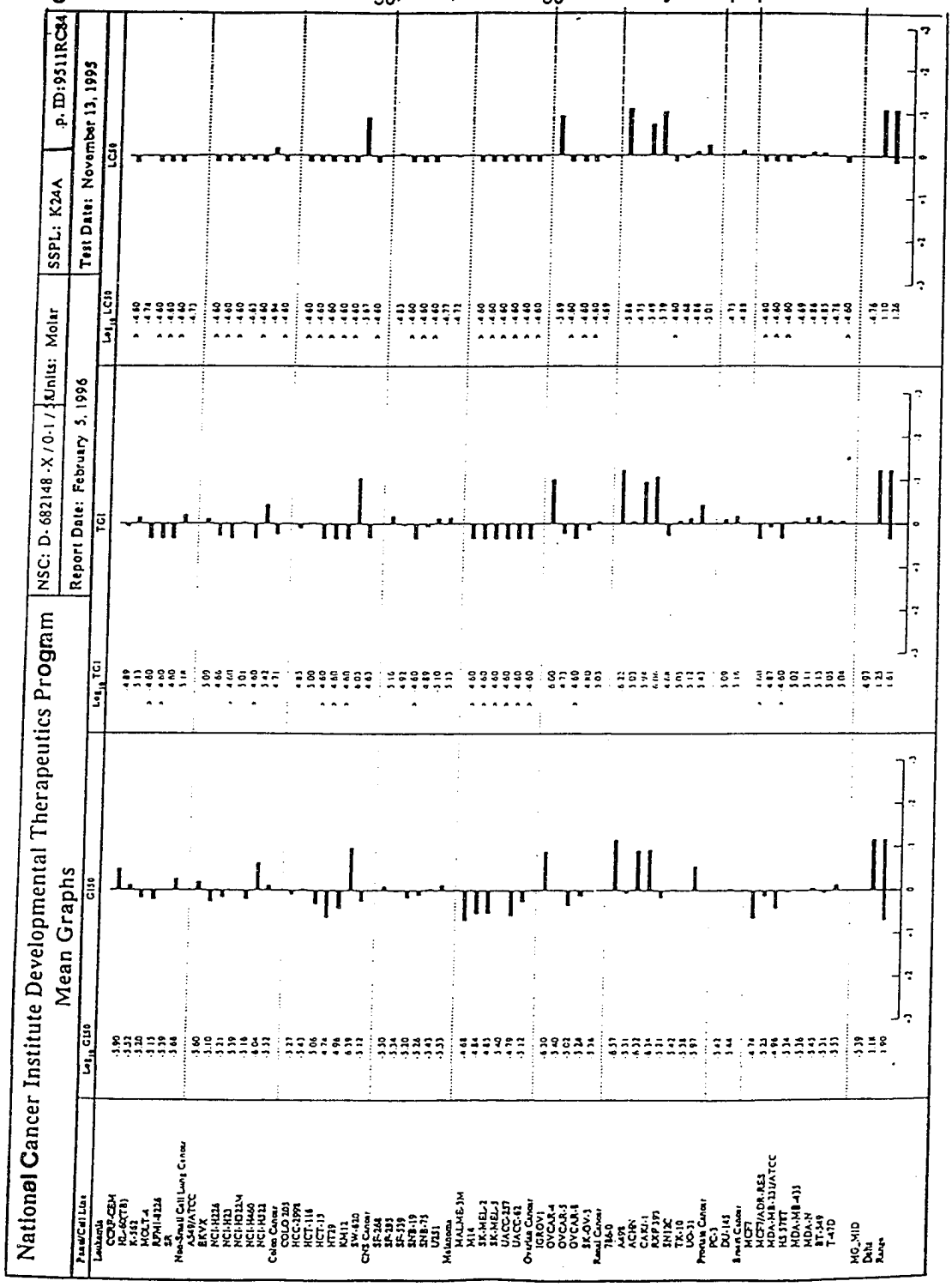


Figure 2.27. NCI 60 cell line GI_{50}, TGI, and LC_{50} for methyl nuapapuoate B.



Plakortolide E (**27**) is representative of the previously observed chemistry and cytoxicity for cyclic butenolide peroxides from *Plakortis* sponges. The simplicity of the structure of these cyclic peroxides coupled with the selective activity that certain compounds display in cancer screens provides promise for the development of new chemotherapeutic leads.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES —The NMR spectra were recorded at 250, 300 or 500 MHz for ^{1}H and 62.9, 75.5 or 125.7 MHz for ^{13}C. Multiplicities of ^{13}C NMR resonances were determined from APT data, DEPT data, or ^{1}H-^{13}C COSY experiments (300 MHz). Low and high resolution electron impact and fast atom bombardment mass spectrometry data were obtained on a magnetic sector instrument at UCSC. High performance liquid chromatography was done with 10μ C_{18} silica columns.

MOLECULAR MODELING —Molecular modeling was performed with the PCMODEL program (version 4.0) on a Silicon Graphic Personal Iris workstation. Molecular mechanics calculations were performed with the MMX force field including pi-VESCF calculations.

COLLECTION AND IDENTIFICATION — The sponges 89134 (0.4 kg) and 92123 (2.4 kg wet weight), collected from Taveuni, Fiji were identified as the same *Plakortis* sp. (order Homosclerophorida, family Plakinidae) by Ms. M. C. Diaz (UCSC) and Dr. R. W. M. van Soest (Institute of Taxonomic Zoology, University of Amsterdam). A voucher sample (92123) is available from the UCSC archives. The sponges are shown in Plates 2.1 and 2,2. The collection was made at a depth of 10 - 30 m. Description: shape - massively encrusting globs 2-3 cm high, oscules had a membrane; color - live specimens ranged in color from brown-green to a dark brown, dry specimens were tan; surface - smooth; consistency - dense and easy to break; ectosome - non-detachable; choanosome - cavernous with a packed arrangement of spicules; spicules - monoaxonic diactineas (100 - 170, 140, 40, 35 μm) x (4, 1, 2 μm), tetraxon-rays (22 x 4 μm), only two observed.

EXTRACTION AND ISOLATION —The sponge was initially preserved by soaking overnight in 1:1 ethanol:water. The solution was then discarded. The damp sponge was transported at ambient temperatures to U.C. Santa Cruz where it was soaked in MeOH (3 L three times for 72 h). The combined MeOH extracts were concentrated and the resultant oil was partitioned between 1:1

CH_2Cl_2:H_2O. The CH_2Cl_2 soluble fraction yielded 5.4 grams of viscous oil which was further partitioned between 10% aqueous MeOH and hexanes. The aqueous MeOH fraction was adjusted to 20% aqueous MeOH and was extracted with CCl_4. The CCl_4 soluble fraction was then evaporated to give a pale yellow viscous oil (0.99 g).

The CCl_4 soluble fraction was applied to a Sephadex LH-20 column (MeOH) and some of the fractions were further purified over a C_{18} silica flash column (eluted with 15% aqueous MeOH, 100% MeOH, then CH_3CN), followed by C_{18} silica HPLC (eluted with 25% aqueous CH_3CN) to yield compounds **27** and **34**. NMR assignments were made with ^1H-^1H COSY, ^1H-^{13}C COSY, APT and DEPT experiments.

PLAKORTOLIDE E [**27**] — A brown waxy solid (76.7 mg, 3%): $[\alpha]_D$ +10.0°, (c = 0.09, CH_2Cl_2); IR (dry film) ν max 2930 (phenyl), 2855 (phenyl), 1763 (C=O, lactone), 1735, 1463, 1281, 1174, 942 cm^{-1}; hreims *m/z*: 372.2664 [M^+-O, $C_{24}H_{36}O_3$, Δ 0.1 of calcd] lreims *m/z* (%) 372 [$M-O_2]^+$ (100), 357 (26), 340 (12), 327 (15), 313 (15); ^1H NMR ($CDCl_3$) δ 7.26 (m, H-19, H-21), 7.18 (m, H-18, H-22, H-20), 4.19 (dd, *J* = 1.8, 6.7 Hz, H-3), 2.15 and 2.09 (AB, *J* = 15.0 Hz, H-5/H-5'), 2.93 (dd, *J* = 6.7, 18.3 Hz, H-2), 2.55 (dd, *J* = 1.8, 18.3 Hz, H-2'), 2.60 (t, *J* = 7.4 Hz, H-16), 1.61 (overlapped m, H-7), 1.55 (overlapped m, H_2-15), 1.44 (s, H_3-24), 1.35 (s, H_3-23), 1.29 (br signal for H-7', and H_2-8 to H_2-14); ^{13}C NMR ($CDCl_3$) δ 175.2 (C-1), 142.9 (C-17), 128.3 (C-19, 21) 128.2 (C-18, 22), 125.5 (C-20), 90.1 (C-4), 73.8 (C-3), 72.9 (C-6), 43.9 (C-5), 43.6 (C-7), 38.1 (C-2), 35.9 (C-16), 31.5 (C-15), 30.0 (C-23), 30.0-29.4 (C-9 to C-14), 26.9 (C-24), 24.4 (C-8). Long range ^1H-^{13}C COSY correlations included correlations from the $H_3$23 to C6 and to C7, from both $H_3$23 and $H_3$24 to C5, from $H_3$24 to C4 and to C3, and from H2' to C1.

PLAKORTOLIDE ETHER [**32**] —A brown waxy solid $[\alpha]_D$ +59.9°, (c = 0.2, CH_2Cl_2); IR (dry film) ν max 2929 (phenyl), 2855 (phenyl), 1777 (C=O, lactone), 1712, 1521, 1264, 1155, 1073, 895, 704 cm^{-1}; hrfabms *m/z*: 373.2702 [M^+, $C_{24}H_{37}O_3$, Δ 4.1 of calcd] lrfabms, (3:1 dithiothreiotol:dithioerythritol) *m/z* (%) 373 (M^+, 100), 355 (75), 337 (45), 313 (85); ^1H NMR

($CDCl_3$) δ 7.26 (m, H-19, H-21), 7.18 (m, H-18, H-22, H-20), 4.35 (d, *J* = 4.2 Hz, H-3), 2.73 (d, *J* = 4.2 Hz, H-2), 2.70 (s, H-2'), 2.60 (t, *J* = 7.7 Hz, H_2-16), 2.40 (d, *J* = 14.3 Hz, H-5), 1.87 (d, *J* = 14.3 Hz, H-5'), 1.60 (m, H_2-15), 1.58 (m, H_2-7), 1.51 (s, H_3-24), 1.26 (br m, H_2-8 to H_2-14), 1.24 (s, H_3-23); ^{13}C NMR ($CDCl_3$) δ 176.0 (C-1), 143.1 (C-17), 128.3 (C-19,21) 128.2 (C-18, 22), 125.5 (C-20), 94.5 (C-4), 84.8 (C-6), 81.0 (C-3), 49.1 (C-5), 41.7 (C-7), 36.5 (C-2), 35.9 (C-16), 31.5 (C-15), 30.0-29.4 (C-9 to C-14), 26.1 (C-23), 24.5 (C-8), 23.6 (C-24); ^1H NMR (C_6D_6) δ 7.24 (m, H-19, H-21), 7.15 (m, H-18, H-22, H-20), 3.70 (d, *J* = 4.9 Hz, H-3), 2.56 (t, *J* = 8.0 Hz, H_2-16), 2.50 (d, *J* = 17.7 Hz, H-2), 2.17 (d, *J* = 13.8 Hz, H-5), 2.16 (dd, *J* = 4.9, 17.7 Hz, H-2'), 1.61 (overlapped m, H_2-15), 1.58 (m, H_2-7), 1.54 (overlapped d, *J* = 13.8 Hz, H-5'), 1.29 (br m, H_2-8 to H_2-14), 0.99 (s, H_3-23), 0.94 (s, H_3-24); ^{13}C NMR (C_6D_6) δ 173.0 (C-1), 142.0 (C-17), 127.2 (C-19, 21), 127.2 (C-18, 22), 125.0 (C-20), 93.1 (C-4), 84.2 (C-6), 81.1 (C-3), 49.3 (C-5), 42.0 (C-7), 36.4 (C-2), 36.3 (C-16), 31.9 (C-15), 30.0-29.4 (C-9 to C-14), 25.9 (C-23), 24.8 (C-8), 23.1 (C-24).

PLAKORIC ACID [**34**] —A brown waxy solid (19.3 mg, 0.8%): $[\alpha]_D$ -18.7°, (c = 1.0, CH_2Cl_2); IR (dry film) ν max, 3200-3400 (acid OH), 1713 cm^{-1} (C=O, carboxylic acid); hrfabms *m/z*: 361.2819 ($[M+1-O]^+$, $C_{23}H_{37}O_3$, Δ 7.6 mmu of calcd); lrfabms (NBA), *m/z* (%): 361 (31), 351 (10), 343 (100), 325 (26), 313 (11); ^1H NMR ($CDCl_3$) δ 7.26 (m, H-18, H-20), 7.18 (m, H-17, H-19, H-21), 2.64 (dd, *J* = 2.5, 17.5 Hz, H-6), 2.61 (t, *J* = 8.0 Hz, H_2-15), 2.40 (d, *J* = 17.5 Hz, H-6'), 1.93 (dd, *J* = 2.5, 14.5 Hz, H-5), 1.78 (d, *J* = 14.5 Hz, H-5'), 1.59 (overlapped m, H_2-3 and H_2-2), 1.54 (s, H_3-22), 1.38 (s, H_3-23), 1.27 (overlapped m, H_2-9 to H_2-14); ^{13}C NMR ($CDCl_3$) δ 170.1 (C-1), 142.9 (C-16), 128.4 (C-18, 20), 128.2 (C-17, 21), 125.6 (C-19), 84.3 (C-7), 69.3 (C-4), 44.6 (C-3), 44.2 (C-6), 43.7 (C-5), 36.0 (C-15), 31.5 (C-2), 31.0 (C-23), 29.8-29.3 (C-8, C-10 to C-14), 28.0 (C-22), 23.4 (C-9).

PREPARATION OF **28** —Compound **27** (20 mg) dissolved in dry THF (4 mL) was placed in a flame dried flask and $LiAlH_4$ (spatula tip amount) was added. After 70 minutes at rt the reaction

was quenched with 10 mL NH_4Cl (10%) and the THF was evaporated. The resulting solution was filtered through celite. The solid residue was washed with 3 mL NaCl (sat, aq.) solution and then with 3 mL CH_2Cl_2. The combined aqueous phases were extracted with CH_2Cl_2 (3 x 0.5 ml). All of the CH_2Cl_2 fractions were combined and evaporated to yield a pale brown waxy solid, (18 mg, 90% yield): lrfabms (NBA) *m/z* (%), 395 ($[M+1]^+$, 23), 377 (23), 359 (100), 341 (15), 329 (6), 307 (97); ^1H NMR ($CDCl_3$) δ 7.26 (m, H-19, H-21), 7.18 (m, H-18, H-20, H-22), 3.84 (m, H_2-1), 3.67 (d, J = 5.3 Hz, H-3), 3.65 (d, J = 5.3 Hz, H-3'), 2.60 (overlapped t, J = 7.5 Hz, H_2-2 and H_2-16), 1.74 (overlapped m, H-5), 1.69 (overlapped m, H_2-8), 1.64 (overlapped m, H_2-9), 1.56 (overlapped m, H-5'), 1.56 (overlapped m, H-7), 1.37 (s, H_3-23 or H_3-24), 1.26 (overlapped m, H-7', H_2-10 to H_2-15), 1.26 (s, H_3-24 or H_3-23); ^{13}C NMR ($CDCl_3$) δ 142.9 (C-17), 128.4 (C-19, 21), 128.2 (C-18, 22), 125.6 (C-20), 78.5 (C-3), 75.3 (C-4 or C-6), 74.1 (C-6 or C-4), 61.4 (C-1), 46.8 (C-5), 45.8 (C-7), 36.0 (C-2, 16), 32.5 (C-9), 31.6 (C-15), 30.2 - 29.4 (C-10 to C-14), 27.9 (C-23 or C-24), 24.0 (C-8), 23.8 (C-23 or C-24).

PREPARATION OF **29** —Compound **28** (8 mg) dissolved in dry CH_2Cl_2 (3 mL) was mixed with *t*-butyl diphenyl silyl chloride (1 drop) and a trace of DMAP. The reaction mixture was stirred for 78 hours at rt then washed with 5 ml HCl (1%). The aqueous layer was extracted (3 x 2 mL) with CH_2Cl_2. The CH_2Cl_2 fractions were combined, dried over $MgSO_4$ and then filtered through celite. The evaporated filtrate yielded a pale yellow oil which was further purified on a silica column eluted with hexanes : EtOAc (9:1) to afford **29** (4 mg, 30% yield): ^1H NMR ($CDCl_3$) δ 7.67 (m, Si-phenyl), 7.44 (m, Si-phenyl), 7.26 (m, H-19, H-21), 7.17 (m, H-18, H-20, H-22), 3.91 (m, H-3), 3.74 (d, J = 2.8 Hz, H-1), 3.71 (d, J = 2.8 Hz, H-1'), 2.66 (t, J = 7.4, H_2-16), 1.66 (AB, J = 14.5, H_2-5), 1.61 (overlapped m, H_2-8, H-7), 1.35 (s, H_3-23 or H_3-24), 1.27 (overlapped m, H-7', H_2-9 to H_2-15, H_3-23 or H_3-24), 1.06 (s, H_9-25); ^{13}C NMR ($CDCl_3$) δ 143.0 (C-17), 135.6, 132.9, 130.0, 128.0, 127.9 (Si-phenyl), 128.4 (C-19, 21), 128.2 (C-18, 22), 125.6 (C-20), 78.8 (C-3), 75.2 (C-6 or 4), 73.4 (C-4 or 6), 63.6 (C-1), 46.9 (C-5), 45.7 (C-7), 36.0 (C-16), 32.7

(C-2), 31.6 (C-9), 30.3-29.3 (C-10 to C-14), 28.2 (C-23 or C-24), 26.9 (C-25), 24.1 (C-24 or C-23), 24.0 (C-8), 19.1 (C-26).

PREPARATION OF MANDELATE ESTERS **30** AND **31** —Both the *R*(+) and *S*(-) mandelic esters **30** and **31** were prepared in the same fashion. Compound **29** (2 mg) was dissolved in CH_2Cl_2 (0.5 ml) and other reagents were added to this solution including DCC (spatula tip amount), DMAP (spatula tip amount) and either the *S*(-)-*O*-methyl mandelic acid or the *R*(+)-*O*-methyl mandelic acid (spatula tip amount). The reaction mixture was stirred for 36 h at rt, then filtered and the CH_2Cl_2 was evaporated. The residue was suspended in 2 mL hexanes and 2 mL water and then washed (3 x 2 mL) with hexanes, followed by HCl (1N, 2 x 0.5 ml), saturated $NaHCO_3$ (2 x 0.5 ml), and saturated NaCl (2 x 0.5 ml). The organic phase was next filtered and the solvent was evaporated to yield the mandelate esters mixed with a small amount of **29**. *R-O*-methyl mandelate ester (**30**): ^{1}H NMR ($CDCl_3$) δ 3.63 (H_2-1), 1.75 (H-2), 1.61 (H-2'), the other signals except for the phenyl signals are overlapped. *S-O*-methyl mandelate ester (**31**): ^{1}H NMR ($CDCl_3$) δ 3.42 (H_2-1), 1.61 (H-2), 1.51 (H-2'), the other signals except for the phenyl signals are overlapped.

PREPARATION OF **33** — Into a flame dried flask was placed compound **32** (5.0 mg) dissolved in 2 mL dry THF. A spatula tip amount of $LiAlH_4$ was added, and after 70 minutes at rt the reaction was quenched with 5 mL NH_4Cl (10% aq.) and the THF was evaporated. The resulting solution was treated, in an analogous manner as in the preparation of **28** described above, to yield **35** as a fine white powder (2.2 mg, 44 % yield): hrfabms *m/z*: 377.3047 ($[M+1]^+$, $C_{24}H_{41}O_3$, Δ 0.9 calcd), 399.2874 ($[M+Na]^+$, $C_{24}H_{40}O_3Na$, Δ 0.1 mmu of calcd); lrfabms *m/z* (%) 377 (M^+, 14), 359 (100), 341 (22); ^{1}H NMR ($CDCl_3$) δ 7.26 (m, H-19, H-21), 7.18 (m, H-18, H-20, H-22), 3.81 (m, H_2-1), 3.71 (d, J = 4.8 Hz, H-3), 2.58 (t, J = 7.2 Hz, H_2-16, H_2-2), 1.98 (d, J = 14.4 Hz, H-5), 1.87 (m, H_2-15), 1.78 (d, J = 14.4 Hz, H-5'), 1.58 (m, H_2-7), 1.29 (m, H_2-8 to H_2-14), 1.22 (s, H_3-24), 1.20 (s, H_3-23); ^{13}C NMR ($CDCl_3$) δ 142.0 (C-17), 128.2 (C-

18, 22), 128.0 (C-19, 21), 126.0 (C-20), 84.1 (C-3), 81.5 (C-6), 79.6 (C-4), 61.4 (C-1), 52.6 (C-5), 43.0 (C-7), 36.0 (C-16), 31.5 (C-15), 30.2 (C-2, 8), 29.6 (C-11 to C-14), 29.4 (C-10), 26.2 (C-23), 24.8 (C-9), 23.4 (C-24).

PREPARATION OF **35** — Into a flame dried flask was placed compound **34** (4.9 mg) dissolved in dry THF. $LiAlH_4$ (spatula tip amount) was added, and after 70 minutes at rt the reaction was quenched with NH_4Cl (10% aq.) and the THF was evaporated. The resultant solution was treated as for **33** to yield **35** as a fine white powder (4.4 mg, 88 % yield): hrfabms (3:1 dithiothreiotol:dithioerythritol) *m/z*: 365.3037 ($[M+1]^+$, $C_{23}H_{40}O_3$, Δ 1.8 mmu of calcd); ^1H NMR ($CDCl_3$) δ 7.26 (m, H-19, H-21), 7.18 (m, H-18, H-20, H-22), 3.94 (m, H_2-1), 2.60 (t, J = 7.5 Hz, H_2-15), 1.87-1.49 (overlapped m, H_2-2 to H_2-6, H_2-8), 1.42 (s, H_3-22 or 23), 1.36 (s, H_3-22 or 23), 1.29 (overlapped m, H_2-9 to H_2-14); ^{13}C NMR ($CDCl_3$) δ 142.9 (C-16), 128.4 (C-18, 20), 128.2 (C-17, 21), 125.6 (C-19), 75.0 (C-7 or C-4), 74.5 (C-4 or C-7), 59.9 (C-1), 49.2 (C-2), 46.2 (C-6), 44.5 (C-3), 36.0 (C-15), 32.8 (C-8), 31.9 (C-14), 30.2 - 29.4 (C-10, C-11 to C-13), 28.7 (C-22 or C-23), 28.6 (C-23 or C-22), 23.3 (C-9).

REFERENCES

(1) Valeriote, F.; Moore, R. E.; Patterson, G. L. M.; Paul, V. J.; Scheuer, P. J.; Corbett, T. In *Anticancer Drug Discovery and Development : Natural Products and New Molecular Models.*; Valeriote, F. A., Corbett, T. H., Baker, L. H., Eds.; Kluwer Academic Publishers: Boston, 1994, pp. 1-26.

(2) Suffness, M. In *Biologically active natural products (Annual Proceedings of the Phytochemical Society of Europe)*; Hostettmann, K., Lea, P., Eds.; Clarendon Press: Oxford, 1987, pp. 85-104.

(3) Swaffar, D. J.; Ireland, C. M.; Barrows, L. R. *Anti-Cancer Drugs* **1994**, *5*, 15.

(4) Johnson, R. K.; Hertzberg, R. P. *Ann. Rep. Med. Chem.* **1990**, *25*, 129.

(5) DeVita, V. T. J. In *Cancer Principles & Practice of Oncology*; 2nd ed.; DeVita, V. T. J., Hellman, S., Rosenberg, S. A., Eds.; J. B. Lippincott Company: New York, 1985, p. 257.

(6) Boyd, M. R. *Principles and Practice of Oncology Updates* **1989**, *3*, 1-12.

(7) Inman, W. D.; O'Neil-Johnson, M.; Crews, P. *J. Am. Chem. Soc.* **1990**, *112*, 1.

(8) West, B. R.; Mayne, C. L.; Ireland, C. M.; Brinen, L. S.; Clardy, J. *Tetrahedron Lett.* **1990**, *31*, 3271-3274.

(9) Higgs, M. D.; Faulkner, D. J. *J. Org. Chem.* **1978**, *43*, 3454-3457.

(10) Stierle, D. B.; Faulkner, D. J. *J. Org. Chem.* **1980**, *45*, 3396-3401.

(11) Davidson, B. S. *Tetrahedron Lett.* **1991**, *32*, 7167-7170.

(12) Horton, P. A.; Longley, R. E.; Kelly-Borges, M.; McConnell, O. J.; Ballas, L. M. *J. Nat. Prod.* **1994**, *57*, 1374-1381.

(13) Phillipson, D. W.; Rinehart, K. L. *J. Am. Chem. Soc.* **1983**, *105*, 7735.

(14) Rudi, A.; Talpir, R.; Kashman, Y.; Benayahu, Y.; Schleyer, M. *J. Nat. Prod.* **1993**, *56*, 2178-2182.

(15) Kobayashi, J.; Naitoh, K.; Sasaki, T.; Shigemori, H. *J. Org. Chem.* **1992**, *57*, 5773-5776.

(16) Tsukamoto, S.; Takeuchi, S.; Ishibashi, M.; Kobayashi, J. *J. Org. Chem.* **1992**, *57*, 5255-5264.

(17) Takeuchi, S.; Ishibashi, M.; Kobayashi, J. *J. Org. Chem.* **1994**, *59*, 3712-3713.

(18) Kashman, Y.; Rotem, M. *Tetrahedron Lett.* **1979**, 1707-1708.

(19) Albericci, M.; Collart-Lempereur, M.; Braekman, J. C.; Daloze, D.; Tursch, J. P.; Declerq, J. P.; Germain, G.; van Meerssche, M. *Tetrahedron Lett.* **1979**, 2687-2690.

(20) Capon, R. J.; MacLeod, J. K.; Willis, A. C. *J. Org. Chem.* **1987**, *52*, 339-342.

(21) Capon, R. J.; MacLeod, J. K. *Tetrahedron* **1985**, *41*, 3391-3404.

(22) Manes, L. V.; Bakus, G. J.; Crews, P. *Tetrahedron Lett.* **1984**, *25*, 931-934.

(23) Capon, R. J. In *Studies in Natural Products Chemistry*; Atta-ur-Rahman, Ed.; Elsevier: Amsterdam, 1991; Vol. 9, pp. 15-33.

(24) Capon, R. J.; Macleod, J. K. *Tetrahedron* **1985**, *41*, 3391-3404.

(25) Gunasekera, S. P.; Gunasekera, M.; Gunawardana, G. P.; McCarthy, P.; Burres, N. *J. Nat. Prod.* **1990**, *54*, 1451-1454.

(26) Quinoa, E.; Kho, E.; Manes, L. V.; Crews, P.; Bakus, G. J. *Tetrahedron Lett.* **1986**, *51*, 4260-4264.

(27) Scharz, H.; Schiebel, H.-M. In *The Chemistry of Peroxides*; Patai, S., Ed.; John Wiley & Sons: New York, 1983, p. 119.

(28) Crews, P.; Jimenez, C.; O'Neil-Johnson, M. *Tetradedron* **1991**, *47*, 3585.

(29) Dale, J. A.; Mosher, H. S. *J. Am. Chem. Soc.* **1973**, *95*, 512.

(30) Trost, B. M.; Curran, D. P. *Tetrahedron Lett.* **1981**, *49*, 4929.

(31) Trost, B. M.; Belletire, J. L.; Godleski, S.; McDougal, P. G.; Balkovec, J. M.; Baldwin, J. J.; Christy, M. E.; Ponticello, G. S.; Varga, S. L.; Springer, J. P. *J. Org. Chem.* **1986**, *51*, 2370.

(32) Ichiba, T.; Scheuer, P. J.; Kelly-Borges, M. *Tetrahedron* **1995**, *51*, 12195-12202.

(33) Look, M. P.; Musch, E. *Chemotherapy* **1994**, *40*, 8-15.

(34) Sperry, S., personal communication, 1996

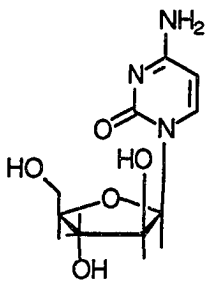
CHAPTER THREE

Hipposulfate, a Matrix Metalloproteinase Inhibitor from a *Hippospongia* sp. Sponge

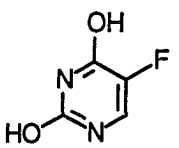
INTRODUCTION

Two complementary strategies have been employed to search for anticancer chemotherapeutics. As discussed in chapter one the use of *in vivo* testing of extracts on cancer cell lines has brought forward the development of anticancer agents by a mechanism blind route.[1] Recently there has been a growing use of *in vitro* enzyme based assays to discover potential anticancer chemotherapeutics with specific mechanisms of action.[2,3]

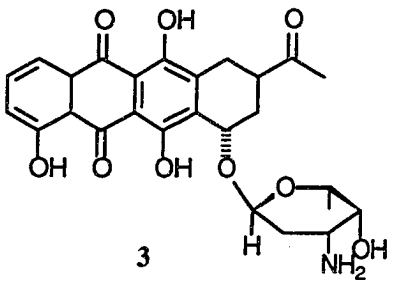
The mechanism of action based assays exploit the heightened events of cell replication and division in tumor cells compared to normal cells. For cell division to proceed it is essential DNA replication take place. Antitumor agents targeting several different aspects of DNA replication have been discovered. The effectiveness of DNA replication is reduced by both DNA analogs, such as ara-C (**1**)[4] and 5-FU (**2**),[5] and DNA intercalators, such as daunomycin (**3**).[6] Alkylation of DNA by cis platin (**4**)[7] prevents the replication of tumor cells.



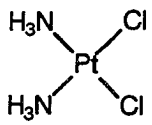
1
ara - C



2
5-FU

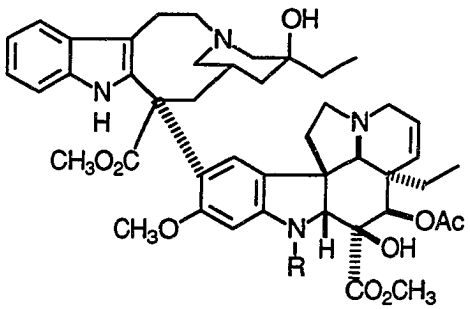


3
daunomycin



4
cis-platin

In addition to attacking the DNA of cancer cells, other compounds exert influence on the tubulin polymers that make up the internal skeleton of cells. Both vincristine (**5**) and vinblastine (**6**) prevent the polymerization of tubulin in order to hamper the correct formation of a skeleton and reduce the mitotic spindle necessary for cell division.[8] By an alternative mechanism taxol (**7**) stabilizes the tubulin skeleton to inhibit tumor growth.[9-12]



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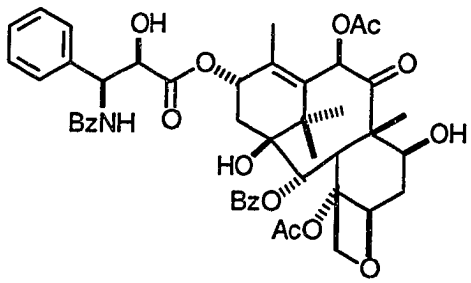
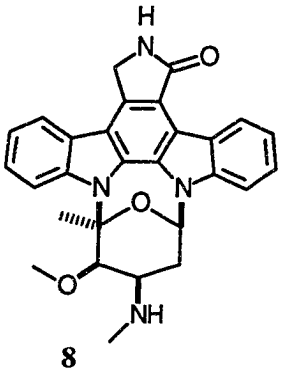
6 vinblastine R = CHO



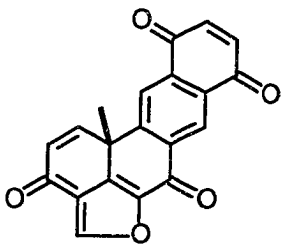
7
taxol

Beyond effecting the ability of tumor cells to reproduce by interfering with DNA replication or the integrity of the tubulin skeleton, new advances have concentrated on the regulation of gene expression necessary for cell replication. The signal transduction pathways containing protein kinase C and tyrosine kinase are important for the regulation of oncogenes. Inhibition of protein kinase C and tyrosine kinase is desired to regulate these over expressed enzymes in cancer cells. Staurosporine (**8**) is the best inhibitor found to date for either enzyme.[13,14]

The marine natural product halenaquinone (**9**) and its derivatives have been found to be inhibitors of tyrosine kinase.[15]



8
staurosporine



9
halenaquinone

Inhibitors of the enzyme targets discussed above are effective for the control of tumor growth, however none of these compounds directly address the spread of tumors from one organ to another. This spread of cancer is termed metastasis. In order for cancer cells to spread throughout the body several events must take place. The tumor cells growing in an organ break through the epithelial cells separating the organ from the adjacent connective and vascular tissue. Some of the tumor cells enter nearby capillaries and eventually arrive at a distant location. At some point in the circulatory system the dislodged cancer cells will attach and breach the vascular tissue. Once they are in the new connective tissue outside of the vascular system these cancer cells may begin to replicate and establish a new tumor site, Figure 3.1.

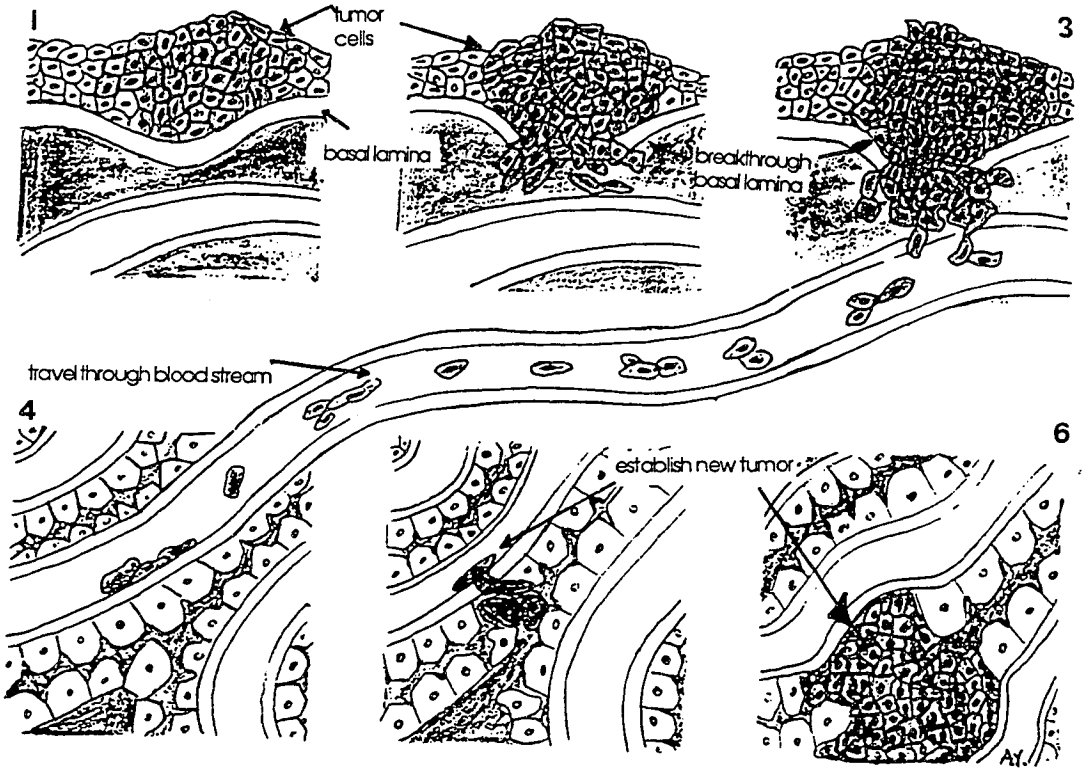


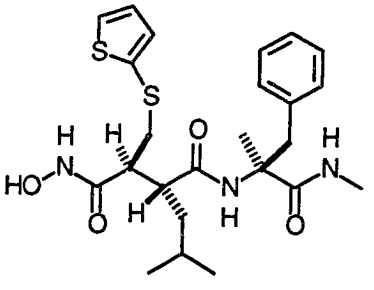
Figure 3.1. Sequence of events of metastasis.

The initial movement of the cancer cells through the basal lamina of the epithelial layer is by action of a group of enzymes called matrix metalloproteinases (MMP). These enzymes are essential in healthy individuals for the breakdown of old tissue to allow the growth of new tissue. However in the case of a metastisizing tumor these enzymes are used to digest healthy tissue for the benefit of the cancer cells. Normal regulation of MMPs are through the action of a group of peptides called tissue inhibitors of matrix metalloproteinases (TIMPS). However when MMPs are employed by cancer cells for metastasis this control is lacking. There are approximately one dozen matrix metalloproteinases currently identified and this number is growing.[16] The MMPs were originally named after the tissue substrates, i.e. collagenase and gelatinase, however, a new nomenclature is in development to provide a consistent series of names, i.e. MMP-1, MMP-2.

Their activity is dependent upon a zinc atom in the active site. Besides cancer, uncontrolled MMPs have been implicated in periodontal disease, rheumatoid arthritis and other degenerative diseases.[17]

A strategy to prevent the spread of cancer is to locate a specific inhibitor of matrix metalloproteinases. Unlike the other mechanism based assays which are aimed at halting or reducing tumor growth the MMP assay will lead to therapeutics for the reduction of tumor metastasis.

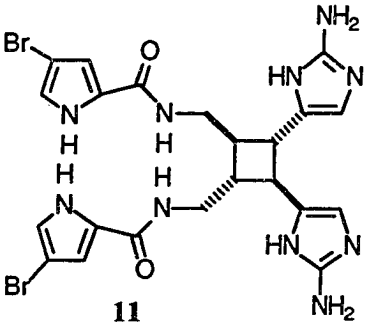
While MMP enzymes are regulated by TIMPS, there is a dearth of chemotherapeutic agents to inhibit matrix metalloproteinases. Only a few inhibitors of MMPs are reported in the literature. As the MMP enzymes are proteisases there are several peptide analogs able to inhibit MMPs. The presence of a hydroxamic acid moiety seems to be responsible for activity in a number of leads for the development of broad spectrum MMP inhibitors.[17] The modified dipeptide batimastat (**10**) is the only agent in clinical trials for use as an MMP inhibitor.[18] Although batimastat (**10**) has the ability to inhibit a wide range of MMPs, it contains an hydroxamic acid unit which may be cleaved to reduce activity.



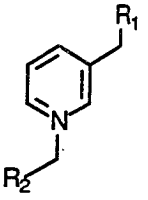
10
batimastat

A series of tetracycline analogs are also in development as MMP inhibitors.[19] The antibiotic effect of the tetracyclines is reduced in the maximization of the MMP inhibitor activity. Several marine natural products have MMP inhibitory activity including sceptrin (**11**), a brominated alkaloid from the sponge *Agelas sceptra*,[20] halenaquinone (**9**),[21] halitoxin (**12**),[22]

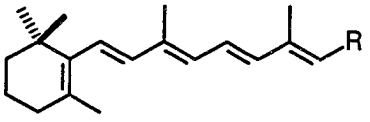
retinol (**13**)[23] and retinoic acid (**14**).[23] Suvanine (**15**), the gaunidium salt of a sulfated terpene from a *Coscinoderma* species was also found to inhibit MMPs .[24]



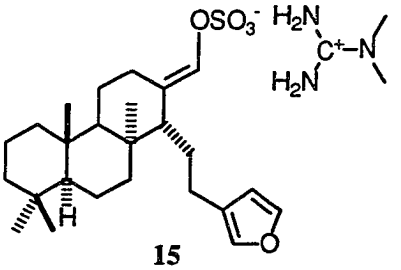
11
sceptrin



12
halitoxin, R_1, R_2 = self polymerizing



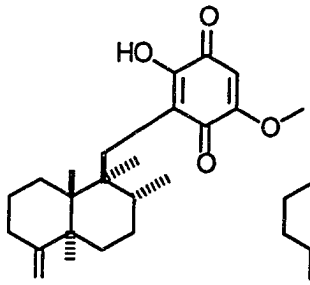
13 retinol R= OH
14 retinoic Acid R = COOH



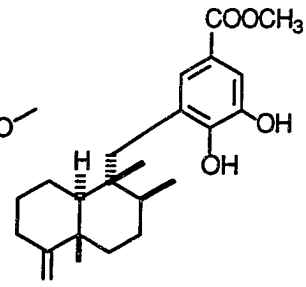
15
suvanine

At the initiation of this project there were no anti MMP agents in clinical trials. With only batimastat (**10**) currently in clinical trials there exists a critical need for novel MMP inhibitors to prevent the spread of cancer. A crude partition of a *Hippospongia* species, collection # 90176, displayed activity in tyrosine kinase and MMP assays performed by Syntex Co. The observed matrix metalloproteinase inhibitory activity became the impetus to examine this collection of *Hippospongia* species.

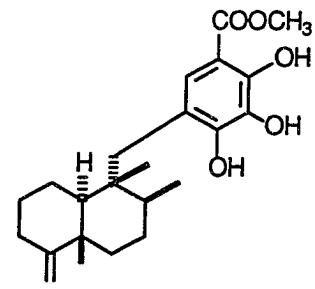
The genus *Hippospongia* is in the class Dictyoceratida and, as is typical of Dictyoceratid sponges, *Hippospongia* species produce several varieties of terpenes. These terpene natural products may be divided into five groups. One of the first compounds isolated from a *Hippospongia* species was the sesquiterpene ilimaquinone (**16**).[25] The related antipodal dictyoceratins (**17-18**)[26] were isolated later.



16
ilimaquinone

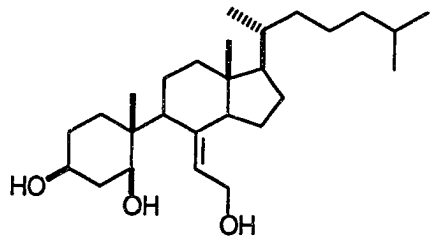


17
dictyoceratin A

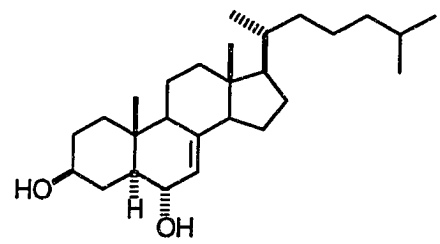


18
dictyoceratin B

A very large family of triterpenes[27] and secosterols[28] have also been discovered from *Hippospongia communis*. Both are large families of compounds with variations in the sidechains of the tetracyclic or cleaved tetracyclic core. The secosterol (**19**) and sterol (**20**) structures shown below are representatives of the compound families.

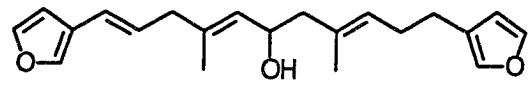


19
5, 6 secosterols 1



20
3β, 6α–dihydroxysterol 1

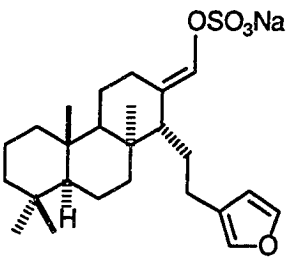
A series of linear furanosesquiterpenes was also isolated from a *Hippospongia* species and is represented by the untenospongine (**21**).[29-32]



21
untenospongine

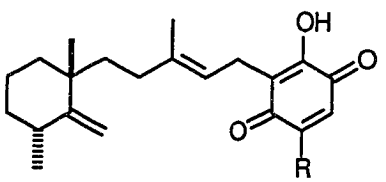
The sodium salt of suvanine (**22**) has been reported from a *Hippospongia* species.[33] As mentioned above the gaunidium salt of suvanine (**15**) was previously isolated from a

Coscinoderma species.[24] Both *Hippospongia* and *Coscinoderma* are related members of the family Spongiidae. This is the only report of a sulfated terpene from a *Hippospongia* species.



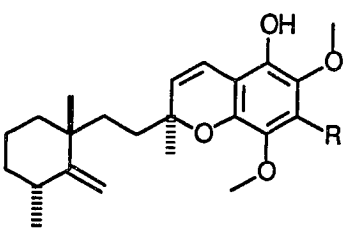
22
suvanine sodium salt

The metachromins (**23-30**), a series of eight benzoquinone bearing sesquiterpenes were isolated from *Hipposongia metachromia.* The metachromins demonstrate various quinol and quinone oxidation levels of the aromatic ring as well as alkylation and alkyl amination of the quinol containing structures.[34-36]

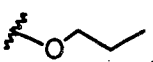


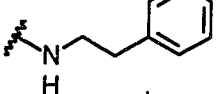
23 metachromin A R = OCH_3

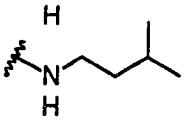
25 metachromin C R = H

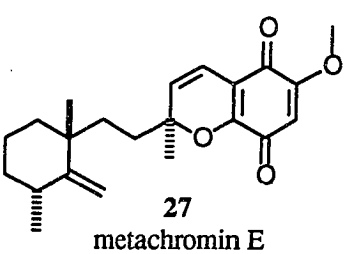


24 metachromin B R = H
26 metachromin D R = OCH_3

28 metachromin F R = 

29 metachromin G R = 

30 metachromin H R = 



27
metachromin E

The compounds from *Hippospongia* species may be summarized as sesqui-, di-, and triterpenes. They often have an aromatic moiety, either furan or benzoqiunone, attached to the

terpene which adds further complexity to the structure. The benzoquinones have been observed in several different oxidation states. There has been only one report of a sulfated terpene from a *Hippospongia* species.[33] This trend of benzoquinone containing and sulfated terpenes is continued in the examination of collection number 90176, *Hippospongia* sp.

RESULTS

Isolation of hipposulfate (31)

A *Hippospongia* sp. sponge, collection # 90176, Plate 3.1, was extracted for 24 hours with methanol three times. A laboratory technician fractionated the crude polar extract by the solvent-solvent partition scheme shown in Figure 3.2. The polar FM fraction displayed potent activity, IC_{50} = 27 µg/mL, in the matrix metalloproteinase assay. Due the scarcity of active extracts in this assay, purification of the active component of the FM partition was pursued.

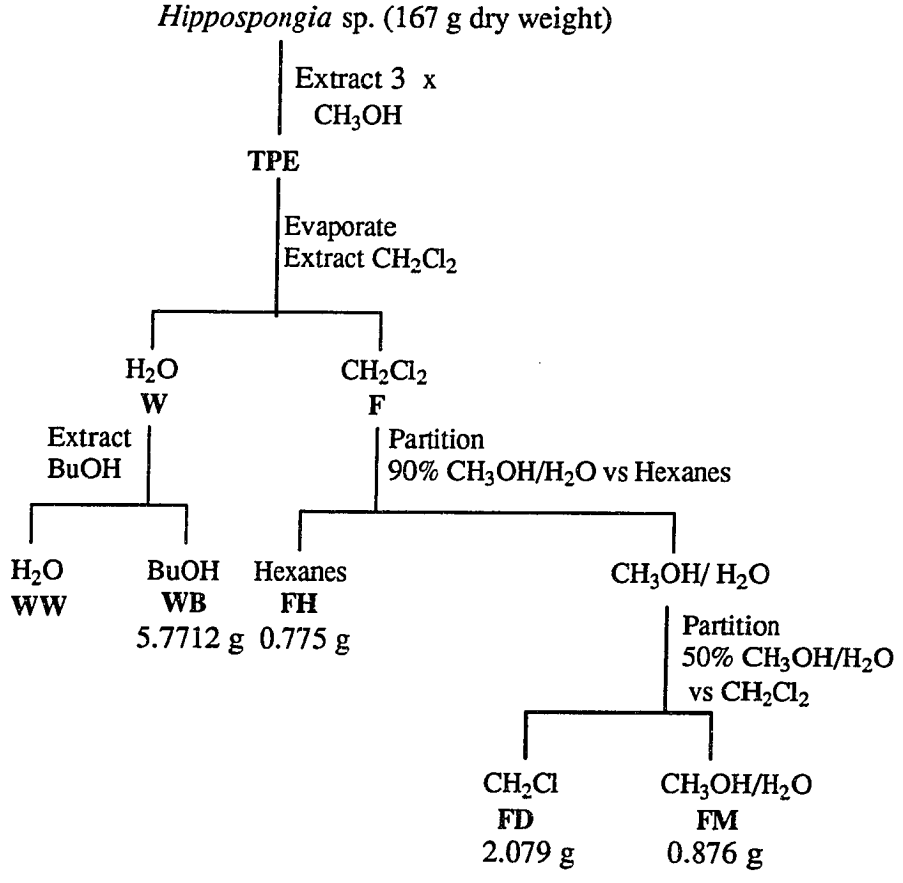
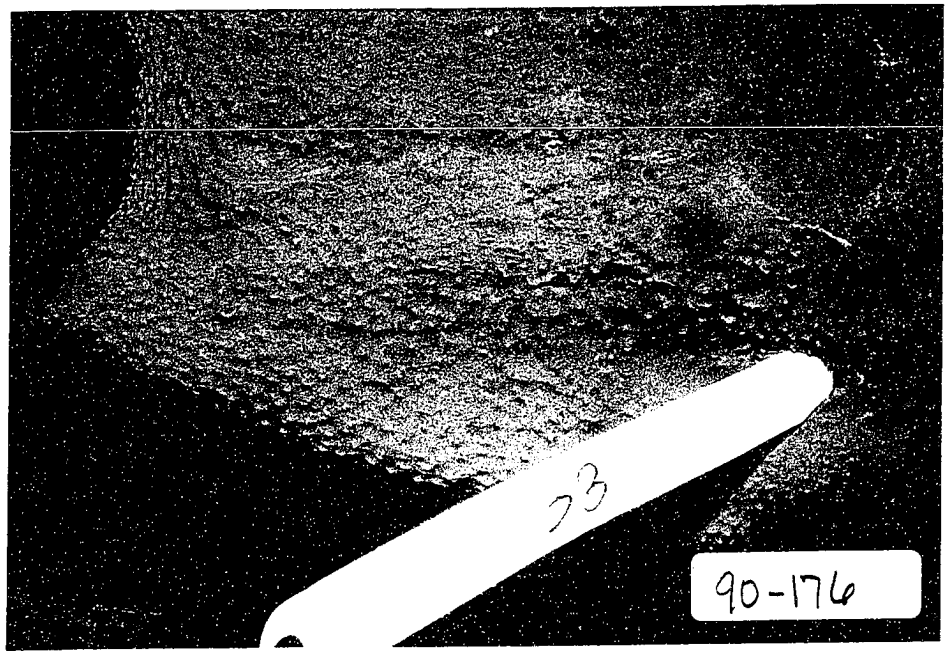


Figure 3.2. Solvent/Solvent extraction scheme for *Hippospongia* sp. (collection # 90176).

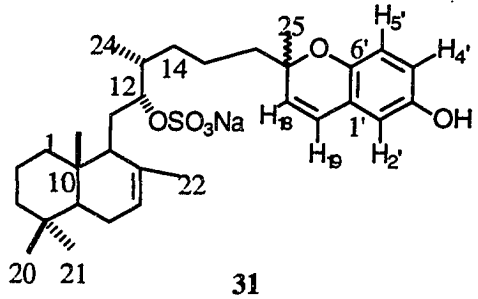
Plate 3.1. Underwater photograph of *Hippospongia* sp., collection # 90176.



A portion of the FM fraction was applied to a Sephadex LH-20 column for a size exclusion separation. The separation was monitored by TLC and column fractions with equal R_f values were combined. As indicated in Figure 3.3 the separation was very poor with the vast majority of the material and significant MMP inhibitory activity in Sephadex fraction S2. This material served as the starting point for three different attempts to locate the active compound. First a gradient normal phase silica flash was used to concentrate most of the activity in fraction F(24-37). Reverse phase octadecyl silane HPLC 60:40, methanol:water resulted in poor separation of this material. Preparative TLC of the largest, most promising fraction, H9, led to the isolation of contaminant phthalates.

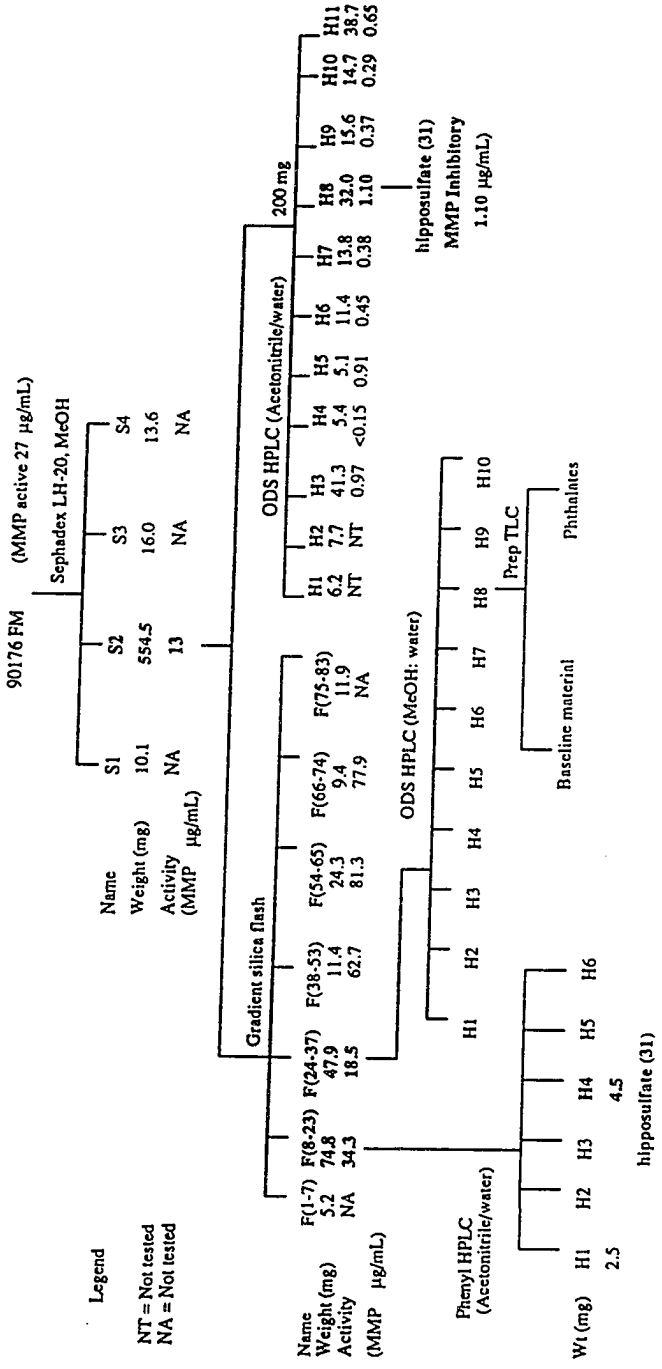
A second attempt was made using Sephadex fraction S2. Instead of methanol:water as the solvent mixture acetonitrile:water, 55:45 was used. Two columns in tandem gave broad but almost acceptable resolution. Partial structure determination was begun at this point and a sulfate group was suspected. It was decided that converting the sulfate to an alcohol would simplify purification of the mixture. While a reaction to solvolyze the sulfate was in progress a third separation was tried.

Fraction F(8-23) was chromatographed on a phenyl bonded reverse phase HPLC column. This provided sufficient resolution to purify hipposulfate (**31**) for the structure determination.



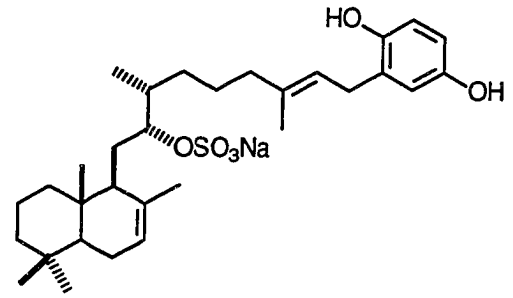
31
hipposulfate

Figure 3.3. Chromatographic separation scheme for the isolation of hipposulfate (**31**).

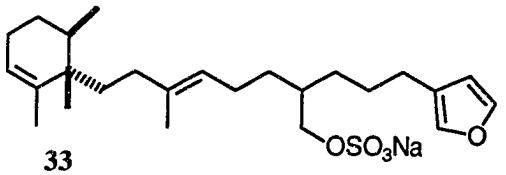


Structure elucidation of hipposulfate (31)

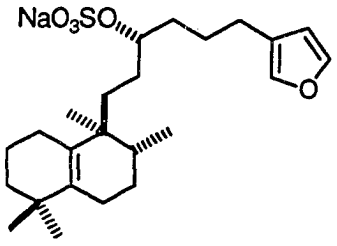
The structure elucidation of hipposulfate (**31**) began with analysis of the ^{1}H and ^{13}C NMR spectra, Figures 3.4 and 3.5. The ^{13}C NMR peak at 85 ppm suggested there may be a sulfate group attached. A double doublet splitting pattern at 5.55 ppm in the ^{1}H NMR spectrum implied the presence of a vinylic ABX spin system, Figure 3.4, although this later proved to be incorrect. The ABX spin system and sulfate functionality were used as criteria in a substructure search on the proprietary Chembase sponge natural products database in the Crews' group. Six compounds (**32-37**) matched the search criteria. Further examination of the ^{1}H and ^{13}C NMR spectra of hipposulfate (**31**) indicated that halisulfate 1 (**32**) was a very close match to hipposulfate (**31**), Table 3.1.[37]



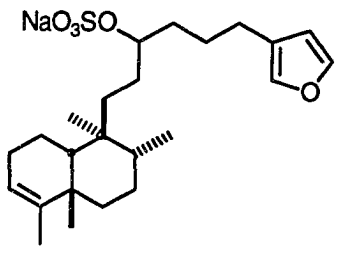
32
halisulfate 1



33
halisulfate 2



34
halisulfate 3



35
halisulfate 4

Figure 3.4. ^{1}H NMR spectrum of hipposulfate (**31**), 250 MHz, CD_3OD.

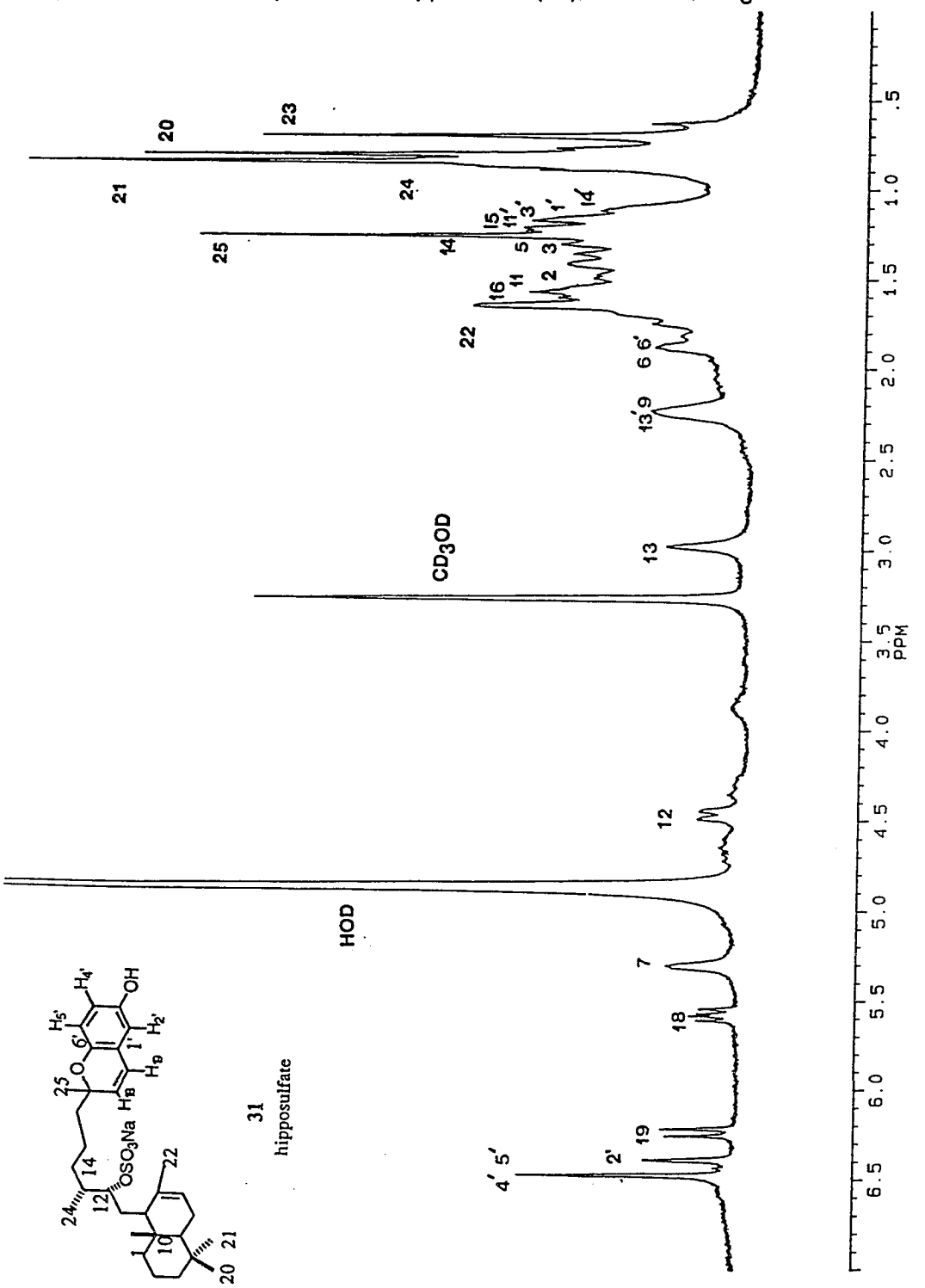
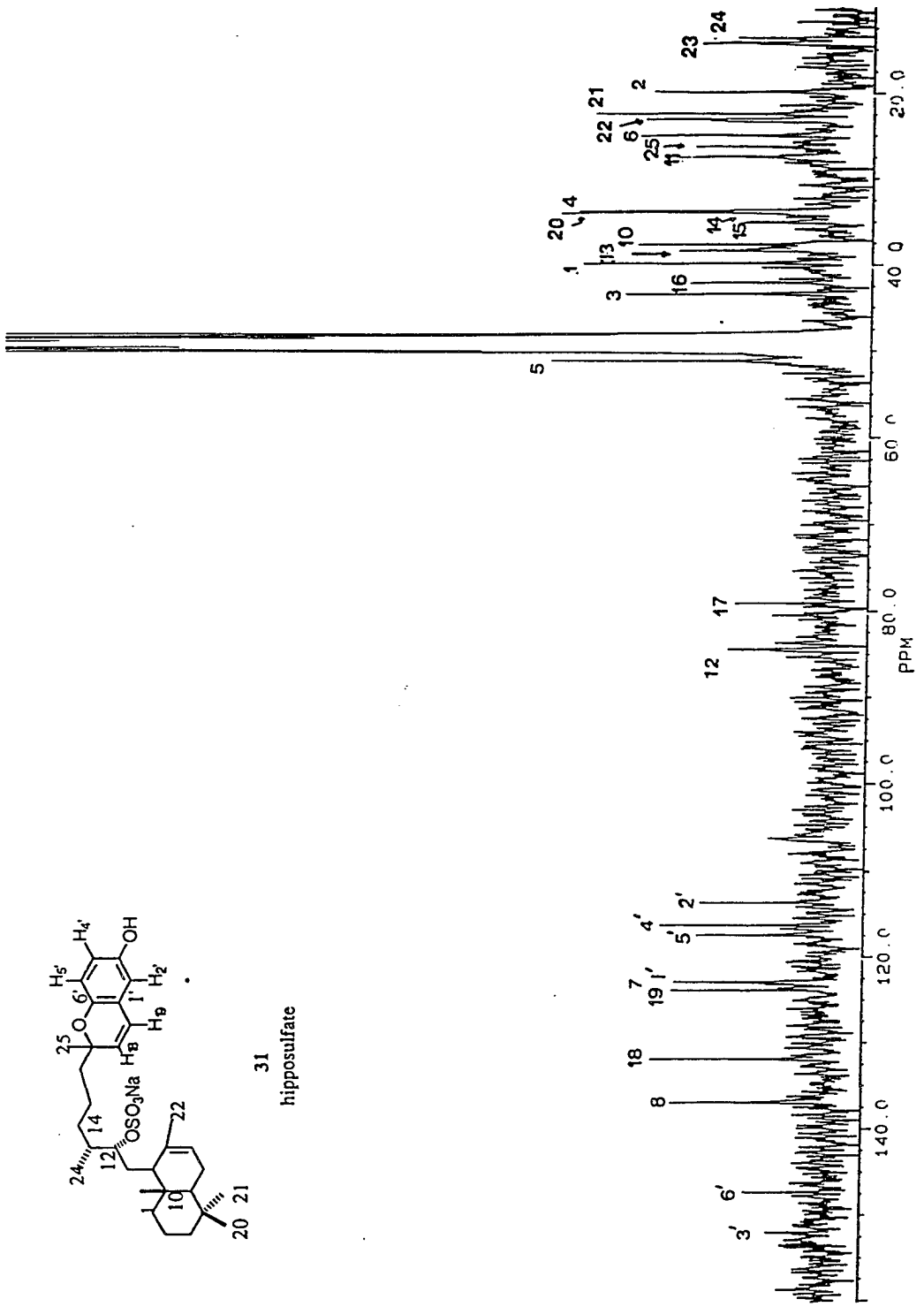


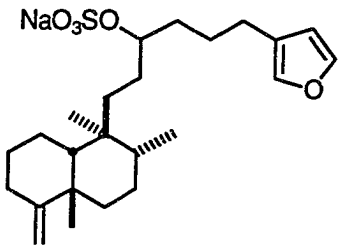
Figure 3.5. ^{13}C NMR spectrum of hipposulfate (**31**), 62.9 MHz, CD_3OD.



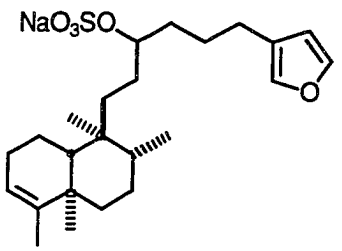
31
hipposulfate

Table 3.1. NMR data of hipposulfate (**31**) and selected NMR data of halisulfate 1 (**32**).

	NMR Data for hipposulfate (**31**)		NMR Data for halisulfate 1 (**32**)	
Atom	^{13}C NMR δ, mult	^{1}H NMR δ, mult, (*J* Hz)	^{13}C NMR, δ	^{1}H NMR δ
1	39.68, t	1.73, m & 1.15, m	39.8	1.70, 1.23
2	19.72, t	1.43, m	19.9	1.43
3	43.27, t	1.35, m & 1.16, m	43.3	1.47 & 1.08
4	33.57, s		33.6	
5	50.99, d	1.24, m	52.0	1.24
6	24.80, t	1.88, m	24.9	1.93
7	122.77, d	5.29, br m	123.9	5.29
8	136.83, s		136.8	
9	49.40, d	2.23, m	49.3	2.23
10	37.48, s		37.4	
11	27.34, t	1.60, m	27.7	1.60
12	84.34, d	4.47, m	85.7	4.48
13	38.03, d	2.98, m & 2.26, m	38.2	2.30
14	34.36, t	1.31, m & 1.13, m	34.3	1.33
15	34.96, t	1.25, m	27.4	1.38
16	41.96, t	1.60, m	41.0	2.00
17	78.99, s		136.7	
18	131.80, d	5.58 / 5.61, dd, (6.6, 9.8)	124.8	5.29
19	123.26, d	6.22, d, (9.8)	29.2	3.16
20	33.57, q	0.78, s	33.7	0.78
21	22.25, q	0.86, s	22.5	0.86
22	22.90, q	1.67, m	23.0	1.67
23	14.00, q	0.70, s	14.2	0.70
24	13.53, q	0.91, d	13.7	0.91
25	26.18, q	1.25, s	16.3	1.67
1'	122.77, s		130.1	
2'	150.96, s		148.6	
3'	116.16, d	6.58, s	117.5	6.56
4'	113.53, d	6.37, s	114.8	6.42
5'	152.00, s		150.8	
6'	117.31, d	6.48, s	118.0	6.60



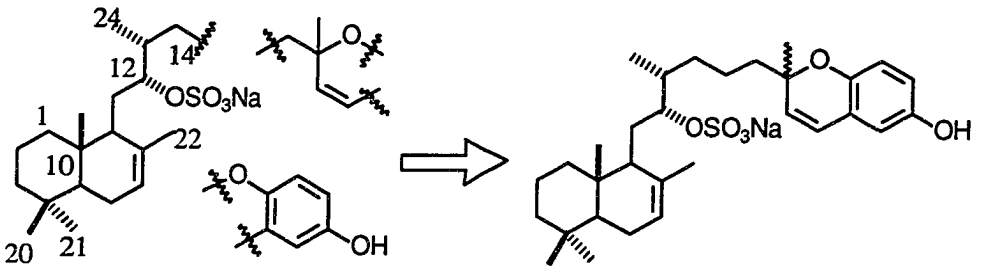
36
halisulfate 5



37
halisulfate 6

The sulfated sesterterpene halisulfate 1 (**32**) was isolated from an unspecified sponge of the family Halichondriidae. Halisulfate 1 (**32**) displayed activity against *Staphylococcus aureus*, *Candida albicans*, *Bacillus subtilis*, inhibited cell division of fertilized sea urchin eggs and inhibited the enzyme phospholipase A. While the other halisulfates 2-7 (**33-37**) were active in a few selected assays none of them possessed the broad spectrum of activity of halisulfate 1 (**32**).

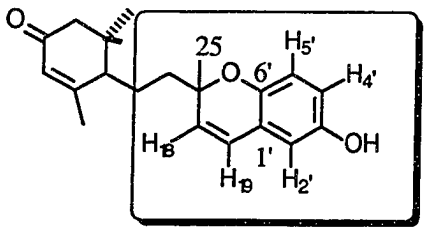
It was apparent from the ^{1}H and ^{13}C NMR shifts reported for **32** that the terpene part of both halisulfate 1 (**32**) and hipposulfate (**31**) was identical, Table 3.1. The difference between **31** and **32** was in the aromatic moiety. Peak matching of the terpene portion of the molecule and analysis of the ^{1}H-^{1}H COSY spectrum resulted in the proposal of three substructures, Figure 3.6. Connection of these substructures provided a tentative structure for hipposulfate (**31**). This structure was also supported by comparison to the NMR data of cyclorenierins A (**38**) and B (**39**), Table 3.2. A close match was found between the hydroquinone portions of hipposulfate (**31**) and the cyclorenierins (**38-39**) .[38]



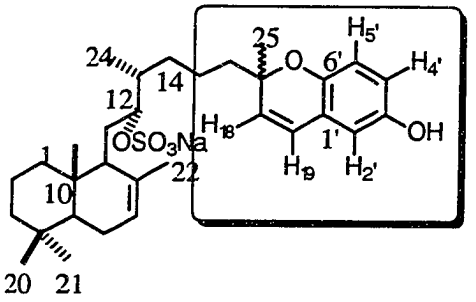
Substructures

Tentative structure of hipposulfate (**31**)

Figure 3.6. Halisulfate 1 (**32**) derived substructures for tentative structure of hipposulfate (**31**).



38 cyclorenierin A methyl 25 up.
39 cylcorenierin B methyl 25 down



31
hipposulfate

Table 3.2. Partial NMR data for cyclorenierins A and B (**38-39**) and hipposulfate (**31**).

	NMR data for cyclorenierin A and B (**38-39**)		NMR Data for hipposulfate (**31**)	
Atom	^{13}C NMR, δ	^{1}H NMR, δ	^{13}C NMR, δ	^{1}H NMR, δ
1'	121.80 /121.75		122.0	
2'	113.10	6.29	113.5	6.37
3'	149.60		152.0	
4'	115.70	6.58	116.2	6.46
5'	116.77 /116.72	6.62	117.3	6.46
6'	146.50 /146.41		147.7	
17	78.16 / 78.04		79.0	
18	130.37 /130.32	5.53 / 5.52	131.8	5.85 / 5.61
19	123.40 /123.32	6.29 / 6.30	123.8	6.22
20	26.20 / 26.08	1.32	26.2	1.25

To provide direct proof for the structure proposed, the HMBC correlations of the hydroquinone portion were analyzed and are summarized in Figures 3.7, 3.8, and 3.9. Methyl-25 was used as the anchor point to analyze the hydroquinone ring. A correlation from methyl-25 to C16, C17 and C18 placed methyl-25 on C17. A correlation from methyl-25 to C18 indicated that C17 was the part of the hydroquinone attached to the oxygen. A correlation from H18 to C17 and from H19 to C17 and H19 to C2' indicated C18 and C19 were attached to the hydroquinone ring. The correlation from H19 to C2' provided an anchor point in the aromatic portion of the hydroquinone ring. From H2' correlations were observed to C1', C3', C4' and C6', and C4' also had a correlation to C6'. These correlations along with shift values matching the cyclorenierins determined the structure of the hydroquinone portion of the hipposulfate (**31**).

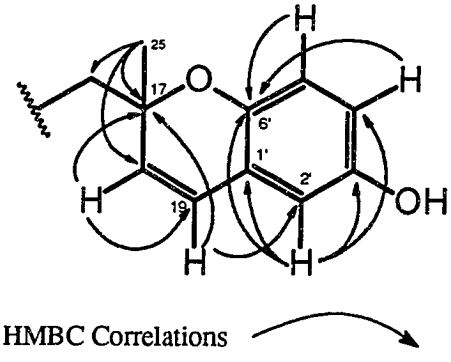


Figure 3.7. HMBC correlations to confirm cyclic hydroquinone moiety of hipposulfate (**31**).

The double doublet at 5.58 of hipposulfate (**31**) could not be accounted for in the structure proposed. The splitting pattern implied coupling to two other nuclei, however there was only one ^{1}H-^{1}H COSY correlation apparent, Figure 3.10. The ^{1}H NMR shift at 5.58 ppm was identified as belonging to H18 by HMQC, Figure 3.11. By HMBC and COSY no other 3 bond vinylic neighbors to H18 other than H19 were observed. Therefore H18 was split for some reason other than an additional coupling. This extra coupling could be accounted for if the stereocenter at C17 was epimerized. This type of peak doubling was observed in the ^{13}C and ^{1}H NMR spectra of the cyclorenierins A and B (**38-39**), Table 3.2.

Figure 3.8. HMBC spectrum of hipposulfate (**31**), expansion of correlations to methyl 25, 500 MHz, CD_3OD.

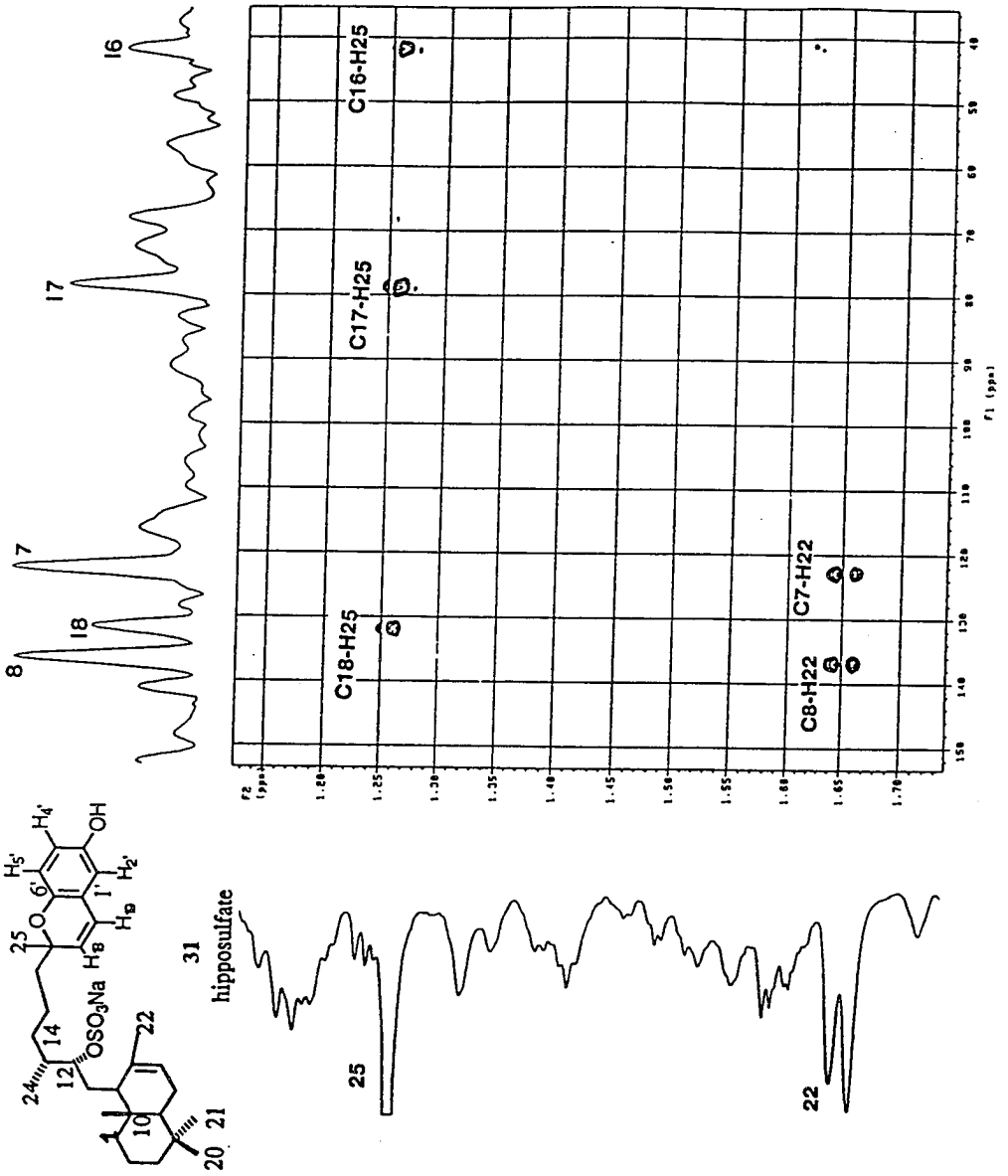


Figure 3.9. HMBC spectrum of hipposulfate (**31**), expansion of correlations to cyclic hydroquinone moiety, 500 MHz, CD_3OD.

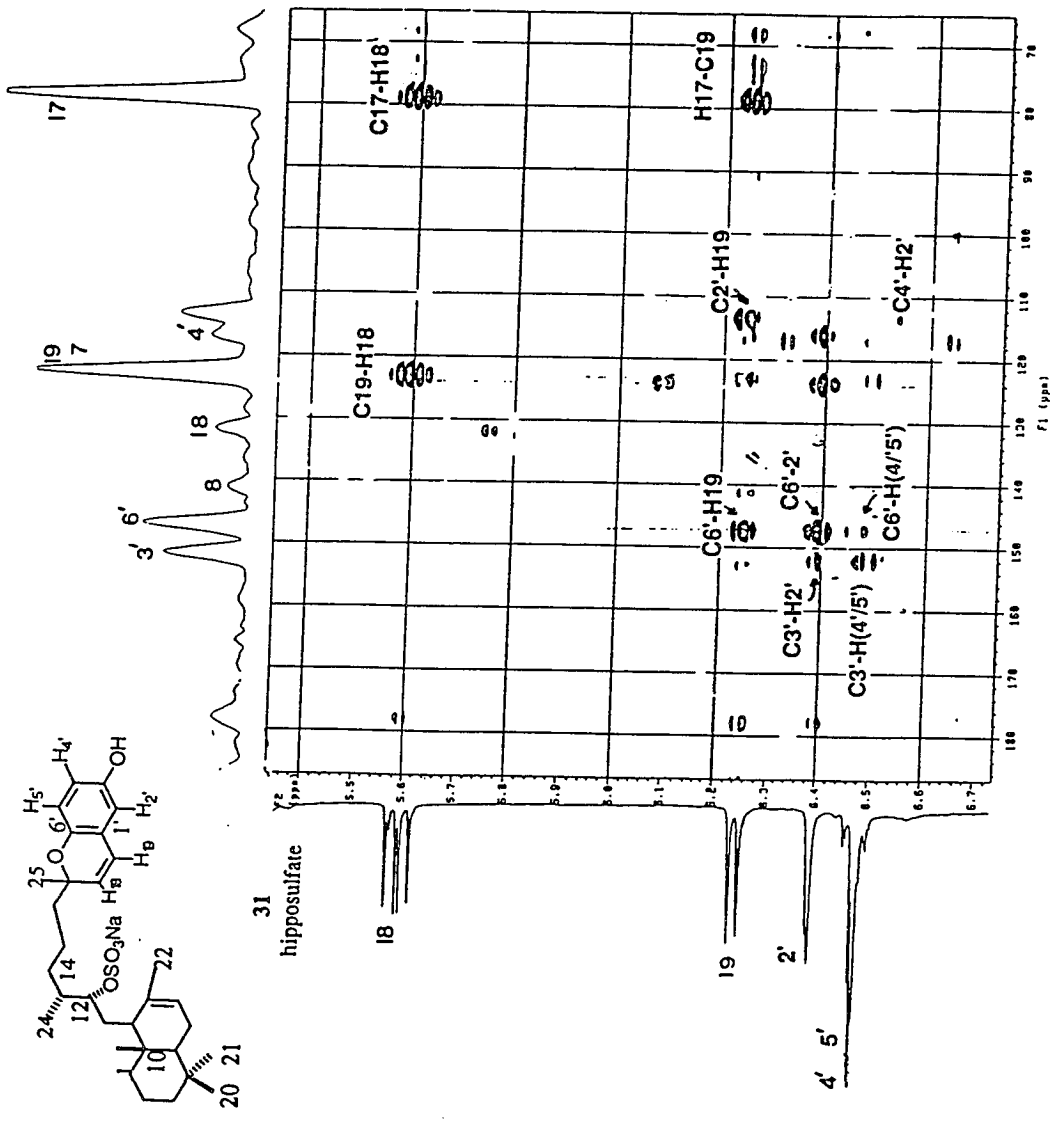


Figure 3.10. ^{1}H-^{1}H COSY spectrum of hipposulfate (**31**), 250 MHz, CD_3OD.

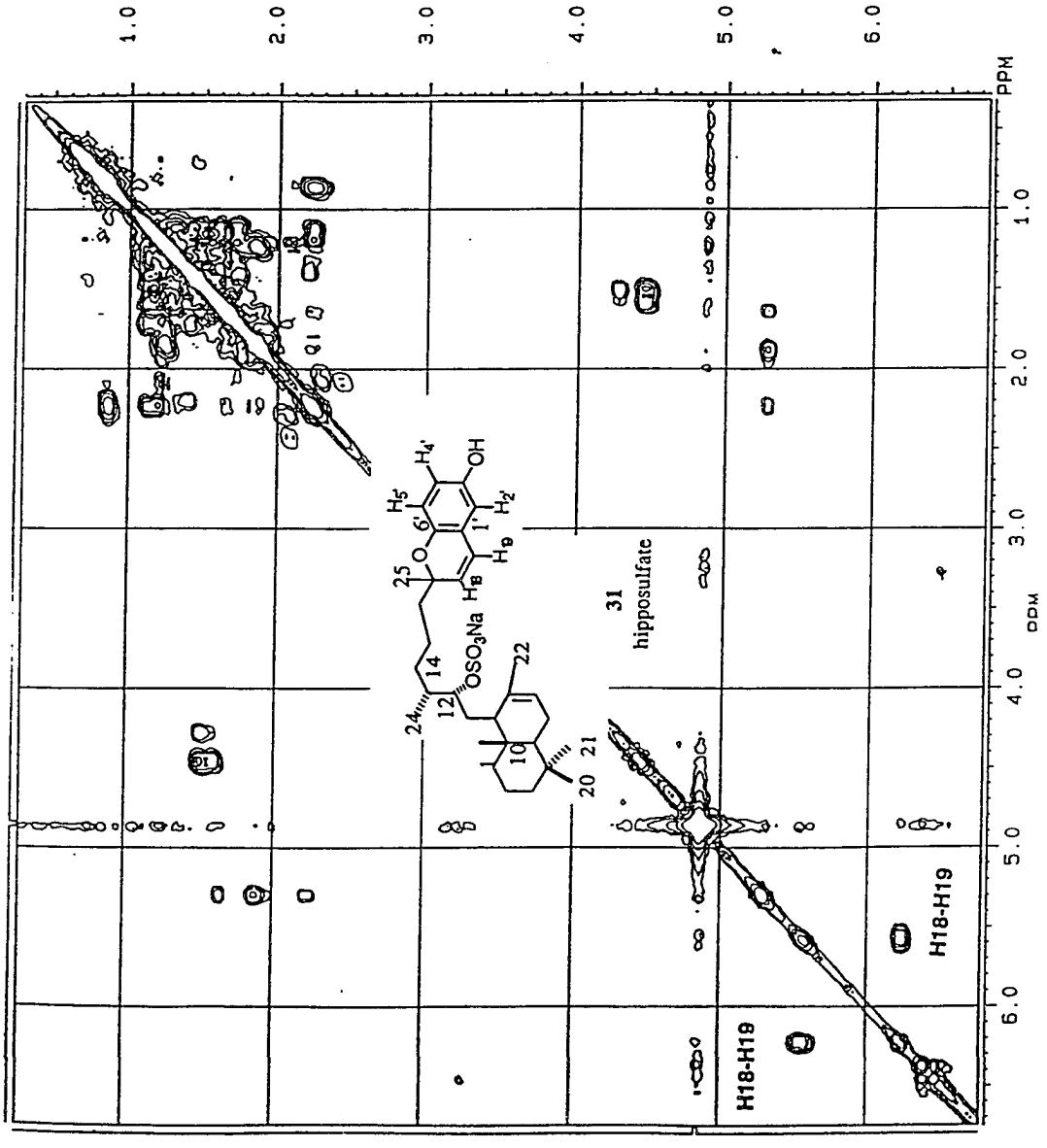
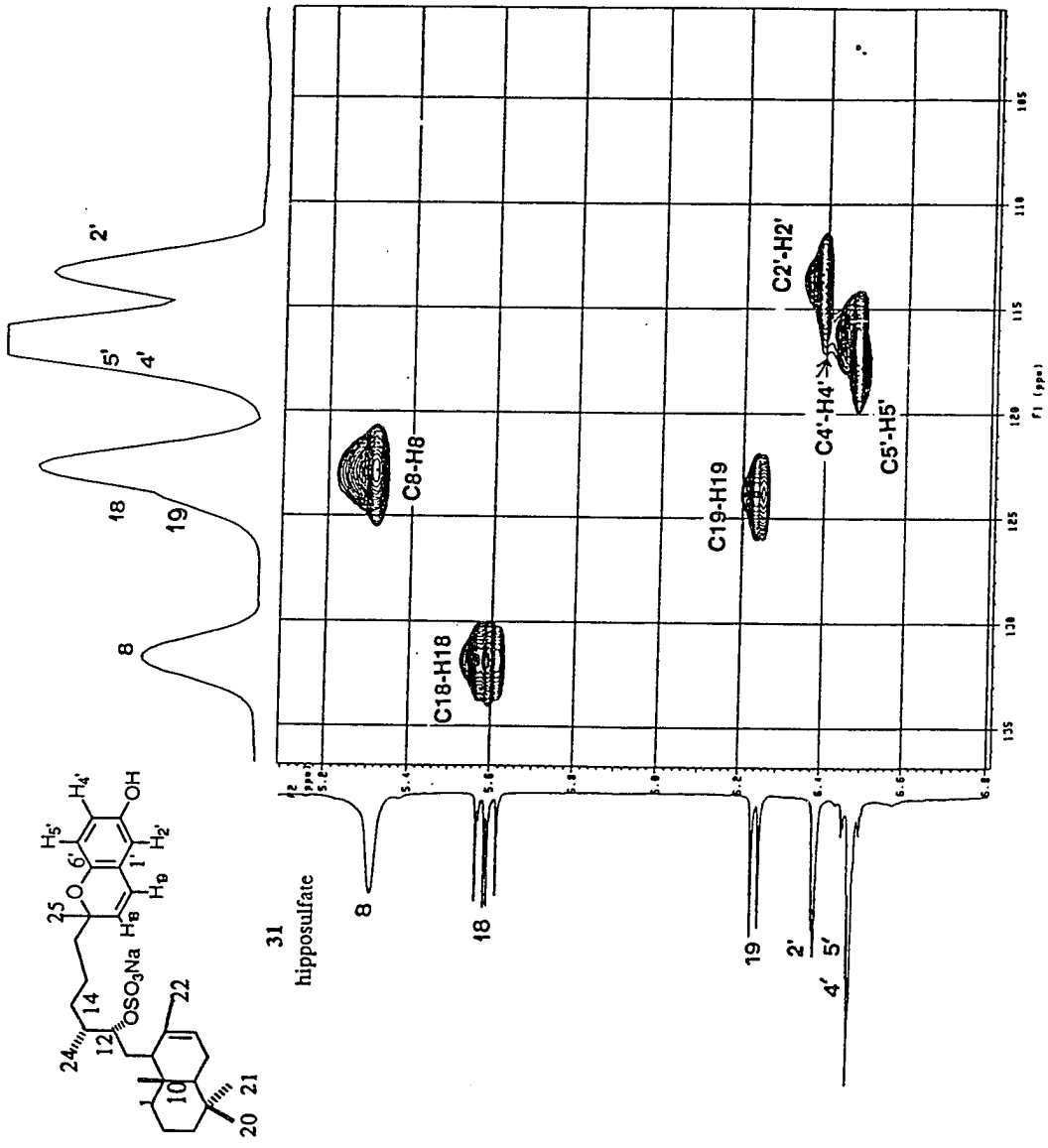
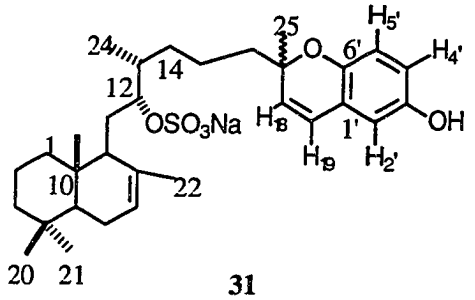


Figure 3.11. HMQC spectrum of hipposulfate (**31**), 500 MHz, CD_3OD.



The stereochemistry of the terpene portion of hipposulfate (**31**) was determined to be the same as the stereochemistry of halisulfate 1 (**32**) due to the very close match in of the ^{1}H and ^{13}C NMR shifts of the terpene moieties of both compounds, Table 3.1. If the terpene portion of hipposulfate (**31**) and halisulfate 1 (**32**) are diastereomeric than it is expected that there would be discrepancies in some of the ^{1}H or ^{13}C NMR resonances of the terpene portions of these molecules, thus hipposulfate (**31**) is assigned the same relative stereochemistry as halisulfate 1 (**32**).

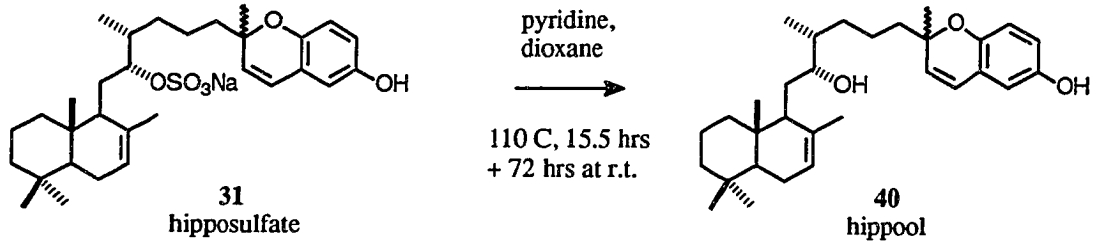


31
hipposulfate

DISCUSSION

The original aim of this project was to identify an MMP inhibitor from the FM solvent solvent partition of the *Hippospongia* sp. (collection # 90176). Isolation of hipposulfate (**31**) IC_{50} = 1.1 μg/mL accomplished this goal. In the HPLC fractions eluting near hipposulfate (**31**) there were other highly MMP inhibitory compounds. After the isolation and structure elucidation of hipposulfate (**31**) it was decided that isolation of the other active compounds was not worthwhile. Examination of the ^1H NMR spectra of these closely eluting fractions indicated that they were further sulfated terpenes similar to hipposulfate (**31**). Although the sulfated sesterterpene hipposulfate (**31**) is active in the MMP assay, sulfates are unfortunately active in a wide variety of enzymes. This may be due to their possessing detergent-like characteristics, The sulfated terpenes, even if MMP inhibitory, will not be developed as drug leads due to the poor enzyme selectivity of sulfated compounds.

The sulfate cleavage[39] product, Scheme 3.1, hippool (**40**) was assayed to determine if the activity of hipposulfate (**31**) was dependent upon the sulfate functionality. A comparison of the MMP activity of hipposulfate (**31**), hippool (**40**), and the simple sulfated compound cycloheptanol sulfate was made. Cycloheptanol sulfate was provided by Mr. Jarrett J. Farias (UCSC). Of these three compounds only hipposulfate (**31**) was active. Both cycloheptanol sulfate and hippool (**40**) did not display any activity at doses as high as 100 μg/mL in the MMP inhibition assay. This indicates that while the sulfate group is essential for the MMP activity of hipposulfate (**31**), there is also a need for the specific carbon framework of hipposulfate (**31**). Hipposulfate (**31**) resembles the guanidium salt of suvanine (**15**), which has been found to have IC_{50} = 27 μg/mL for matrix metalloproteinases. While more data is needed, it is possible that the similar terpene subunits in suvanine (**15**) and hipposulfate (**31**) confer activity against MMPs for these compounds.



31
hipposulfate

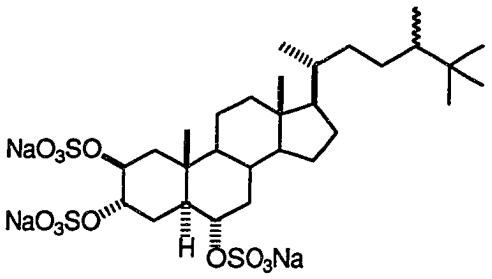
40
hippool

Scheme 3.1. Solvolysis of hipposulfate (**31**).

From a chemotaxonomic perspective hipposulfate (**31**) has some interesting features. The similarity in the structure of hipposulfate (**31**) and halisulfate 1 (**32**) is intriguing as the compounds come from sponges that differ taxonomically at the order level. While mistakes may occur in the taxonomy determination, there are gross differences between the order Dictyoceratida, which contains the family Spongiidae, genus *Hippospongia*, and the order Halichondrida which contains the family Halichondriidae. Dictyoceratid sponges do not contain any spicules while the Halichondriidae have a very well defined spicule skeleton.[40] Both orders of sponges produce terpenes. In this case it appears that both a *Hippospongia* sp. sponge and a sponge from the family Halichondriidae were able to produce the same terpene backbone with the same functionalization.

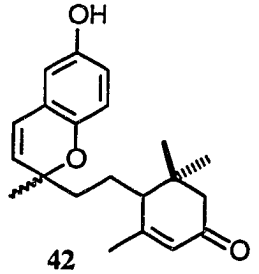
The production of the sesterterpene sulfates is not unprecedented in *Hippospongia*. Suvanine has been reported from the genera *Hippospongia* and *Coscinoderma* in the family Spongiidae. This incorporation of a sulfate moiety and an aromatic ring in suvanine are reminiscent of both hipposulfate (**31**) and halisulfate 1 (**32**). Hipposulfate (**31**) is also similar to the metachromins (**23-30**) in the cyclization of the hydroquinone moiety. It appears hipposulfate (**31**) is the type of compound one would expect from a sponge in the genus *Hippospongia*. While sponges of the family Halichondriidae produce terpenes along with compounds derived from a variety of other biosynthetic pathways, only one other sulfated compound, halistanol sulfate (**41**) is known. Halistanol sulfate (**41**) is a trisulfated steroid produced by *Halichondria* c.f. *moorea*.[41] There are no other reports of sesterterpenoid aromatic compounds similar to the halisulfates 1- 7 (**32-37**). It

appears the isolation of the original halisulfates 1-7 (**32-37**) from the family Halichondriidae is unique and unexpected.



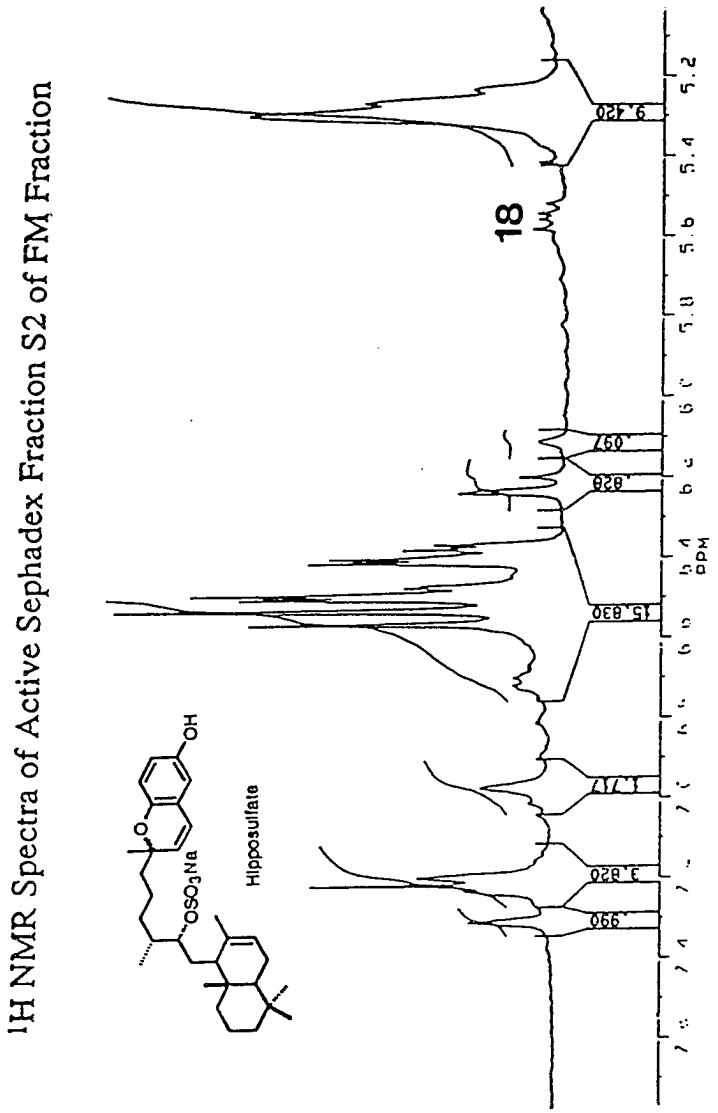
41
halistanol sulfate

The source of the cyclization at C17 in hipposulfate (**31**) may either be enzymatic or it may be an artifact of isolation. In a similar structure, the hydroquinone of panicein A has been cyclized to yield racemic panicein A_2 (**42**).[42] This cyclization was accomplished with K_2CO_3 in refluxing acetone. The crude FM S2 fraction ^1H NMR spectrum of the *Hippospongia* sp. studied shows the doubled peaks of H18, Figure 3.12. The conditions to reach the FM S2 fraction are very mild and consist of the solvents shown in Figure 3.2. The size exclusion column is considered to be completely unreactive.



42
panicein A_2

Figure 3.12. Observation of ^1H NMR signal of H18 double doublet in Sephadex S2 fraction. 250 MHz, CD_3OD.



The epimerization of C17 in hipposulfate (**31**) may be enzymatic or it may have occurred during extraction. The site of epimerization is a vinylogously benzylic, allylic tertiary ether which would form a highly stabilized cation on cleavage of the C17 oxygen bond. The cylcorenierins A and B (**38-39**) also exhibited a similar epimerization of the cyclic hydroquinone attachment position and the doubling of the peaks in the pure cyclorenierins was apparent in the crude solvent partition. The reports of the metachromins B (**24**), D (**26**), and E (**27**) did not mention any epimerization of the cyclic hydroquinones. While there is no conclusive proof, the mildness of the extraction and partition conditions to obtain the FM S2 fraction suggest hipposulfate (**31**) is not an artifact of halisulfate 1 (**32**) and was produced by the sponge.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES —The NMR spectra were recorded at 250, 300 or 500 MHz for ^{1}H and 62.9, 75.5 or 125.7 MHz for ^{13}C. Multiplicities of ^{13}C NMR resonances were determined from APT data, DEPT data, or HMQC experiments (250, 300 and 500 MHz). Low and high resolution fast atom bombardment mass spectrometry data were obtained on a magnetic sector instrument. High performance liquid chromatography was done with 10μ C_{18} silica columns and phenyl bonded reverse phase columns.

COLLECTION AND IDENTIFICATION — The sponge (1.7 kg wet weight), collected from the Solomon Islands at Boia-Boia Waga Is. (collection # 90176) was identified as *Hippospongia* sp. (order Dictyoceratida, family Spongiidae) by Ms. M. C. Diaz (UCSC). A voucher sample (90176) is available from the UCSC archives and a photo is presented in Plate 3.1. The collection was made at a depth of 15 m. Description: shape - massive oblong to fan; color - live specimens were black, dry specimens are dark brown to black; surface - regularly conulose; consistency - compressible; ectosome - non-specialized, but foreign material is concentrated ; choanosome - whorls of wool-like secondary reticle, (fibers 15-30 μm); spicules - none.

ISOLATION OF HIPPOSULFATE (**31**) — The freshly collected sponge was preserved by soaking overnight in a 50/50 mixture of alcohol and water. This solvent solution was discarded and the damp sponge was brought to UCSC at ambient temperatures. A laboratory technician performed the initial extraction and solvent solvent partition of the sponge. The sponge was extracted with methanol for three times 24 hours each time. The methanol from this crude polar extract was evaporated and the residue was resuspended between water and methylene chloride. The water fraction was extracted with sec-butanol while the methylene chloride fraction was evaporated under reduced pressure and the residue resuspended between 10% aqueous methanol and hexanes. The 10% aqueous methanol layer was extracted with hexanes. The 10% aqueous

methanol solution was diluted to form a 40% aqueous layer and this new methanol water solution was extracted with methylene chloride to yield a 2.079 g oil from the methylene chloride layer (FD) and 0.876 g oil in the 40% aqueous methanol layer (FM). This process is summarized in Figure 3.2.

The chromatographic separation of the active compounds in the FM was performed by myself. A portion of the FM fraction was applied to a Sephadex LH-20 column (methanol) for a size exclusion separation. The separation was monitored by TLC and column fractions with equal R_f values were combined. As indicated in Figure 3.3 the separation was very poor with the vast majority of the material in the Sephadex fraction S2. MMP bioassay of the material indicated that the second fraction was the active fraction with an MMP IC_{50} = 13 μg/mL activity level. This material was applied to a normal phase silica flash column eluted with successively more polar solvents starting with 5%:95% methanol:ethyl acetate and ending with 100% methanol. The MMP inhibition assay of these fractions indicated the most active fraction was F(24-37). Several attempts were made to separate this F(24-37) fraction. The first attempt was with a reverse phase HPLC method using octadecyl silane (C18) as the stationary phase and methanol:water as the solvent. Unfortunately this did not lead to a pure compound but the fractions with sufficient material for bioassay were submitted to the MMP assay and fraction H9 possessed an MMP IC_{50} <0.8μg/mL activity. Preparative TLC was used in an attempt to separate this material on normal phase silica with 67%:33% hexanes:ethylacetate as the solvent system, Figure 3.3. Phthalates were identified as one of the bands and it was apparent that the majority of the applied material was left on the baseline. The phthalates were most likely a contaminant from tygone tubing used in the flash column in the previous step.

A second attempt at arriving at a pure compound was made with a portion of the remaining S2 fraction. A 200 mg portion of this material was purified by HPLC with an ODS reverse phase silica column, Figure 3.3. Instead of the methanol:water solvent system used previously, a

55%:45% mixture of acetonitrile:water gave better resolution and peak shape. However with one column the peak shape was very poor. To improve the peak shape a second ODS silica column was added in tandem. While the peak shape was still poor it was adequate for the isolation of the major peak, hipposulfate (**31**), as a fairly pure (~90 %) sample. At this point it was believed a sulfate group was present on the molecule and it was hypothesized that the sulfate group was hampering the separation process. A reaction to cleave the sulfate group from hipposulfate (**31**) was started, as it was believed separation of the resulting alcohols would be easier to purify. As the sulfate cleavage reaction was running more efficient methods for the purification of the crude flash partitions were explored. Reverse phase HPLC of fraction F(8-23) with a phenyl bonded reverse phase column was explored for enhanced separation. Elution with 40:60 acetonitrile:water provided much better resolution and peak shape for hipposulfate (**31**) than the tandem ODS columns. Complete structure elucidation was performed on this pure sample of hipposulfate (**31**).

HIPPOSULFATE (**31**) —A brown waxy solid : $[\alpha]_D$ + 60.74, (c = 0.27, CH_3OH); IR (dry film) 3455. 2956, 1729, 1707, 1640, 1453, 1221, 1056 cm^{-1} UV λ_{max} 300 sh., 280 sh., 230; hrfabms *m/z*: 591.2772 ($[M+Na+Na-H]^+$, $C_{31}H_{45}O_6SNa_2$, Δ 4.0 mmu of calcd); lrfabms (NBA), *m/z* (%): 591 (11), 471 (16), 329 (11), 215 (16), 176 (100); ^1H NMR, ^{13}C NMR. See Table 3.1.

HYDROLYSIS OF HIPPOSULFATE (**31**) TO HIPPOOL (**40**) — Equal amounts (2 mL) of 1,4-dioxane and pyridine were added to 24 mg of mostly pure (~ 90%) hipposulfate (**31**) in a dry 10 mL round bottom flask. This solution was allowed to reflux for 15.5 hours. The reaction mixture was evaporated under reduced pressure and the remaining solid was triturated in ethyl acetate (6 mL). After washing the ethyl acetate with 2 x 5 mL water, the ethyl acetate layer was dried over $MgSO_4$ and passed through filter paper to give a golden brown solution. The ethyl acetate was removed under reduced pressure to afford 10.9 mg of a mixture containing impure hippool (**40**). Pyridine peaks were present in the ^1H NMR of this mixture. It is believed the pyridium salt of hipposulfate (**31**) was formed. On standing for an additional 72 hours the pyridine

signals decreased. Hippool (**40**) was purified from the mixture by HPLC (normal phase, 20:80 hexanes:ethyl acetate) to yield 3.4 mg of hippool (**40**) as a brown waxy solid: hrfabms *m/z*: 505.3045 ($[M+K]^+$, $C_{31}H_{46}O_3K$, Δ 3.9 mmu of calcd); lrfabms (NBA), *m/z* (%): 466($[M]^+$, 8), 307 (10), 279 (20), 205 (6), 161 (100); ^1H NMR ($CDCl_3$) δ 6.61 (d, J = 9 Hz, H5'), 6.57 (dd, J = 3, 8.5 Hz, H4'), 6.48 (d, J = 3 Hz, H3'), 6.25 (d, J = 10 Hz, H19), 5.57 & 5.55 (d, J = 10 Hz, H18), 5.40 (br m, H7); 3.51 (m, H12), 1.96 (m, H9, H6a), 1.83 (m, H6b), 1.80 (m, H1a), 1.63 (m, H16a), 1.58 (m, H16b), 1.59 (br s, Me22), 1.52 (m, H13), 1.48 (m, H11a), 1.46 (m, H14a), 1.42 (m, H2a/2b), 1.37 (m, H3a), 1.33 (s, Me25), 1.33 (m, H15a/15b), 1.27 (m, H5), 1.12 (m, H3b), 1.08 (m, H14b), 0.96 (m, H1b), 0.88 (s, Me23), 0.85 (s, Me21), 0.84 (s, Me20), 0.71 (s, Me24); ^{13}C nmr ($CDCl_3$) δ 149.3 (C3'), 147.1 (C6'), 135.2 (C8), 131.2 (C18), 122.6 (C19), 122.3 (C7), 122.0 (C1'), 116.7 (C5'), 115.5 (C4'), 112.9 (C2'), 78.3 (C17), 76.2 (C12), 50.4 (C9), 50.2 (C5), 42.3 (C3), 41.3 (C16), 40.1 (C13), 39.4 (C1), 36.4 (C10), 33.1 (C20), 32.4 (C4), 32.4 (C14), 31.4 (C15), 26.1 (C25), 24.0 (C6), 22.6 (C22), 21.9 (C21), 21.8 (C11), 18.6 (C2), 15.2 (C23) 13.7 (C24).

REFERENCES

(1) Suffness, M. In *Biologically active natural products (Annual Proceedings of the Phytochemical Society of Europe)*; Hostettmann, K., Lea, P., Eds.; Clarendon Press: Oxford, 1987, pp. 85-104.

(2) Swaffar, D. J.; Ireland, C. M.; Barrows, L. R. *Anti-Cancer Drugs* **1994**, *5*, 15.

(3) Johnson, R. K.; Hertzberg, R. P. *Ann. Rep. Med. Chem.* **1990**, *25*, 129.

(4) Cohen, S. S. *Med. Biol.* **1976**, *54*, 299-326.

(5) Heidelberger, C.; Chandhau, N. K.; Dannenberg, P.; Mooren, D.; Griesbach, L.; Duschinsky, R.; Schintzer, J. R.; Pleven, E.; Scheiner, J. *Nature* **1957**, *179*, 663-666.

(6) Crooke, S. T.; DuVernay, V. H.; Galvan, L.; Prestayko, A. W. *Mol. Pharmacol.* **1978**, *14*, 290-298.

(7) Rodenberg, B.; Van Camp, L.; Trosho, J. E.; Mansour, V. H. *Nature* **1965**, *222*, 385-386.

(8) Owellen, R. J.; Hartke, C. A.; Dickerson, R. M.; Hains, F. O. *Cancer Res.* **1976**, *36*, 1499-1502.

(9) Schiff, P. B.; Fant, J.; Horwitz, S. B. *Nature* **1979**, *277*, 665.

(10) Manfredi, J. J.; Harwitz, S. B. *Pharmacol. Ther.* **1984**, *25*, 83.

(11) Mandelkow, E.; Mandelkow, E.-M. *Curr. Opin. Struct. Biol.* **1994**, *4*, 171.

(12) Avila, J. *Life Sci.* **1991**, *50*, 327.

(13) Takahashi, I.; Saitoh, Y.; Yoshida, M. *J. Antibio.* **1989**, *52*, 571.

(14) Powis, G. *Trends Pharmacol. Sci.* **1991**, *12*, 188.

(15) Alvi, K. A.; Rodriguez, J.; Diaz, M. C.; Moretti, R. S.; Lee, R. H.; Slate, D. L.; Crews, P. *J. Org. Chem.* **1993**, *58*, 4871-4880.

(16) Woessner, J. W. *Ann. N. Y. Acad. Sci.* **1994**, *732*, 11-21.

(17) Hodgson, J. *Biotechnology* **1995**, *13*, 554-557.

(18) Brown, P. D. *Ann. N.Y. Acad. Sci.* **1994**, *732*, 217-221.

(19) Greenwald, R. A. *Ann. N.Y. Acad. Sci.* **1994**, *732*, 181.

(20) Walker, R. P.; Faulkner, D. J.; Van Engen, D.; Clardy, J. *J. Org. Chem.* **1981**, *103*, 6772-6773.

(21) Lee, R. H.; Slate, D.; Alvi, K.; Crews, P. *BBRC* **1992**, *184*, 765-772.

(22) Schmitz, F. J.; Hollenbeak, K. H.; Campbell, D. C. *J. Org. Chem.* **1978**, *43*, 3916-3922.

(23) Braunhut, S. J.; Mosses, M. A. *J. Biol. Chem.* **1994**, *269*, 13472-13479.

(24) Manes, L. V.; Crews, P.; Kernan., M. R.; Faulkner, D. J.; Fronczek, F. R; Gandour, R. D. *J. Org. Chem.* **1988**, *53*, 570-575.

(25) Luibrand, R. T.; Erdman, T. R.; Vollmer, J. J.; Scheuer, P. J.; Finer, J.; Clardy, J. *Tetrahedron* **1978**, *35*, 609-612.

(26) Nakamura, H.; Deng, S.; Kobayashi, J.; Ohizumi, Y.; Hirata, Y. *Tetrahedron* **1986**, *42*, 4197-4201.

(27) Madaio, A.; Peccialli, V.; Sica, D.; Corriero, G. *J. Nat. Prod.* **1989**, *52*, 952-961.

(28) Madaio, A.; Notaro, G.; Piccialli, V.; Sica, D. *J. Nat Prod.* **1990**, *53*, 565-572.

(29) Cimino, G.; de Stefano, S.; Minale, L.; Fattorusso, E. *Tetrahedron* **1972**, *28*, 267-273.

(30) Cimino, G.; de Stefano, S.; Minale, L.; Fattorusso, E. *Tetrahedron* **1971**, *27*, 4673-4679.

(31) Kobayashi, J.; Ohizumi, Y.; Nakamura, H.; Hirata, Y. *Tetrahedron* **1986**, *27*, 2113-2116.

(32) Kobayashi, J.; Shononaga, H.; Shigemori, H.; Sasaki, T. *Chem. Pharm. Bull.* **1993**, *41*, 381-382.

(33) Nakagawa, M.; Ishihama, M. *Tennen Yuki Kagobutsu Toronkai* **1987**, *29*, 552-559.

(34) Ishibashi, M.; Ohizumi, Y.; Cheng, Y.; Nakamura, H.; Hirata, Y.; Sasaki, T.; Kobayashi, J. *J. Org. Chem.* **1988**, *53*, 2855-2858.

(35) Kobayashi, J.; Murayama, T.; Ohizumi, Y.; Ohta, T.; Nozoe, S.; Sasaki, T. *J. Nat. Prod.* **1989**, *52*, 1173-1176.

(36) Kobayashi, J.; Naitoh, K.; Sasaki, T.; Shigemori, H. *J. Org. Chem.* **1992**, *57*, 5773-5776.

(37) Kernan, M. R.; Faulkner, D. J. *J. Org. Chem.* **1988**, *53*, 4574-4578.

(38) Jaspars, M.; Horton, P. A.; Madrid, L. H.; Crews, P. *J. Nat. Prod.* **1995**, *58*, 609-612.

(39) McKenna, J.; Norymberski, J. K. *J. Am. Chem. Soc.* **1957**, 3889-3893.

(40) Diaz, C., personal communication, 1996.

(41) Fusetani, N.; Matsunaga, S.; Konosu, S. *Tetrahedron Lett.* **1981**, *22*, 1985-1988.

(42) Zubia, E.; Ortega, M. J.; Carballo, J. L.; Salva, J. *Tetrahedron* **1994**, *50*, 8153.

CHAPTER FOUR

Marine Fungal Natural Products : The Chloriolins, Coriolins and Analysis of the Field of Marine Fungal Natural Products

INTRODUCTION

At the initiation of this study, in 1992, there were very few reports of marine fungal natural products. In contrast, a rich history exists for terrestrial fungal natural products.[1-3] The best known fungal natural products may be those from the poisonous or hallucinogenic mushrooms (Basidiomycetes) such as *Amanita muscaria* or *Psilocybe coprophila*. Other non-Basidiomycetes fungal natural products, including the ergot alkaloids from *Claviceps purpurea* growing on rye grass[4] and the aflatoxins from *Aspergillus flavus*, have a history of affecting human food supplies.[1] The discovery of the first natural product antibiotic, penicillin, has led to the study of terrestrial fungi as a source of beneficial natural products.[5] Terrestrial fungi have demonstrated the ability to produce natural products by a very wide range of biosynthetic pathways.[2,3] The structural diversity present in fungal natural products has yielded a great breadth of biological activities. Despite the demonstrated biosynthetic ability of terrestrial fungi, the investigation of marine fungi as a source of novel natural products is only just gaining interest.

It is postulated that marine fungi will produce new and unknown structural classes of natural products compared to their terrestrial counterparts. The ability of the fungi to grow in the ocean suggests that there may be alternative metabolic pathways present to confront the challenges of a marine environment. The basis of biodiversity is that different habitats will host different species. Also, in keeping with the principles of biodiversity, different species are expected to possess different biosynthetic abilities to produce novel secondary metabolites. Even if the species found in a marine habitat are the same as terrestrial species, it has been shown that the habitat of

fungi strongly control the distribution of compounds isolated.[6] Thus, marine fungi, whether they are of the same or different species as terrestrial fungi, are expected to produce different compounds than their terrestrial analogs.

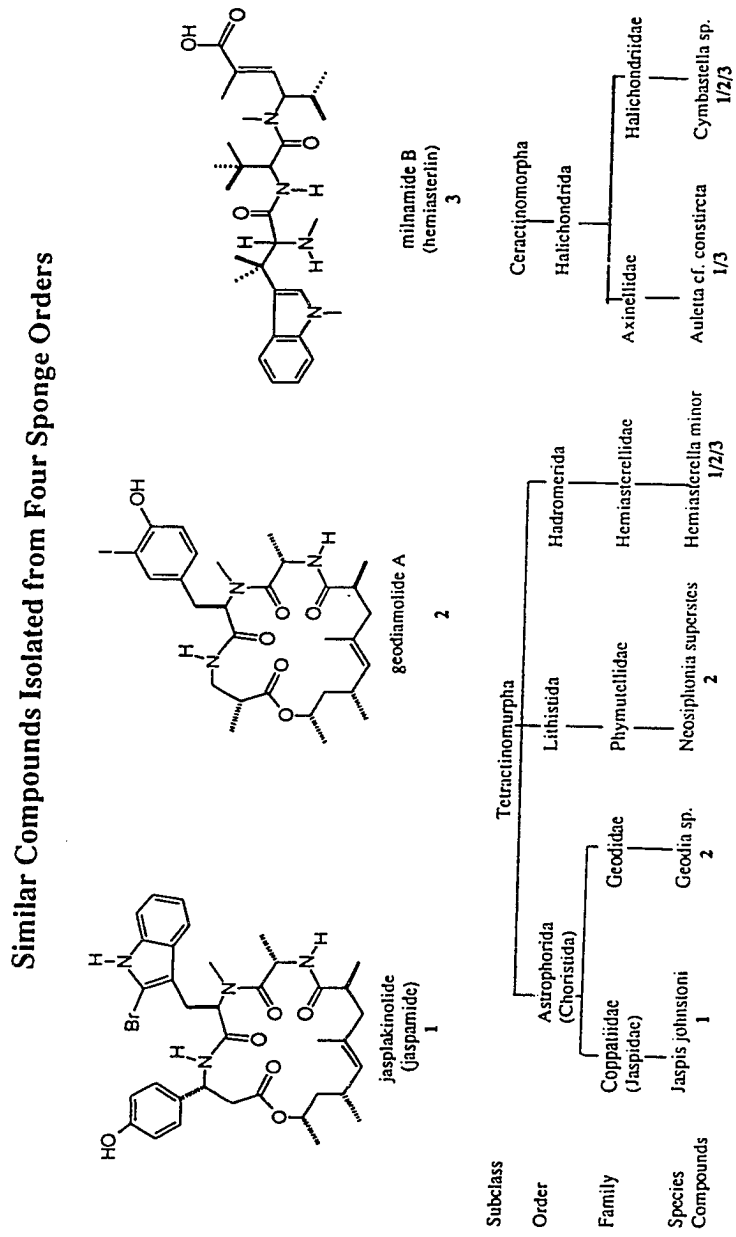
The microbial origin of compounds isolated from marine macroorganisms may best be demonstrated by tetrodotoxin. Three different marine bacteria[7] isolated from a red alga,[8] a xanthid crab,[9] and from the skin of a pufferfish[10] each produce tetrodotoxin that was also isolated from their host organisms. Similarly, the microbial origin of sponge metabolites has been demonstrated. Okadaic acid was isolated from the sponge *Halichondria okadai*[11] and later isolated from the dinoflagellate *Prorocentrum lima*.[12]

Recently, there have been examples to confirm that bacteria are the ultimate source of sponge-derived compounds. Cell sorting has been used to separate bacterial, cyanobacterial and sponge cells; examination of the chemical constituents has shown cyanobacteria that grow within *Dysidea herbacea* sponges are responsible for the production of compounds isolated from the sponges.[13,14]

Examples of identical or similar sponge metabolites isolated from sponge species that are taxonomically very distant have been observed. The isolation of jasplakinolide (**1**) from three orders of sponges,[15-18] the isolation of the biosynthetically similar compound geodiamolide (**2**) from two different orders,[19-21] and the isolation of the milnamides (**3**) from two different orders,[16,22] are summarized in Figure 4.1. The possibility of fungi as the source of sponge-derived compounds had not been explored at the start of this project. However, to have such complex compounds produced by such taxonomically different sponges suggested that a common microorganism may be responsible.

To address the points raised above the chemistry of a fungal culture derived from a *Jaspis johnstonii* sponge was explored. Identification of the secondary metabolites isolated from a marine fungal culture would begin to provide answers regarding the novelty of marine fungal natural

Figure 4.1. Similar compounds isolated from four different sponge orders.



products compared to their terrestrial counterparts. The use of a sponge inoculum would allow a comparison to be made between the utility of sponges as fungal culture sources for natural products compared to other substrates in the ocean. Specifically, using the *Jaspis johnstonii* sponge as the fungal source provided a means to search for a possible microbial source of jasplakinolide. To understand the novelty of marine fungal metabolites and the various definitions of marine fungi, a summary of the field of marine fungal natural products is presented.

Marine fungi - Biology

The classification of indigenous marine fungi has been accepted since 1944[23] when Barghoom and Linder described the presence of fungi which spent their entire life cycle underwater. During the past 20-30 years Jan Kohlmeyer[24] and E. B. Garth Jones[25] have led the field of marine mycology. Kohlmeyer has classified marine fungi into two groups, obligate and facultative, based on the isolation source of the fungi.[26] Obligate marine fungi are those that grow and sporulate exclusively in marine or estuarine habitats. The facultative marine fungi are those that are either of marine or terrestrial origin that are able to grow (without a demonstrated ability to reproduce) in a marine habitat. Whether a facultative or obligate marine fungi is examined depends on the method of isolation. The only method to ensure that an obligate marine fungi is cultured is by inoculation from a fruiting body directly in sea water. This method ensures that the fungi will be able to grow and reproduce in the marine habitat. Unfortunately, the fruiting bodies in marine habitat are very small. Most of the marine fungi are microscopic and the ascocarps of Ascomycetes or the basidiocarps of Basidiomycetes are in general no more than 3-4 mm in size. An alternative method of isolation is by using a portion of a marine substrate as an inoculum to begin a culture. This substrate may be submerged wood, sand, alga or aquatic organisms such as sponges. However, any fungi isolated and grown in marine media are considered facultative marine fungi until it is shown that these fungi can grow and reproduce in a marine habitat. The presence of a mycostatic factor has been postulated in seawater that prevents terrestrial fungi from growing.[27]

This mycostatic factor is destroyed by heat. Kohlemeyer reports that the conidia of terrestrial *Pennicillium* sp. are able to germinate in sterilized (autoclaved) or aged seawater but not in the natural habitat.[24] Thus, to prove that fungi isolated from the marine environment is an obligate marine fungi it must be grown and reproduce in either the ocean or in newly collected unmodified seawater.

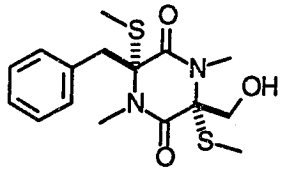
Marine fungi - Chemistry

The chemistry of marine-derived fungi has continually expanded during the course of this work. The compounds isolated are considered to be from marine fungi if the fungal culture examined was from a marine medium or if an established culture of an obligate marine fungi was examined. Researchers have cultured marine-derived fungi in both freshwater and saltwater. In a few select cases the organism was cultured in both deionized water and seawater. In some cases (see below) the same compounds were produced in seawater and freshwater. In contrast, in other cases different compounds were produced as the culture conditions were changed from marine to freshwater (see below). It would be beneficial if culture in both marine and freshwater was performed to determine the dependence of the various fungi on marine conditions for natural products production.

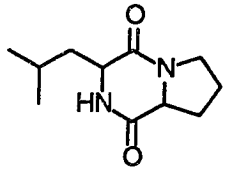
Alkaloids

While the majority of the marine fungal alkaloids are diketopiperazines, there are examples of linear peptides as well as other unclassifiable alkaloids. The diketopiperazines isolated may be considered as simple two amino acid diketopiperazines or as more complex structures containing more than one diketopiperazine ring, or they may have extra amino acids attached to a diketopiperazine ring. The variations reported in marine fungal diketopiperazines have been previously observed in terrestrial fungal diketopiperazines.

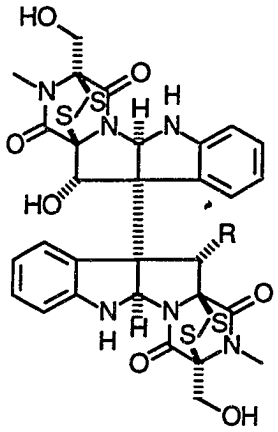
The compounds isolated from obligate marine fungi include gliovictin (**4**),[28] gancidin W (**5**) and the melinicidins III (**6**) and IV (**7**).[29] Gliovictin (**4**) and melinicidins III (**6**) and IV (**7**) are sulfur-containing diketopiperazines while gancidin W (**5**) is a non-sulfur containing diketopiperazine. Gliovictin (**4**) has been previously isolated from the terrestrial fungi *Hyalodendron* sp.[30] and *Penicillium turbatum*,[31] and the enantiomer of gliovictin (**4**) has been identified from *Helminthosporium victoriae*.[32] Gliovictin (**4**) was isolated from a culture of the obligate marine fungus *Asteromyces cruciatus* obtained from Chesapeake Bay. *A. cruciatus* is an imperfect fungi that has been isolated from wood, alga and sand. There are no other compounds reported for *Asteromyces cruciatus*. Melinicidins III (**6**) and IV (**7**) were isolated with gancidin W (**5**) from the obligate marine fungi *Corrollospora pulchella* and have been previously isolated from the terrestrial fungus *Acrostalagmus cinnabarinus* var. *melinicidicus*.[33-35] The melinicidins (**6-7**) are very similar to the terrestrial chaetocins (**8**) and verticillins (**9**).[36] They are dimers of tryptophan and serine possessing an additional ring formed from the indole of the tryptophan moiety. Gancidin W (**5**) was also previously isolated from an actinomycete *Streptomyces gancidus*.[37] The discovery of these compounds in 1985 marked the first published production of antibiotic and antitumor compounds from marine fungi. It is interesting to note that the marine fungi, *Corrollospora pulchella*, and a terrestrial filamentous bacteria are able to biosynthesize the same secondary metabolite **5**.



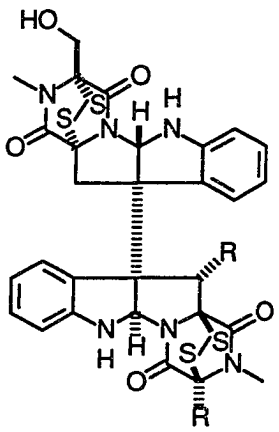
4
gliovictin



5
gancidin W

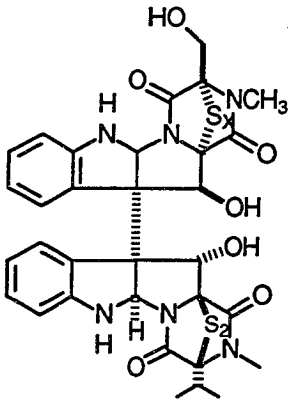


6 melinicidin III R=H
7 melinicidin IV R=OH

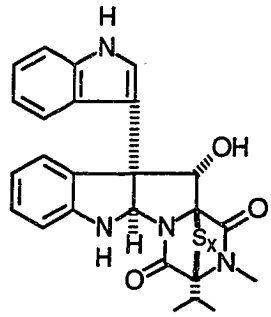


8 chaetocin R = CH_2OH
9 verticillin A R = CH_3

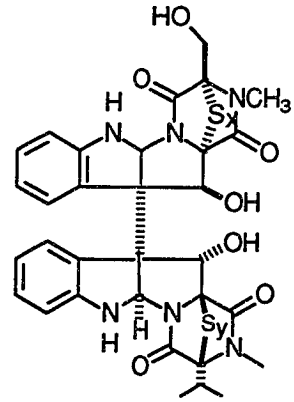
The discovery of the leptosins (**10-24**) introduced the first examples of novel diketopiperazine dimers from obligate marine fungi.[38,39] The leptosins (**10-24**) were discovered from the marine fungi *Leptosphaeria* sp. isolated from sea grass in the Bay of Naples. This culture has produced 15 different compounds with varying numbers of bridging sulfurs. Two major types of dimers exist with the majority of the structures possessing extra annulations from the tryptophan indole to the diketopiperazine rings: the leptosins A-C (**10-12**), G (**16**), G_1 (**17**), G_2 (**18**), H-K (**19-22**), K_1 (**23**),K_2 (**24**). The three examples of the unsymmetrical dimers without the extra annulation are the leptosins D-F (**13-15**). The leptosins (**10-24**) resemble the melinicidins (**6-7**) as well as the chaetocins (**8**) and verticillins (**9**) which are produced by *Chaetomium* sp.[35] and *Verticillium* sp.,[40,41] respectively. The leptosins (**10-24**) display *in vivo* antitumor activity against Sarcoma 180 ascites. The genus *Leptosphaeria* has many marine members which produce a wide variety of compounds (further examples are described below). The terrestrial members of this genus produce only terpene natural products.[42]



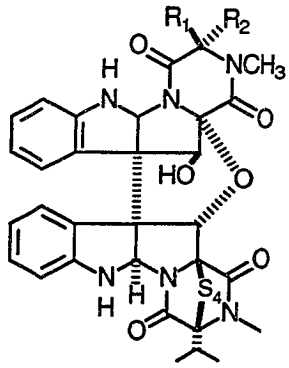
10 leptosin A, x = 4
11 leptosin B, x = 3
12 leptosin C, x = 2



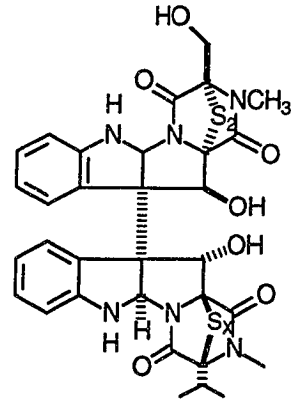
13 leptosin D, x = 2
14 leptosin E, x = 3
15 leptosin F, x = 4



16 leptosin G, x = 4, y = 3
17 leptosin G_1, x = 3, y = 3
18 leptosin G_2, x = 2, y = 3
19 leptosin H, x = 3, y = 4

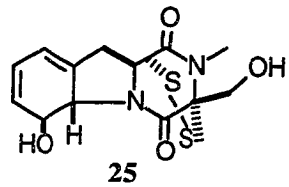


20 leptosin I, R1 = CH_2OH, R2 = R3 = H,
21 leptosin J, R1 = R3 = H, R1 = CH_2OH



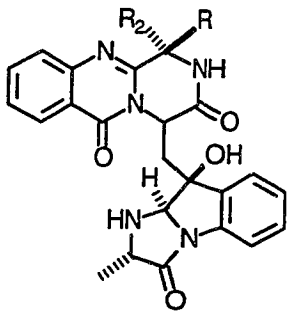
22 leptosin K, x = 2
23 leptosin K_1 , x = 3
24 leptosin K_2 , x = 4

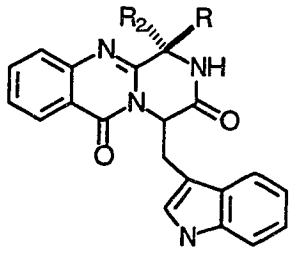
An *Aspergillus* culture isolated from marine mud produced the known diketopiperazine gliotoxin (**25**).[43] Occurrence of gliotoxin (**25**) is widespread from deuteromycetes[44] including other *Aspergillus, Gliocladium,* and *Pennicillium* species.



25
gliotoxin

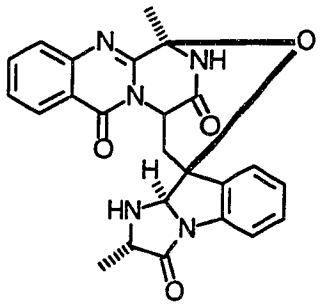
The fumiquinalozines A-G (**26-32**) were isolated from an *Aspergillus fumigatus* culture derived from the gastrointestinal tract of a marine fish (*Pseudolabrus japonicus*).[45] The fumiquinalozines A-G (**26-32**) are tryptoquivaline type compounds consisting of an anthranillic acid, two alanine units and a modified tryptophan arranged as modified diketopiperazines. The terrestrial fungus *Aspergillus fumigatus* produces a wide variety of compounds including other tryptoquivalines.[46,47] The fumiquinalozines (**26-32**) are mildly cytotoxic against the P388 leukemia cell line.

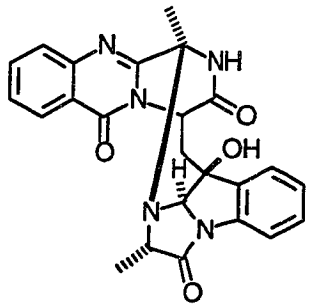




26 fumiquinazoline A $R_1 = CH_3$, R_2=H
27 fumiquinazoline B R_1 = H, R_2=CH_3
28 fumiquinazoline C $R_1 = CH_3$, R_2=OCH_3

29 fumiquinazoline D $R_1 = CH_3$, R_2=H
30 fumiquinazoline E R_1 = H, R_2=CH_3



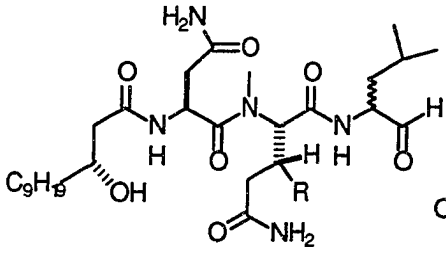


31
fumiquinazoline F

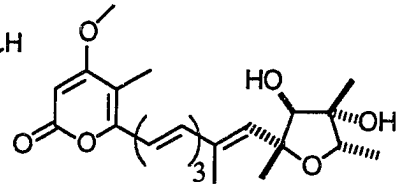
32
fumiquinazoline G

The only non-diketopiperazine amino acid-derived natural products reported to date from a marine fungi are the fellutamides A (**33**) and B (**34**).[48] The fellutamides (**33-34**) producing culture

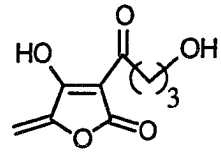
of *Pennicillium fellutanum* was isolated from the intestinal tract of the marine fish *Apogon endidtaenia*. The fellutamides (**33-34**) consist of three modified amino acids linked to a 3-hydroxydodecanoic acid moiety. The unusual amino acids consist of a leucanal portion for both fellutamides (**33-34**) along with a β-hydroxy glutamine for fellutamide A. *Pennicillium fellutanum* is reported to produce the polyketide citreoviridin (**35**)[49] and the tricarboxylic acid (TCA) cycle derived dehydrocarolic acid (**36**).[50] The fellutamides (**33-34**) resemble the terrestrial leupeptins, which are a series of tripeptides containing small polyketide chains and an arginal termini isolated from actinomycetes.[51]



33 fellutamide A, R=OH
34 fellutamide B, R=H

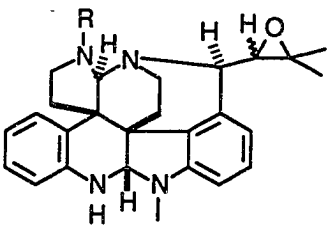


35
citreoviridin

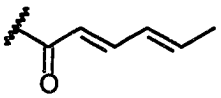


36
dehydrocarolic acid

Communesins A (**37**) and B (**38**) are the only non-peptide alkaloids found from marine fungi to date.[52] The communesins (**37-38**) producing culture was isolated from a *Pennicillium* sp. derived from the marine alga *Enteromorpha intestinalis*. Members of the *Pennicillium* genus are considered a terrestrial fungi and are able to biosynthesize a large number of compounds. The framework of the communesins (**37-38**) appears to be unique, and this compound may be the most original alkaloid isolated from a marine fungi to date.



37 communesin A , R = $COCH_3$

38 communesin B, R= 

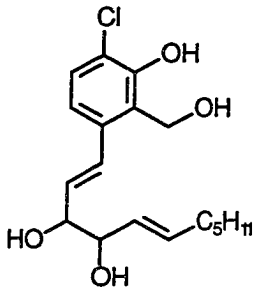
Aspergillus fumigatus which produces the fellutamides A (**33**) and B (**34**) and the *Penicillium* sp. responsible for the communesins (**37-38**) can be considered facultative marine fungi as neither culture was started with a fruiting body. Apparently, neither culture has been tested for its ability to reproduce in natural seawater.

Polyketides

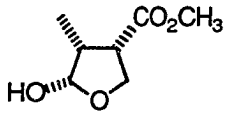
Marine fungi parallel the ability of terrestrial fungi in the production of polyketide natural products. Both obligate and facultative marine fungi are able to produce a wide variety of polyketide metabolites. Fused polyaromatic, pyrone, depsidones and repeating ester polyketides have been isolated.

The obligate marine fungi *Zopfiella marina*, *Zofiella marina* and *Leptosphaeriae obiones* have all produced novel polyketide metabolites. *Zopfiella marina* is responsible for the production of zopfinol (**39**).[53] The terrestrial fungus *Zopfiella matsushimae* of this genus is known to produce botryodiplodin (**40**),[54] a compound of unclear biogenesis unrelated to zopfinol (**39**). The terrestrial fungus *Hypocrea citrinin* produces aurocitrin which has the same polyketide chain carbon framework as zopfinol (**39**).[55] A report in the Japanese patent literature reveals *Zofiella marina* produces the antibiotic zofimarin (**41**).[56] While the exact biosynthetic nature of this compound is not clear, it resembles a polyketide metabolite. There are no other reports of metabolites produced

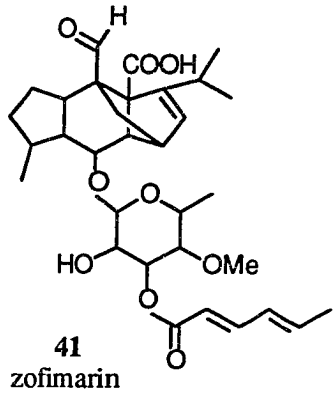
by the genus *Zofiella*. It is possible that the genus *Zofiella* is the same as *Zopfiella* and a misspelling occurred in the patent application.



39
zopfinol

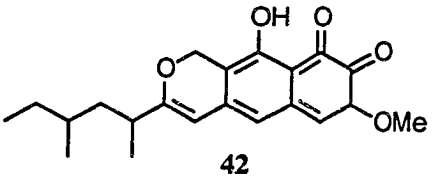


40
botryodiplodin

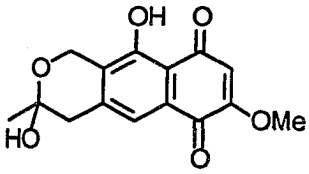


41
zofimarin

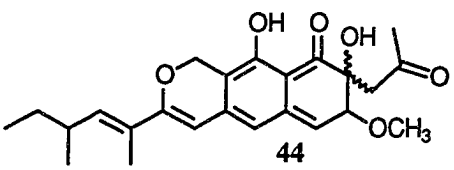
Among the variety of compounds the obligate marine fungal genus *Leptosphaeriae* produces, the species *L. obiones* is responsible for the polyketide obionin A (**42**).[57] The structure of obionin A (**42**) nonaketide represents the first occurrence of the naptho[2,3-c] pyran 8,9 dione system, however 6,9 pyrones represented by 5-deoxyfusarubin (**43**) are known.[58] In another study of *Leptosphaeriae oraemaris* the polyketide lactone leptosphaerolide (**44**) and its putative precursor leptosphaerodione (**45**) were isolated. Leptosphaerodione (**45**) is the C11-C12 saturated version of obionin A (**42**). *Leptosphaeriae* species are able to produce a variety of metabolites from a variety of biosynthetic pathways in saltwater culture. This is in contrast to the terrestrial *Leptosphaeriae typhae* which produces only terpenes.[3]



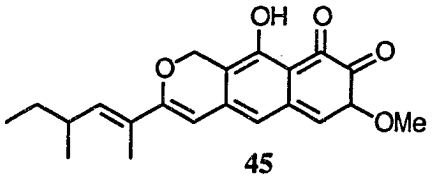
42
obionin A



43
5-deoxyfusarubin

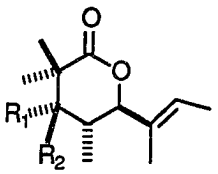


44
leptosphaerolide

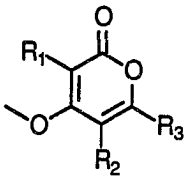


45
leptosphaerodione

There are two reports of pyrone lactones produced from marine fungi. The helicascolides A (**46**) and B (**47**) were isolated from the obligate marine fungi *Helicascus kanaloanus*,[59] and the related compounds demethyl nectriapyrone A (**48**) and B (**49**) were isolated by our group from an unidentified fungi that was cultured from a sponge.[60] In addition to the helicascolides (**46-47**) the same culture of *Helicascus kanaloanus* produced the known compound mellein (= ochracin) (**50**). Mellein (**50**) has been isolated from *Aspergillus ochraceus* and *A. melleus*,[61,62] both of which are considered terrestrial fungi. The closest analogy to the helicascolides (**46-47**) and demethylnectriapyrones A (**48**) and B (**49**) is nectriapyrone isolated from the terrestrial fungus *Gyrostroma missouriense* (Seeler).[63] There are no other reports of secondary metabolites from terrestrial *Helicascus* sp.

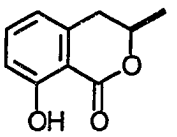


46 helicascolide A R_1 = OH, R_2 = H
47 helicascolide B R_1 = H, R_2 = OH



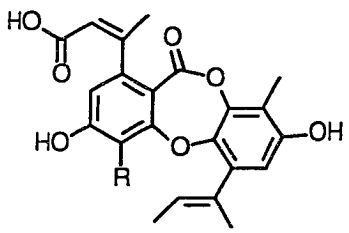
48 demethylnectriapyrone A, R_1 = H, R_2 = H, R_3 =

49 demethylnectriapyrone B, R_1 = H, R_2 = CH_3, R_3 = CH_2CH_3

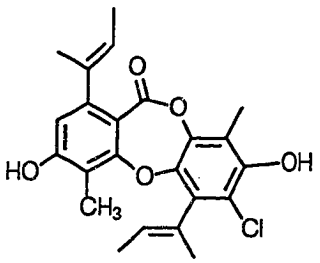


50 (-) -*R* mellein

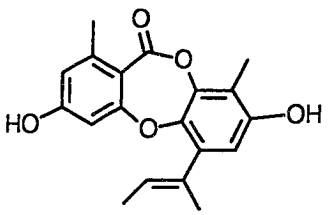
The facultative marine fungi *Preussia aurantiaca* produces the auranticins A (**51**) and B (**52**).[64] Related depsidones, emeguisins A-C, represented by emeguisin A (**53**) and trisdechloronornidulin (**54**) are known from the terrestrial fungi *Emericella unguis* and *Aspergillus nidulans*, respectively.[65,66] Various species of the *Preussia* are known to make the polyketide preussomerins.[67,68] *Preussia* is considered to be a terrestrial fungi and the authors believed that the isolation of a *Preussia* species from a mangrove root was coincidental. They also state that the culture of *Preussia aurantiaca* in fresh or seawater results in the same compounds.[64]



51 auranticin A, R = CH_2OH
52 auranticin B, R = CHO

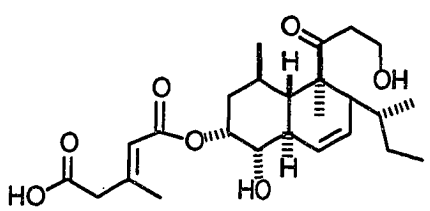


53
emeguisin A $R_1 = R_2 = R_3 = H$

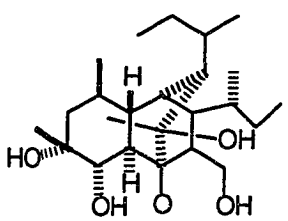


54
trisdechloronornidulin

A culture of the deuteromycete *Trichodermia harzium* separated from the marine sponge *Mycale cecilia* produced the polyketide trichoharzin (**55**).[69] This marked the first publication of a natural product from a sponge-derived fungi. Cultures of the fungi were performed in both salt and freshwater. Trichoharzin (**55**) was only present in the seawater culture of the fungus. The skeleton of trichoharzin (**55**) is similar to the betaenones, represented by betaenone A (**56**), isolated from a terrestrial *Phoma* sp.[70]

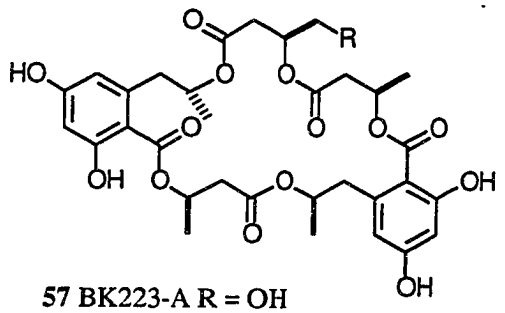


55
trichoharzin

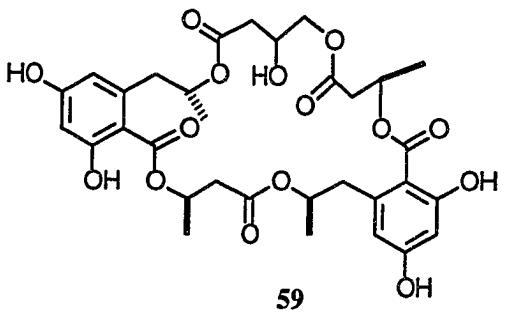


56
betaenone A

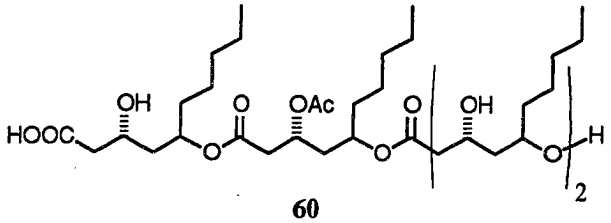
There have been reports of three macrocyclic lactides, BK 223 A-C (**57-59**) from the obligate marine fungi *Hypoxylon oceanicum.*[71] These compounds have also been isolated from a terrestrial *Pennicillium verruculosum* species and were named NG-010 through NG-012.[72] There is no primary literature available on the production of the BK 233 A-C (**57-59**) from *Hypoxylum* therefore it is unclear if the *Hypoxylum* cultures were incubated in the same manner as the *Penicillium* cultures. There is also a preliminary report of halymecin (**60**), from a *Fusarium* sp.[71] *Fusarium* is a well known terrestrial genus that produces a wide variety of compounds from many different biosynthetic pathways.[3] The polyketides produced by terrestrial *Fusarium* sp. are cyclized aromatic polyketides as opposed to the open chain ester linked polyketides demonstrated in halymecin (**60**). The diacid hymenoscyphin A (**61**) from a salt marsh species of *Hymenoscyphus* sp. was described in a review,[73] however, no further information is available on this compound at present. The genus *Hymenoscyphus* has not been reported to produce any other secondary metabolites.



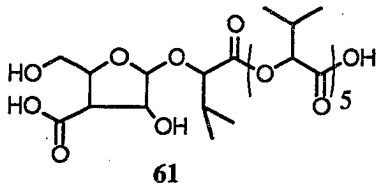
57 BK223-A R = OH
58 BK223-B R = H



59
BK223-C



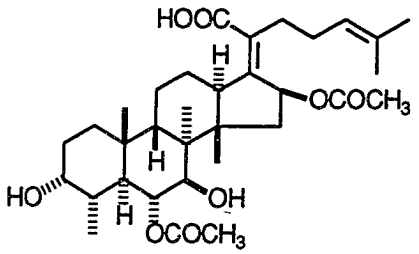
60
halymecin



61
hymenoscyphin A

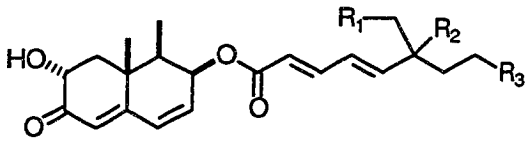
Terpenes

The production of terpenes by marine fungi is well documented. The discovery of the triterpene cephalosporin P_1 (**62**) in 1957 marks the first compound isolated from a marine fungus.[74] Since that time terrestrial members of the genus *Cephalosporium* have produced other triterpenes as well as polyketides and amino acid-derived natural products.[3] While the inoculum for the cephalosporin P_1 (**62**) producing fungus was from the sea, it is assumed that the fungus was cultured in deionized water, as no special note is made of seawater in the procedure.

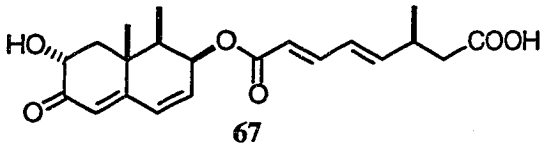


62
cephalosporin P_1

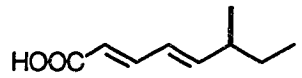
The dendryphiellins A-D (**63-67**) are rare trinor-eremophilane sesquiterpenes isolated from the obligate marine fungi *Dendryphiella salina*.[75] While trinor-eremophilanes have been previously discovered in plants,[76,77] the dendryphiellins A-D (**63-67**) represent the first discovery of this structural class in fungi. The original discovery was followed-up with the discovery of other members of the class with varying hydroxylation patterns on the ester side chain of the terpene.[78] The side chains, dendryphiellic acids (**68-69**), and the complete sesquiterpene, dendryphiellins E-G (**70-74**) were also isolated, showing conclusively that the original structures were degraded sesquiterpenes.[79] There are a wide variety of eremophilane sesquiterpenes from terrestrial *Phoma exigua* and *Pennicillium* species, however there are no trinor-eremophilanes known.[3] Only one other series of natural products has been reported from the genus *Dendryphiella*. These are the polyketide dendryols A-D (**75-78**) from a weed pathogenic fungus.[80]



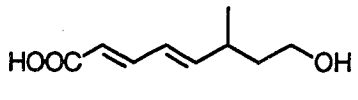
63 dendryphiellin A : $R_1 = R_2 = H$, $R_3 = OH$
64 dendryphiellin B : $R_1 = R_3 = H$, $R_2 = OH$
65 dendryphiellin C : $R_1 = R_2 = R_3 = H$
66 dendryphiellin D : $R_1 = OH$, $R_2 = R_3 = H$



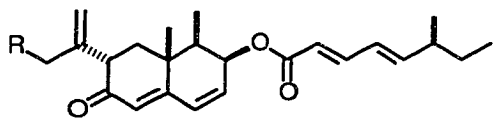
67
dendryphiellin A_1

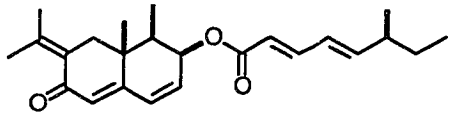


68
dendryphiellic acid A



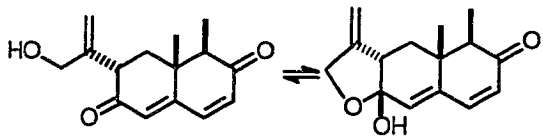
69
dendryphiellic acid B



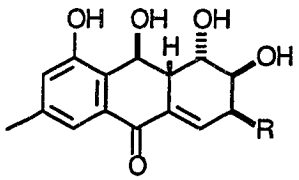


71
dendryphiellin E_2

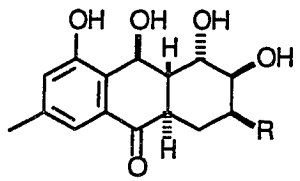
70 dendryphiellin E : R = OH
72 dendryphiellin E_1 : R = H
73 dendryphiellin F - formed during isolation



74
dendryphiellin G - exists as two equilibrium forms



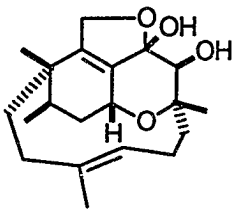
75 dendryol A R = H
76 dendryol B R = OH



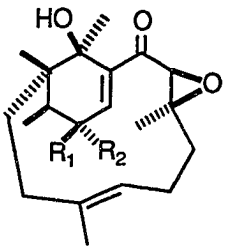
77 dendryol C R = OH
78 dendryol D R = H

A *Phoma* sp. fungi isolated from the shell of a crab is responsible for the production of the platelet activating factor antagonists the phomactins (**79-87**).[81-83] The phomactins (**79-87**) are sesquiterpenes with a macrocyclic furochroman skeleton. While the pentadecane ring has been found in plants,[84] the presence of the macrocycle was unprecedented when phomactin A (**79**) was

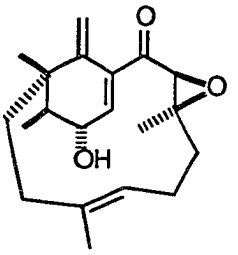
isolated. Before the report of phomactins B-G (**80-83, 85-87**) a similar compound Sch 47918 (= phomactin C) (**84**) was found from a *Phoma* sp. cultured from a piece of wood. The terrestrial *Phoma* species are prolific producers of polyketides and amino acid-derived compounds, however, there were no reports of terpenes until the discovery of phomactin C (**84**) from a terrestrial fungus.[3]



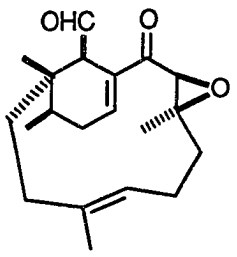
79
phomactin A



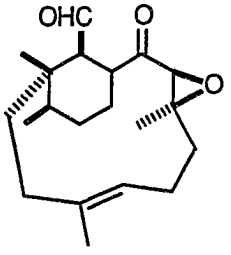
80 phomactin B : R_1 = H, R_2 = OH
81 phomactin B_1 : R_1 = OH, R_2 = H
82 phomactin E : R_1 = R_2 = H



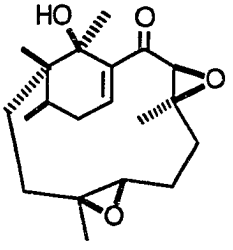
83
phomactin B_3



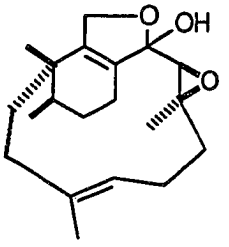
84
Sch 47918 (phomactin C)



85
phomactin D

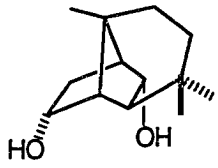


86
phomactin F



87
phomactin G

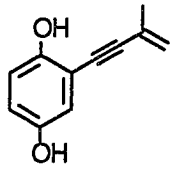
The known sesquiterpene culmorin (**88**) was isolated from the obligate marine fungi *Leptosphaeria oraemaris* when a series of lignincolous fungi were assayed for antifungal activity.[85] Culmorin (**88**) was initially isolated from the terrestrial fungus *Fusarium roseum*[86] cultured in deionized water. The seawater cultures of *Leptosphaeria oraemaris* also produced culmorin (**88**) indicating the biosynthesis of **88** is not salinity dependent.



88
culmorin

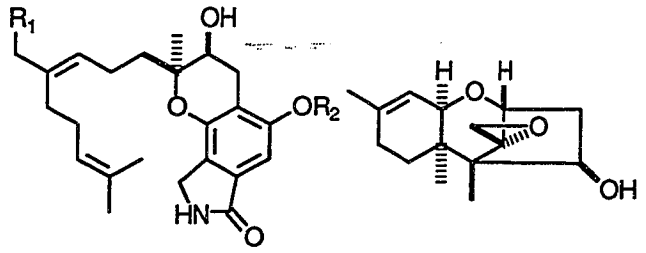
Mixed Biogenetic Pathways

Marine fungi have also produced a number of compounds of mixed biogenesis containing terpenoids. Siccayne (**89**), a prenylated hydroquinone, was originally isolated from the terrestrial fungus *Helminthsporium siccans*.[87] In the reisolation of siccayne (**89**) from the obligate marine basidiomycetes *Halocyphina villosa*, the culture conditions were carried out in freshwater.[88] There are no other reports of compounds from the genus *Halocyphina*. While *Helminthsporium* species produce many polyketides and terpenes, there are no other reports of compounds resembling siccayne (**89**).[3]



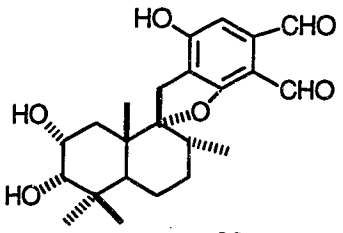
89
siccayne

The stachybotrins A (**90**) and B (**91**) were isolated from the facultative marine fungi *Stachybotrys* sp.[89] The original culture was isolated from a twig submerged in the ocean, however, the culture conditions that produced the stachybotrins (**90-91**) were in freshwater. The genus *Stachybotrys* has a well-explored natural products history and is responsible for the production of trichothecine mycotoxins, such as trichodermol (**92**)[90] and sterols.[3] The stachybotrins (**90-91**) are composed of a sesquiterpene chain attached to an indole ring. The closest analogies to the stachybotrins (**90-91**) may be triprenylated phenols such as K-76 (**93**).[91] The presence of a nitrogen containing unit is unprecedented in these compounds.



90 stachybotrin A R_1 = OH, R_2 = H
91 stachybotrin B R_1 = R_2 = H

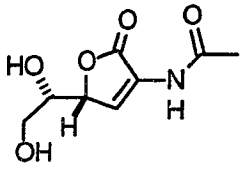
92
trichodermol



93
K-76

Other biogenetic pathways

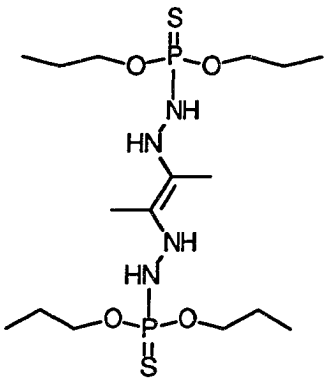
Leptosphaerin (**94**) was the first novel natural product isolated from an obligate marine fungi, *Leptosphaeria oraemaris*. There are no other precedents for this amino hexose type structure.[92] The novel compound leptosphaerin (**94**) was produced in freshwater culture of *L. oraemaris*.[93]



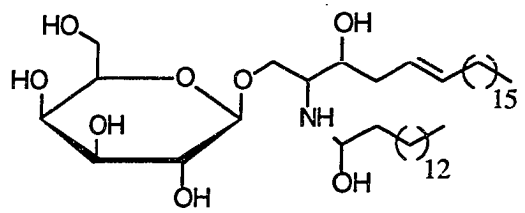
94
leptosphaerin

The obligate marine fungus *Lignicola laevis* was cultured to provide three dissimilar compounds which have been published unnamed.[94] A unique phosphorohydrazide thioate (**95**), a ceramide (**96**) and 7-hydroxyergosterol (**97**) were isolated from culture of the fungus in seawater. The structure reported for 7-hydroxyergosterol (**97**) is questionable as the reported enone would be expected to tautomerize to the ketone. Other problems with the structure elucidation of the 7-hydroxyergosterol (**97**) include the absence of a ^{13}C NMR resonance consistent with the site of attachment of the hydroxyl group. The structure of the phosphorohydrazide thioate (**95**) is also somewhat suspect as the structure elucidation does not explain how the presence of phosphorous

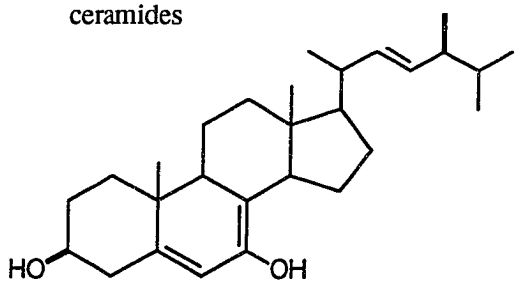
and sulfur was established nor is there an explanation for the sequence of heteroatoms in the structure. Though the phosphorohydrazide thioate (**95**) was thought to a biotransformation product of an insecticide from the culture seawater, no insecticide resembling it was found in the chemical registry. The authors did not report if the same compounds were found when the culture was grown in deionized water. There are no compounds resembling the phosphorohydrazide thioate (**95**) known from terrestrial fungi.



95
phosphorohydrizide thiolate

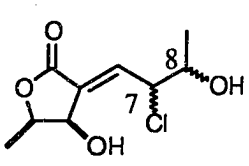


96
ceramides

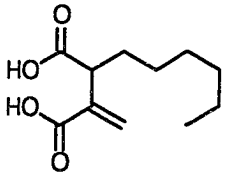


97
7- hydroxyergosterol

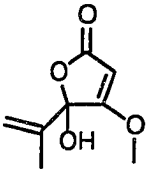
The novel diastereomeric compounds chlorocarolides A (**98**) and B (**99**), along with mellein (**50**), hexylitaconic acid (**100**) and penicillic acid (**101**) were isolated from a fungus putatively identified as *Aspergillus ochraceus* which was derived from a *Jaspis* cf. *coricea* sponge. This sponge is the source of the bengamides[95] and the bengazoles.[96] The chlorocarolides (**98-99**) are similar to carolic acid (**102**), a compound of oxoloacetate and C_6 polyketide biogenesis.[97] The previously isolated hexylitaconic acid (**100**) may share the same biosynthetic pathway as carolic acid (**102**).[98] Neither the chlorocarolides (**98-99**) nor hexylitaconic acid (**100**) were produced when the culture was grown in freshwater, however, penicillic acid (**101**) and mellein (**50**) were observed.



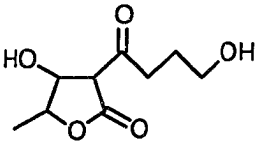
98 chlorocarolide A 7*R** 8*R**
99 chlorocarolide B 7*S** 8*S**



100
hexylitaconic acid



101
pennicillic Acid



102
carolic acid

As the preceeding discussion indicates, the marine fungi are able to produce a wide range of compounds by paralleling and duplicating the biosynthetic abilities of terrestrial fungi. The summary of marine natural products presented here provides the background information necessary to qualitatively determine the uniqueness of marine fungal natural products. The research performed on a *Jaspis johnstonii* derived marine fungi will add additional information on the suitability of marine sponges as a source of biosynthetically capable marine fungi.

To aid in organizing the data presented in this introduction, the marine fungal natural products classes are summarized in Table 4.1. Relationships between marine fungal sources and related terrestrial fungi, marine fungal compounds and related terrestrial fungal compounds may be observed in Table 4.1.

Table 4.1. Summary of compound classes isolated from marine fungi, related terrestrial compounds and related terrestrial fungi.

Compound	Fungal source	Obligate/ Facultative	Marine/ fresh culture	Related terrestrial fungi	Related/ same compounds	Terrestrial source of related compounds
gliovictin (**4**)	*Asteromyces cruciatus*	obligate	marine	None	gliovictin (**4**)	*Hyalodendron* sp *Penicillium turbatum* *Helminthosporium victoriae*
melinicidins (**6-7**)	*Corollospora pulchella*	obligate	marine	None	mellinicidins	*Acrosstalagmus cinnabarinus*
gancidin W (**5**)	*Corollospora pulchella*	obligate	marine	None	gancidin W (**5**)	*Streptomyces gancicidus*
leptosins (**10-24**)	*Leptosphaeria* sp	obligate	marine	*Leptosphaeriae typhae*	chaetocin verticillin	*Chaetomium* sp *Verticillium* sp
gliotoxin (**25**)	*Aspergillus* sp.	facultative	marine	*Aspergillus*	gliotoxin	widespread
fellutamides (**33-34**)	*Penicillium fellutanum*	facultative	marine	*Penicillium*	leupeptins	
fumiquinalozines (**26-32**)	*Aspergillus fumigatus*	facultative	marine	*Aspergillus fumigatus*	trytoquivalines	*A. fumigatus* *A. clavulatus*
communesins (**37-38**)	*Penicillium* sp	facultative	marine	*Penicillium*		
zopfinol (**39**)	*Zopfiella marina*	obligate	marine	*Zopfiella matsushimae*	aurocitrin	*Hypocrea citrinin*
zofimarin (**41**)	*Zofiela marina*	?	?	?		
obionin A (**42**)	*Leptosphaeriae obiones*	obligate	marine	*Leptosphaeriae typhae*	deoxyfusarubin	*Nectria haemotococca* *Emericella unguis*
leptosphaerolide (**44**) leptosphaerodione (**45**)	*Leptosphaeriae oraemaris*	obligate	not specified	*Leptosphaeriae typhae*	deoxyfusarubin	*Nectria haemotococca*
helicascolides (**46-47**)	*Helicascus kanaloanus*	obligate	marine	none	nectriapyrone	*Gyrostroma missouriense*
mellein (ochracin) (**50**)	*Helicascus kanaloanus*	obligate	marine	none	mellien	*Aspergillus ochraceus* *A. melleus*
demethylnectriapyrones (**48-49**)	unknown	facultative	marine		nectriapyrone	*Gyrostroma missouriense*
auranticins (**50-51**)	*Preussia aurantiaca*	facultative	marine & fresh	*Preussia* sp.	emeguisins A-C	*Emerricella unguis*

Table 4.1 (cont.). Summary of compound classes isolated from marine fungi, related terrestrial compounds and related terrestrial fungi.

Compound	Marine fungal source	Obligate/ Facultative	Marine/ fresh culture	Related terrestrial fungi	Related/ same compounds	Terrestrial source of related compounds
trichoharzin (55)	*Trichodermia harzium*	facultative	both compound from marine	*Trichoderma* sp.	betaenone B	*Phoma* sp.
BK 223 - A-C (57-59) (NG-011 -013)	*Hypoxylon oceanicum*	obligate	not stated	none	BK 223 A-C (NG-011, NG-013	*Penicillium verruculosum*
halymecin (60)	*Fusarium* sp.	facultative	not stated	*Fusarium* sp.		
hymenoscyphin A (61)	*Hymenoscyphus* sp.	facultative	not stated	*Hymenoscyphus*		
cephalosporin P_1 (62)	*Cephalosporium sp.*	facultative	fresh	*Cephalosporium*	tetracyclic triterpenes	*Aspergillus, Cephalosporium*
dendryphiellins (63-74)	*Dendryphiella salina*	obligate	fresh ?	*Dendryphiella* sp.	eremophilanes	*Phoma*
phomactins (79-87)	*Phoma* sp.	facultative	marine	*Phoma* sp.	Sch 47918	*Phoma* sp.
culmorin (88)	*Leptosphaeria oraemaris*	obligate	marine	*Leptosphaeriae typhae*	culmorin	*Fusarium roseum*
siccayne (89)	*Halocyphina villosa*	obligate	fresh		siccayne	*Helminthsporium*
stachybotrins (90-91)	*Stachybotrys*	facultative	fresh	*Stachybotrin* sp.	K-76	*Stachybotris complementi*
leptosphaerin (94)	*Leptosphaeria oraemaris*	obligate	fresh	*Leptosphaeriae typhae*		
phosphorohydrizide thioate (95)	*Lignicola laevis*	obligate	marine	none	insecticides ?	
ceramide (96)	*Lignicola laevis*	obligate	marine		ceramides	widespread
7-hydroxyergosterol (97)	*Lignicola laevis*	obligate	marine		ergosterols	widespread
chlorocarolides (98-99)	*Aspergillus ochraceus*	facultative	marine	*Aspergillus*	carolic acid	widespread
penicillic acid (101)	*Aspergillus ochraceus*	facultative	marine	*Aspergillus*	carolic acid	widespread

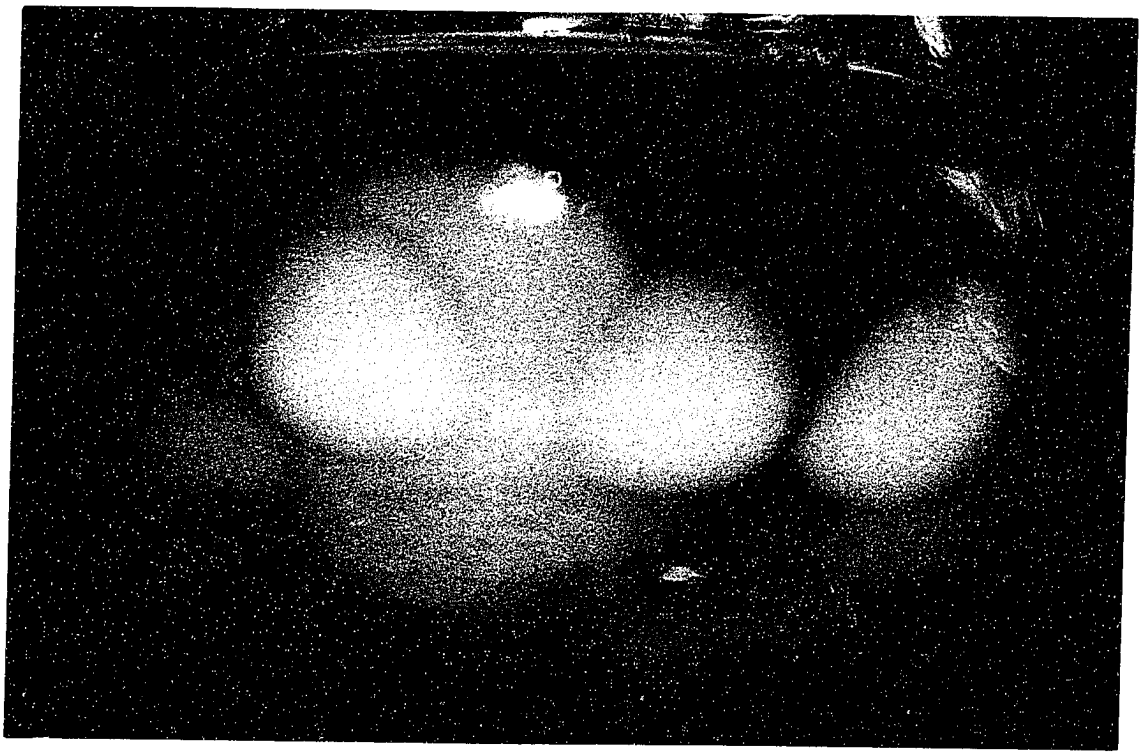
RESULTS

The fungal culture, 92902, Plate 4.1, derived from a *Jaspis johnstonii* sponge was examined to test whether jasplakinolide might be of fungal origin, and to explore the biosynthetic ability of marine sponge-derived fungi. The cooperative efforts of several people were necessary for the completion of this project. Mr. Leif Abrell, a Ph.D. candidate, contributed in the initial isolation and culture of the fungi. Dr. Xing Cheng, a former postdoctoral fellow, was responsible for the isolation of the novel chloriolins from the fungal broth. This thesis work involved the isolation and dereplication of the known coriolins. The structure of chloriolin A (**105**) was solved in a group effort between myself, Xing Cheng and Professor Phil Crews while the unequivocal structures of chloriolins B and C were determined by Phil Crews and myself after Xing Cheng departed from UCSC.

Culture of fungi

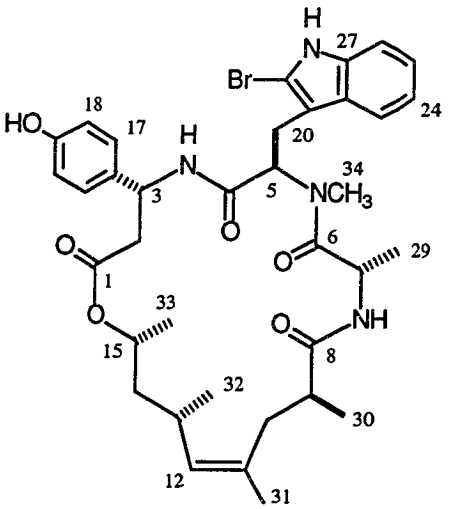
Leif Abrell collected a piece of the jasplakinolide (**1**) producing sponge, *Jaspis johnstonii*, in Benga Lagoon, Fiji by SCUBA. A piece of the sponge was removed from a large colony of *Jaspis johnstonii* and placed in a sterile whirl pack bag. On the surface the piece of sponge was cut into smaller portions in a sterile environment and placed on four different fungal culture plates containing antibacterials. In approximately seven days a fungal culture was observed to be growing from the sponge placed on the cellulose powder plate. Leif subcultured this emerging fungi from the cellulose plate onto a new malt extract agar plate without any antibacterial agents. When a pure culture of fungi was established on the malt extract agar plate it was used for the initiation of the liquid malt extract cultures (4x500 mL). Leif Abrell started these with a small portion of pure fungi culture growing on the malt extract agar plate. Subsequent liquid cultures were initiated from the most recent liquid cultures.

Plate 4.1. Photograph of malt extract broth liquid culture of 92902, (500 mL broth in 1L erlenmeyer flask).



Extraction and purification of coriolins and chloriolins

I extracted the mycelium three times with methanol for 24 hours while the broth was extracted by Xing Cheng, three times with ethyl acetate to generate two crude oils. Both the crude mycelium and broth extracts underwent a separate but equal solvent/solvent partition, Figure 4.2, carried out by myself and Xing Cheng, respectively. When the crude ^{1}H NMR of the broth FD partition, Figure 4.3, and the mycelium FD partition, Figure 4.4, were examined it was seen that they were dissimilar to each other and did not contain the same ^{1}H NMR resonances as jasplakinolide (**1**), Figure 4.5.[15,17,18]



1
jasplakinolide

Figure 4.3. ^{1}H NMR spectrum of fungal broth FD partition, 250 MHz, $CDCl_3$.

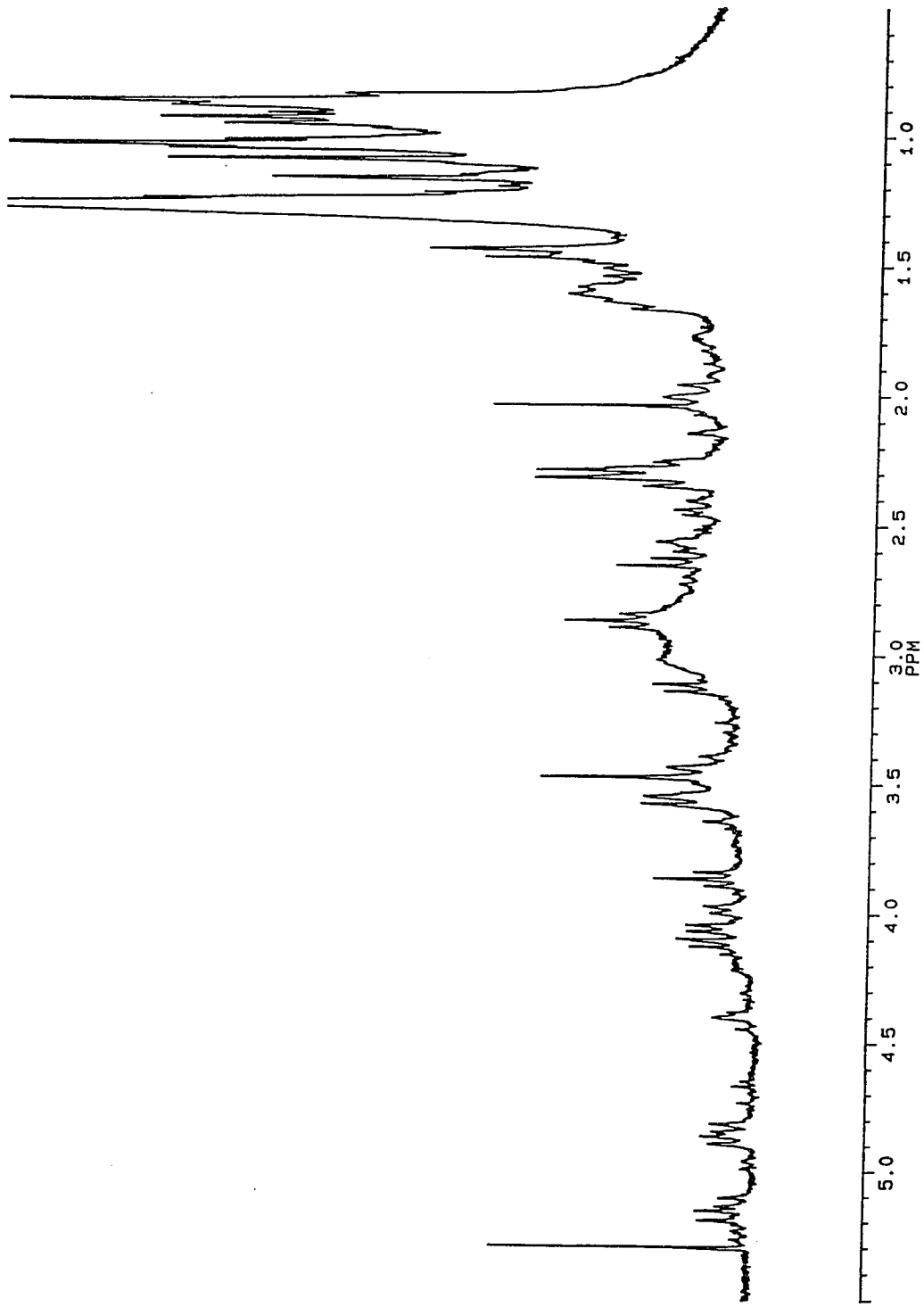


Figure 4.4. ^{1}H NMR spectrum of fungal mycelium FD partition, 250 MHz, $CDCl_3$.

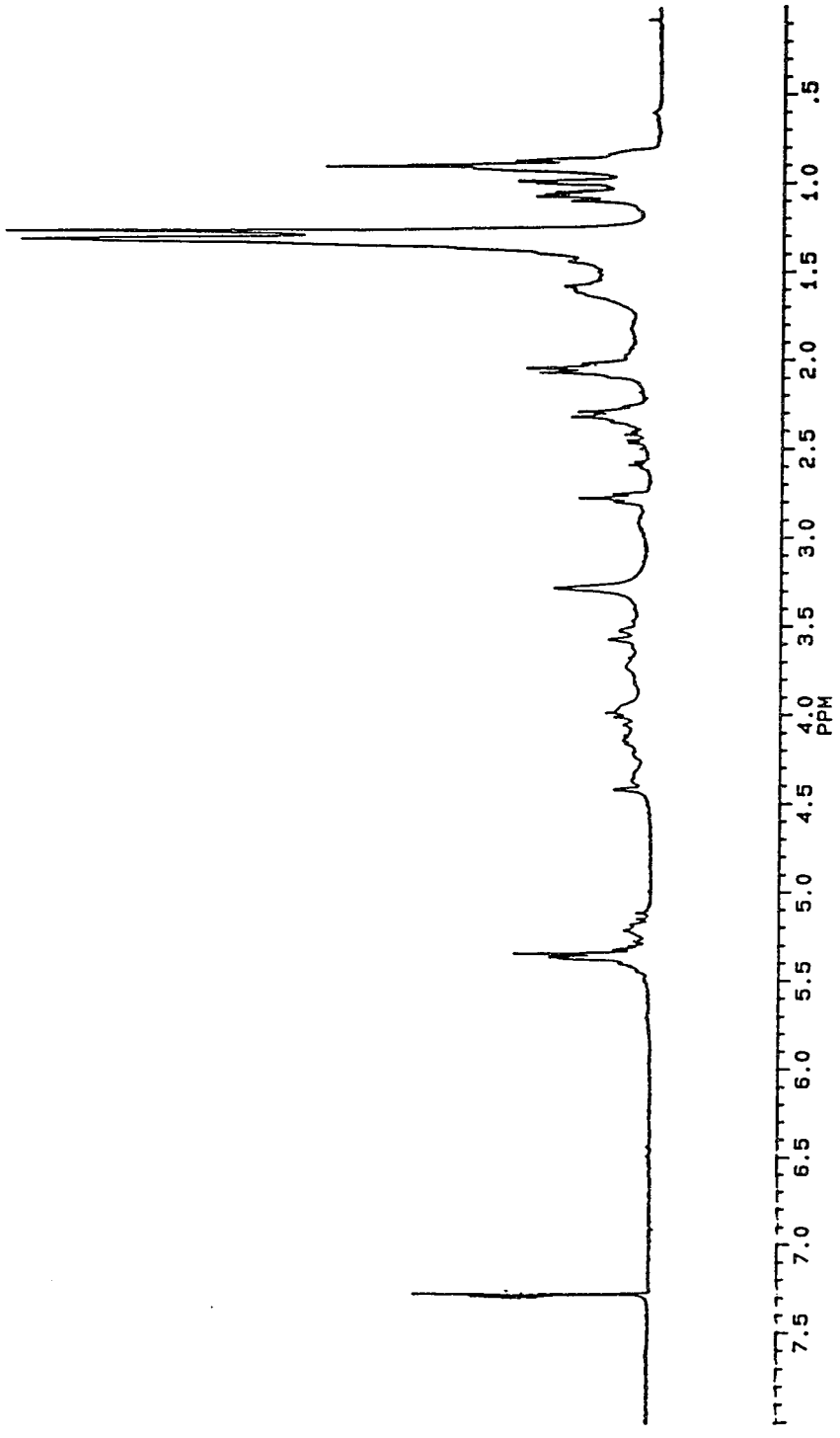
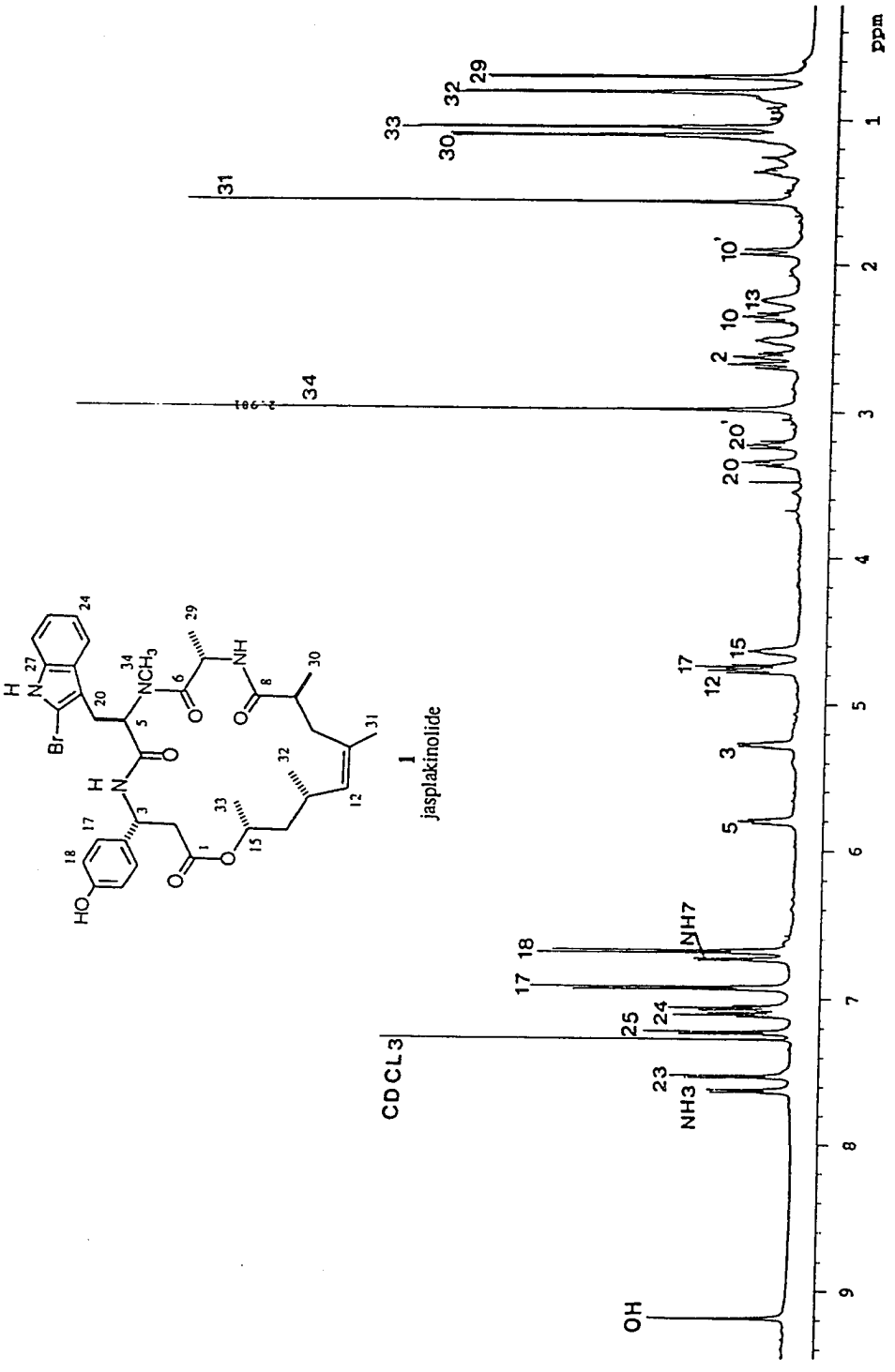


Figure 4.5. ^{1}H NMR spectrum of jasplakinolide (**1**), 500 MHz, $CDCl_3$.



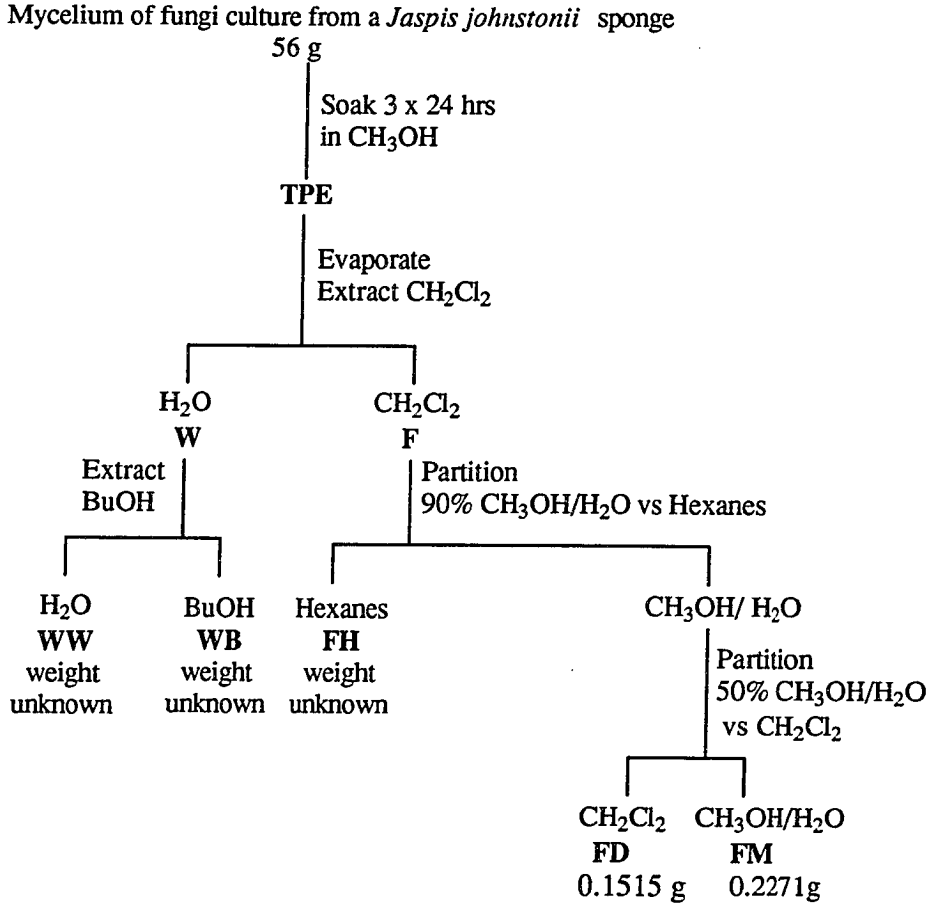
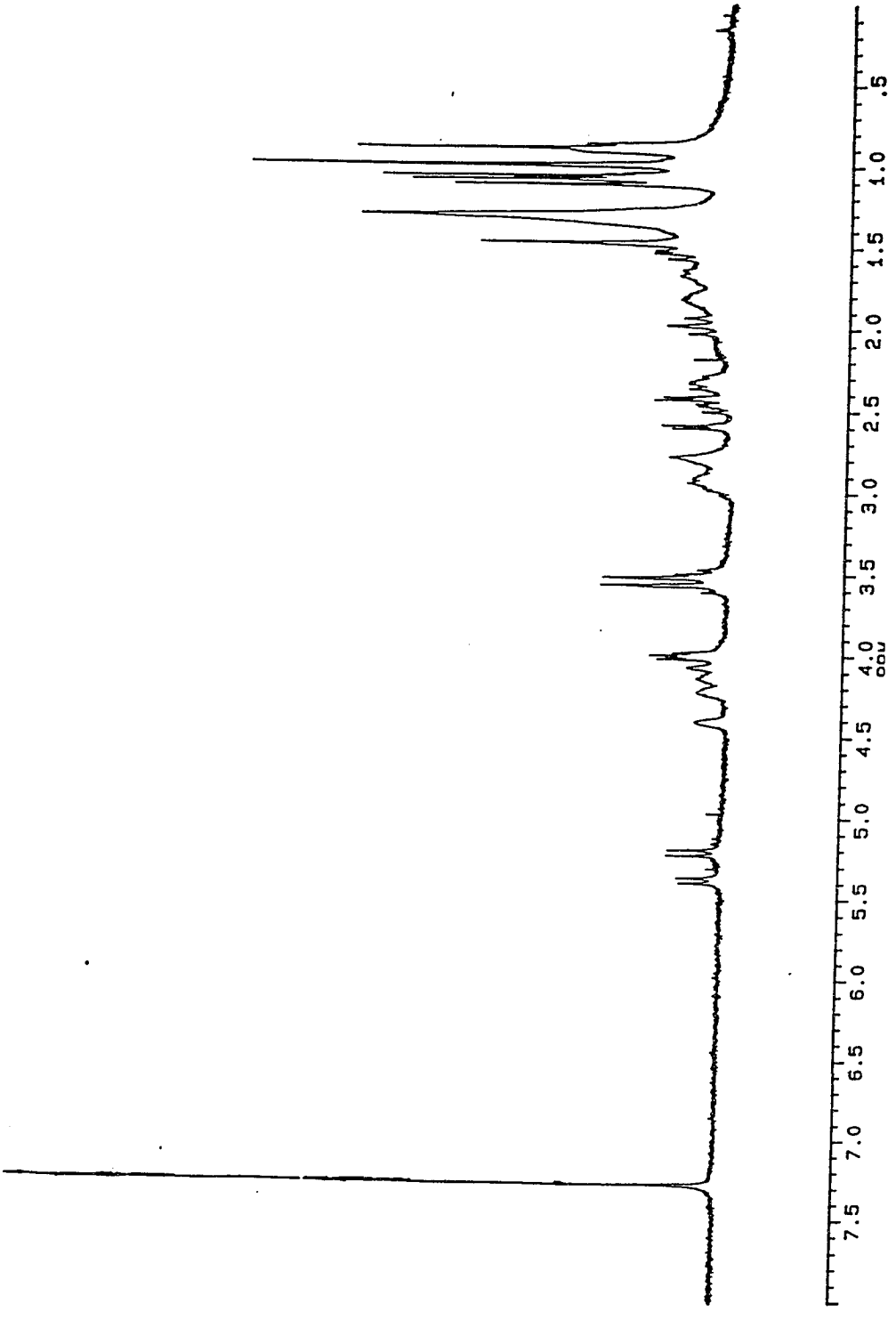
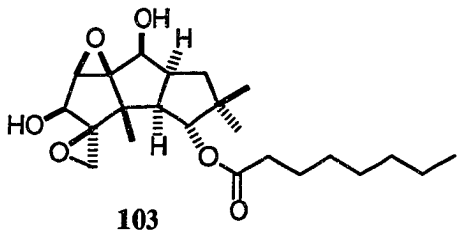


Figure 4.2. Extraction and partition scheme of fungal mycelium.

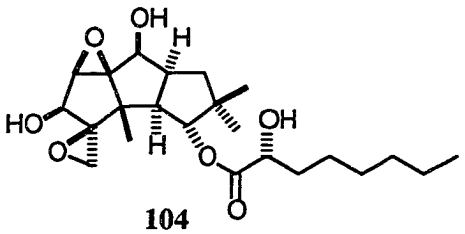
Separate and different purification methods were employed for the broth and the mycelium FD partitions. I applied the mycelium FD partition (227 mg) to a Sephadex LH-20 size exclusion column (methanol); a band was observed to form a dry white powder on the rims of the collection test tubes. These test tubes were combined and this material was chromatographed by isocratic HPLC (eluted 20% aqueous methanol) to yield the known compounds coriolin B (**103**)[99] (17 mg) and dihydrocoriolin C (**104**)[100] (16.5 mg). A mixture of two compounds was isolated as well. The ^1H NMR shifts, Figure 4.6, of this binary mixture did not correspond to any known coriolins. Unfortunately the NMR tube containing the entire supply of this fraction was stolen from the NMR laboratory and never recovered.

Figure 4.6. ^{1}H NMR spectrum of stolen binary mixture of mycelium, 250MHz, $CDCl_3$.



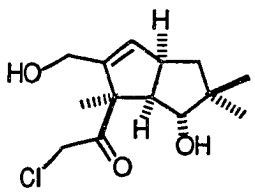


103
coriolin B

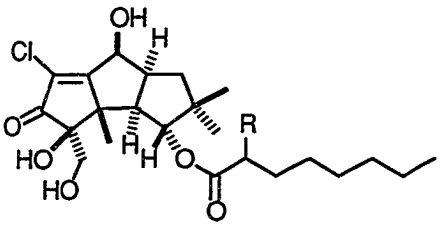


104
dihydrocoriolin C

Xing Cheng applied the CH_2Cl_2 portion of the broth extract (714 mg) to a silica gel flash column eluted with hexanes/EtOAc (the solvent was varied from 2:1 hexanes:EtOAc to 1:2 hexanes: EtOAc). One of the flash fractions was further purified by normal phase HPLC (hexanes:EtOAc 3:7) to yield the novel compound chloriolin A (**105**) (18.0 mg). Other flash fractions were also purified by HPLC (hexanes:EtOAc 1:1) to yield novel chloriolin B (**106**) (16.0 mg) and chloriolin C (**107**) (3.0 mg).



105
chloriolin A



106 chloriolin B R = OH
107 chloriolin C R = H

Structure elucidation of coriolin B (103) and dihydrocoriolin C (104)

The structure elucidation of coriolin B was undertaken first. Examination of the ^{13}C NMR of coriolin B (**103**), Figure 4.7, revealed the presence of 23 carbons. The APT spectra, Figure 4.8, and the ^{1}H NMR, Figure 4.9, suggested a partial molecular formula of $C_{23}H_{34}$. Using the carbon and hydrogen count established from the NMR spectra and the low and high resolution FAB mass spectra, Figure 4.10, a potential formula of $C_{23}H_{36}O_6$ was obtained. The single carbonyl, δ 172-C16, accounted for one of the six unsaturations in the molecule, therefore there were five rings present in the molecule.

Figure 4.7. ^{13}C NMR spectrum of coriolin B (**103**), 62.9 MHz, $CDCl_3$.

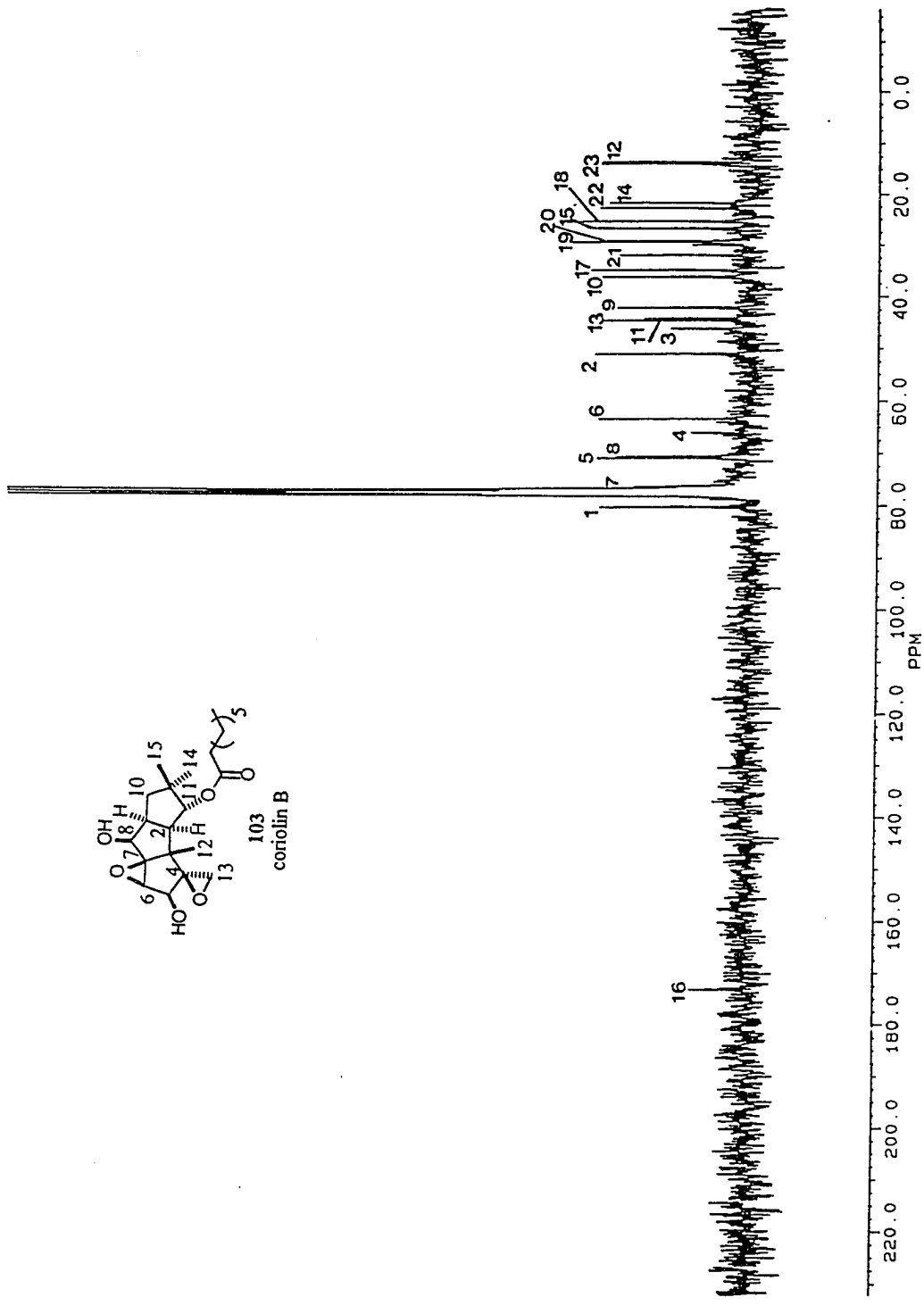


Figure 4.8. APT spectrum of coriolin B (**103**), 62.9 MHz, $CDCl_3$.

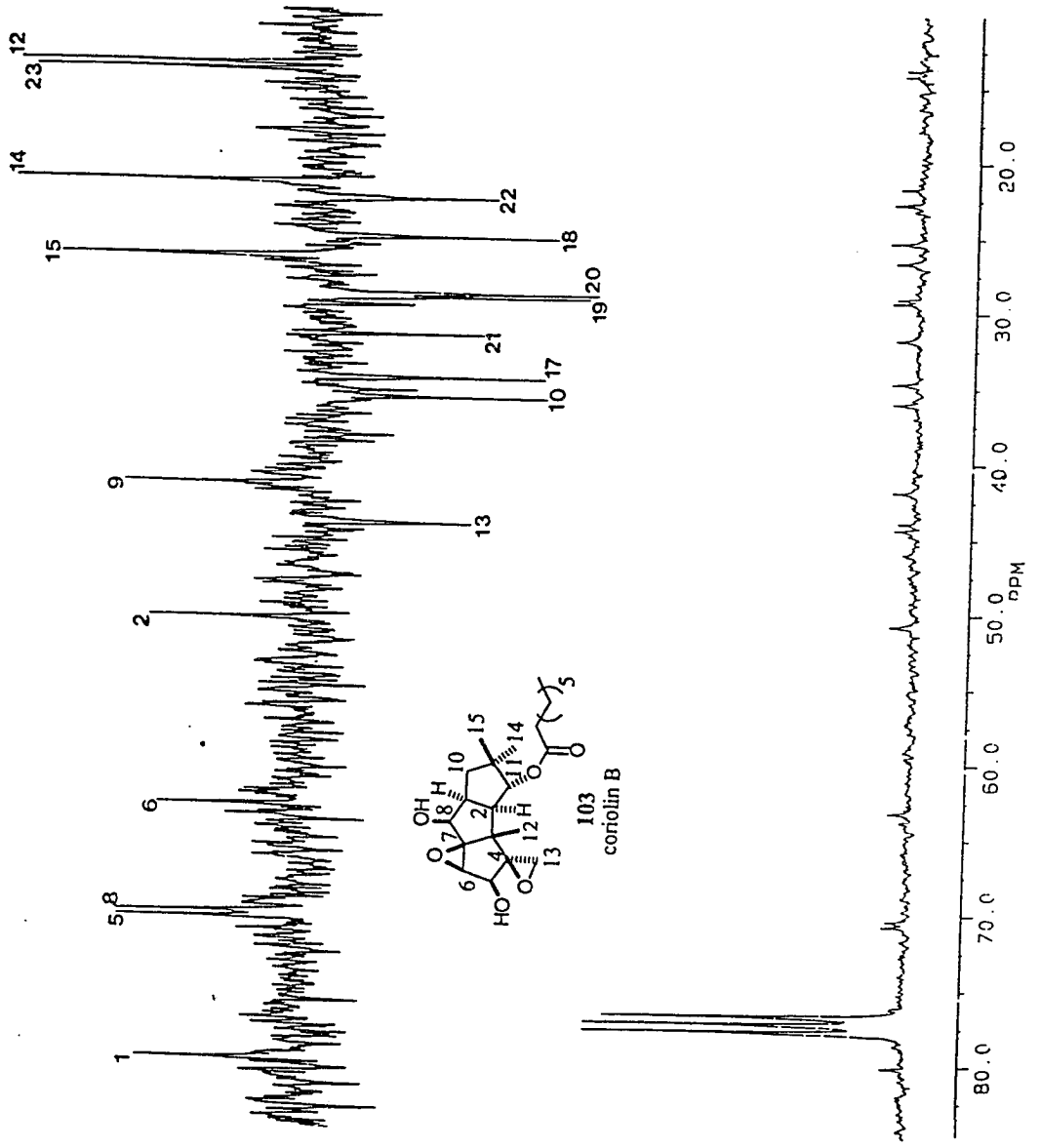


Figure 4.9. ^{1}H NMR spectrum of coriolin B (**103**), 250 MHz, $CDCl_3$.

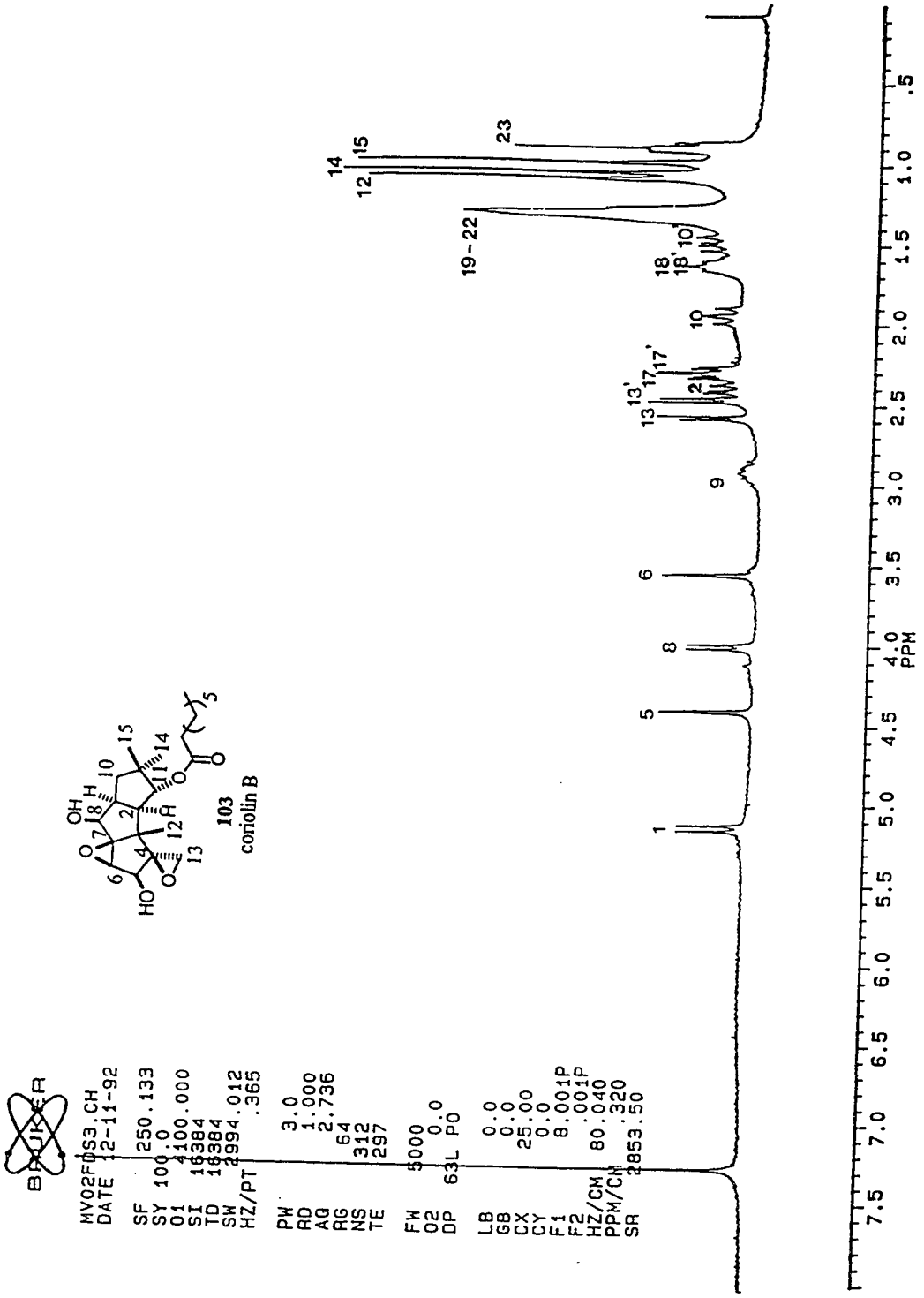
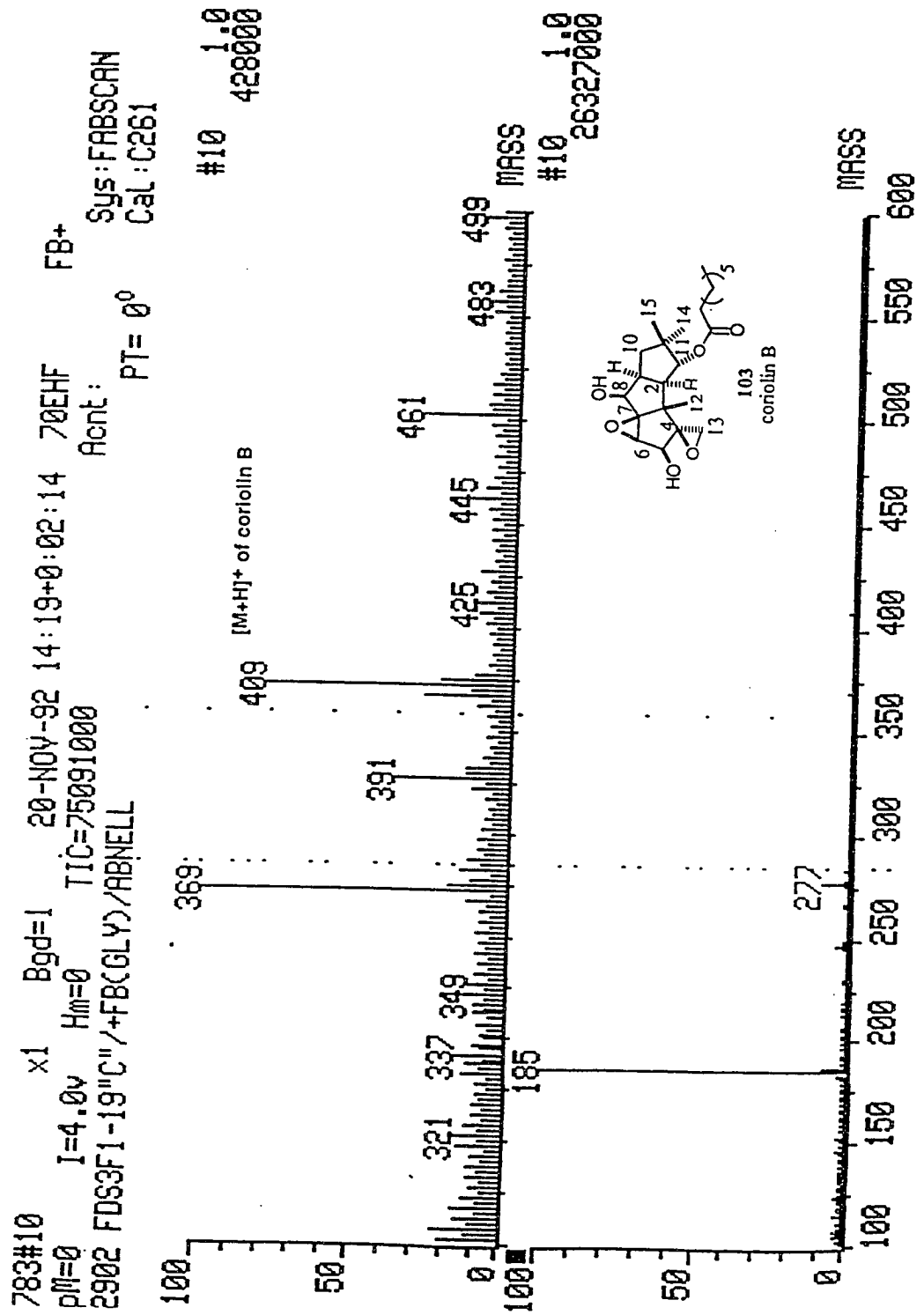


Figure 4.10. LR FAB MS of coriolin B (**103**), glycine matrix.



Examination of the ^{1}H-^{1}H COSY spectrum provided the large spin system H1-H2-H8-H9-H10/10', Figure 4.11. A further spin system consisting of a seven carbon chain was observed from ^{1}H-^{1}H COSY correlations; the observed ^{13}C NMR shifts matched the literature ^{13}C NMR shifts for straight chain esters.[101] Two further two proton spin systems were identified. The H5-H6 vicinal hydrogens were found as well as the diastereotopic geminal hydrogens H13 and H13' that correlated to C13 at 44.2 ppm. An epoxide at C13 was postulated due to the small 4.8 Hz geminal coupling observed. There were also three singlet methyls, as well as four quaternary carbons left after the analysis of the ^{1}H-^{1}H COSY spectrum. These substructures could be summarized as shown in Figure 4.12.

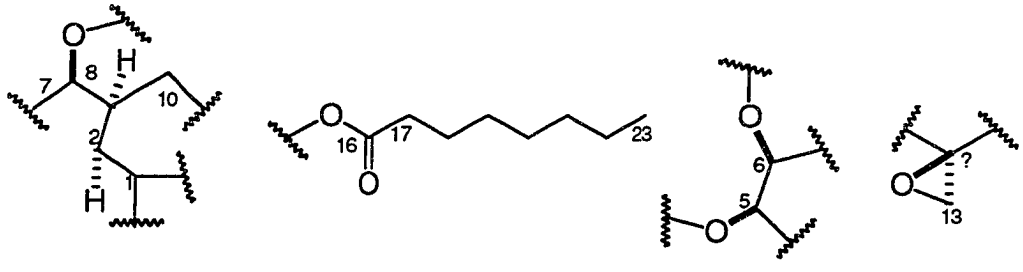
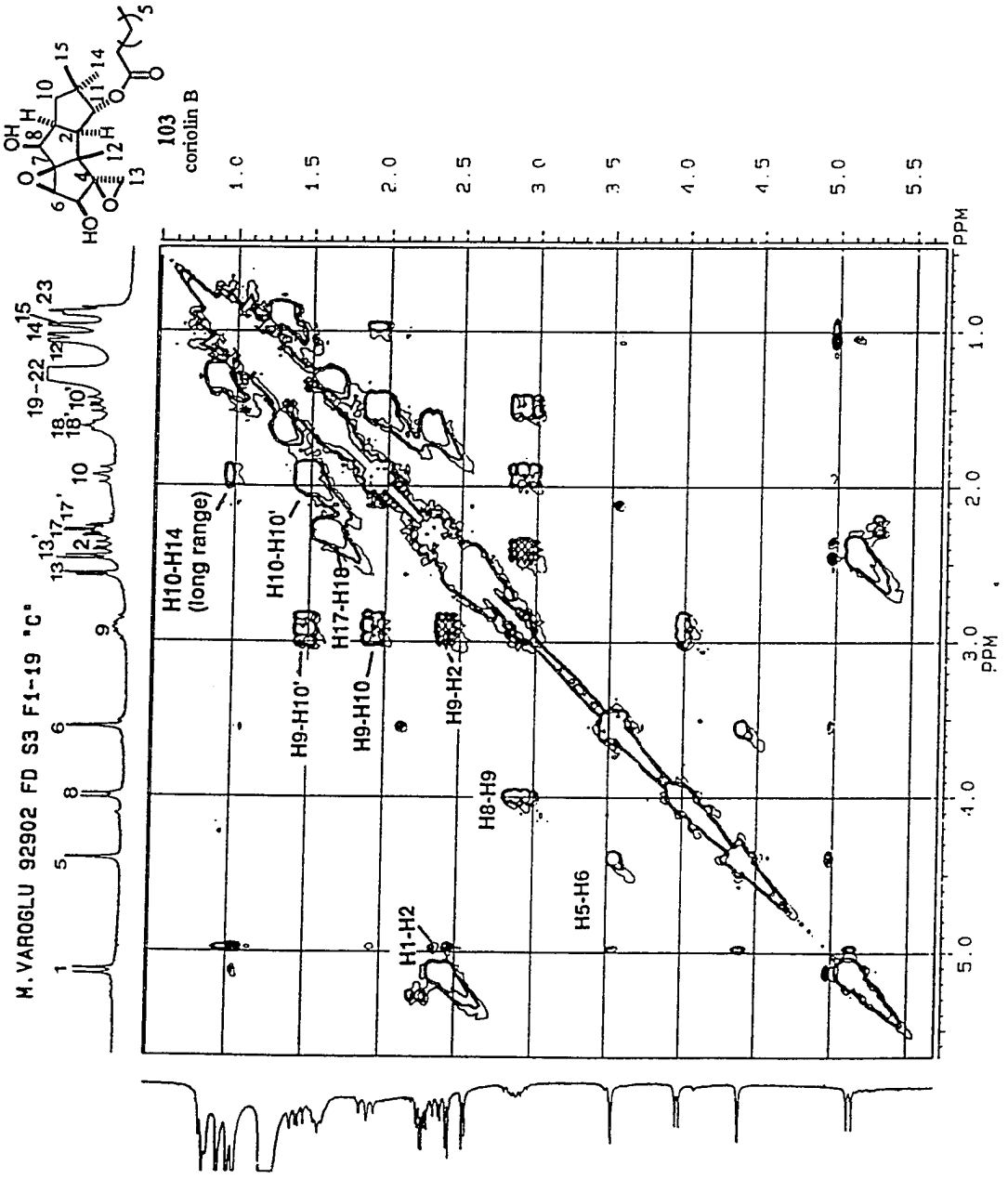
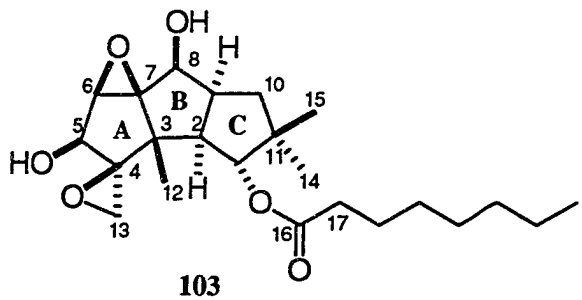


Figure 4.12. Coriolin B (**103**) ^{1}H-^{1}H COSY derived substructures.

Before a long-range ^{13}C-^{1}H COSY ($J_{C\text{-}H}$= 9 Hz) could be acquired to connect the substructures, a molecular formula search on the Bérdy database was performed.[102] The only result returned was the structure of coriolin B (**103**). All of the substructures deduced above could be located in the published structure of coriolin B (**103**). Comparison of the reported literature ^{1}H NMR for coriolin B (**103**)[99] and the measured ^{1}H NMR data indicated the sponge-derived fungal culture did produce the known compound coriolin B (**103**).

Figure 4.11. ^{1}H-^{1}H COSY spectrum of coriolin B (**103**), 250 MHz, $CDCl_3$.





103
coriolin B

Coriolin B (**103**) was previously isolated from a culture of the Japanese wood rotting fungus *Coriolus consors* in 1970.[99] The structure of coriolin B (**103**) was revised and corrected in 1971,[103] and the absolute stereochemistry was determined by comparison to the absolute stereochemistry of coriolin A (**110**), determined by X-ray crystallography.[104] Coriolin B (**103**) was found to have antibacterial and cytotoxic properties.

Comparison of the ^{1}H NMR of coriolin B (**103**) with the ^{1}H NMR of dihydrocoriolin C (**104**) indicated they shared many similar features. The same pentacyclic core with the similar attached functionality was apparent in the ^{1}H NMR, Figure 4.13, and ^{13}C NMR, Figure 4.14, of dihydrocoriolin C (**104**). Low and high resolution EIMS indicated an extra 16 amu, Figure 4.15, compared to the molecular weight of coriolin B (**103**), due to an extra oxygen in dihydrocoriolin C (**104**). This oxygen was placed on the octanyl side chain of coriolin B (**103**) to provide the structure of **104**. Dihydrocoriolin C (**104**) was isolated in 1974 during the course of biosynthetic studies on *Coriolus consors*.[100] The NMR data of dihydrocoriolin C (**104**) matched the NMR data of the isolated compound and the second marine fungal compound was determined to be dihydrocoriolin C (**104**).

Figure 4.13. ^{1}H NMR spectrum of dihydrocoriolin C (**104**), 250 MHz, $CDCl_3$.

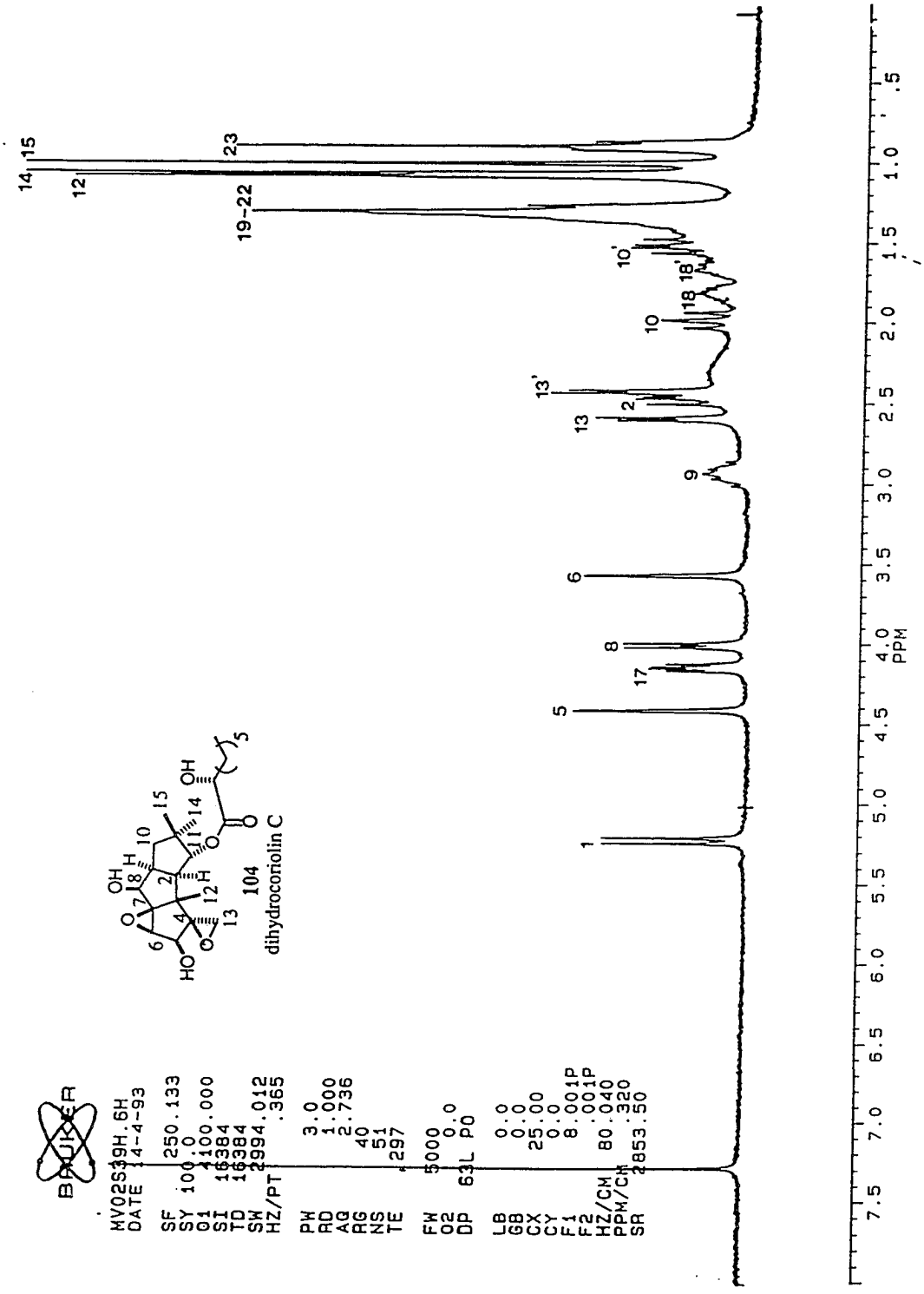


Figure 4.14. ^{13}C NMR spectrum of dihydrocoriolin C (**104**), 62.9 MHz, $CDCl_3$.

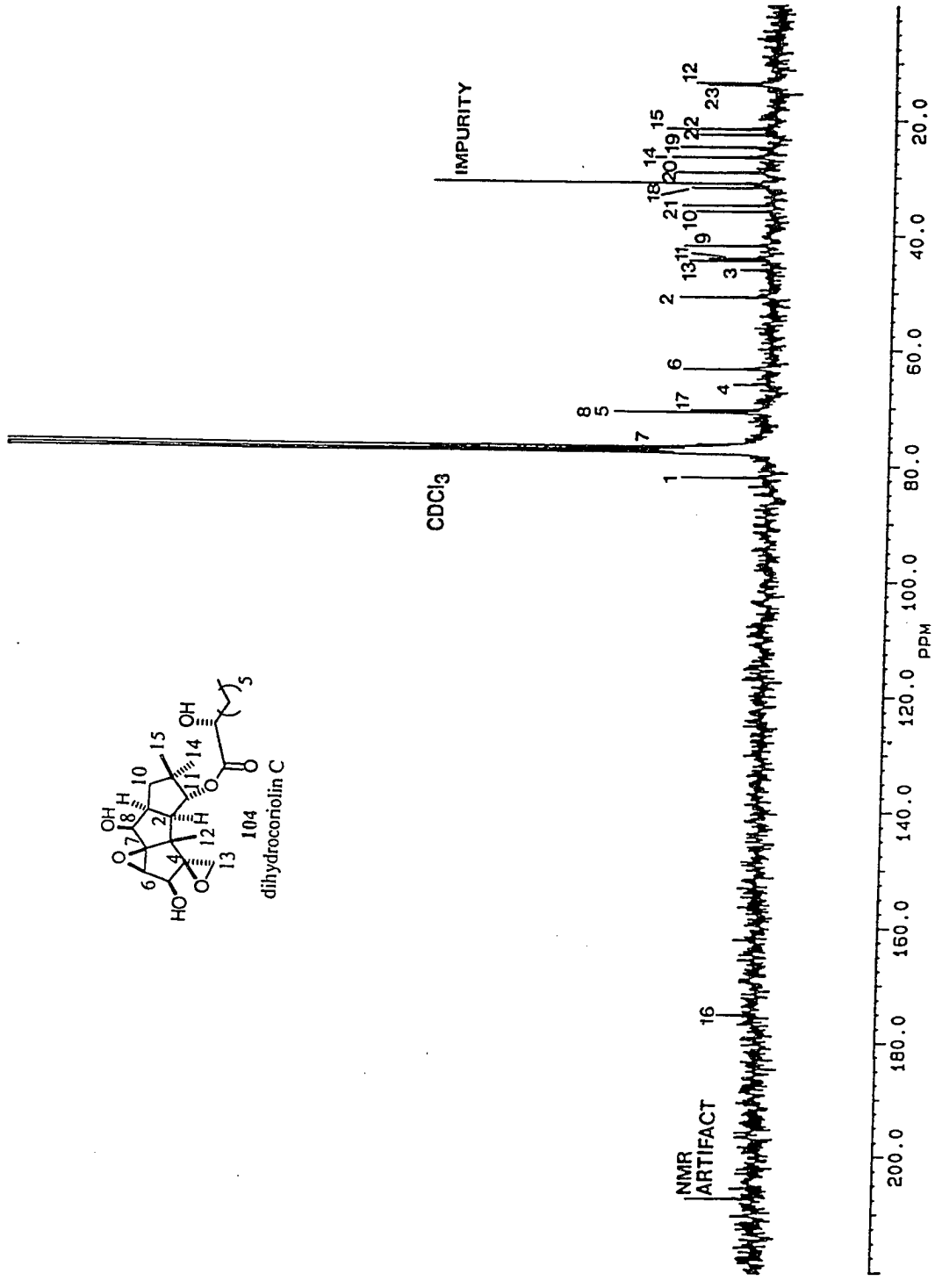
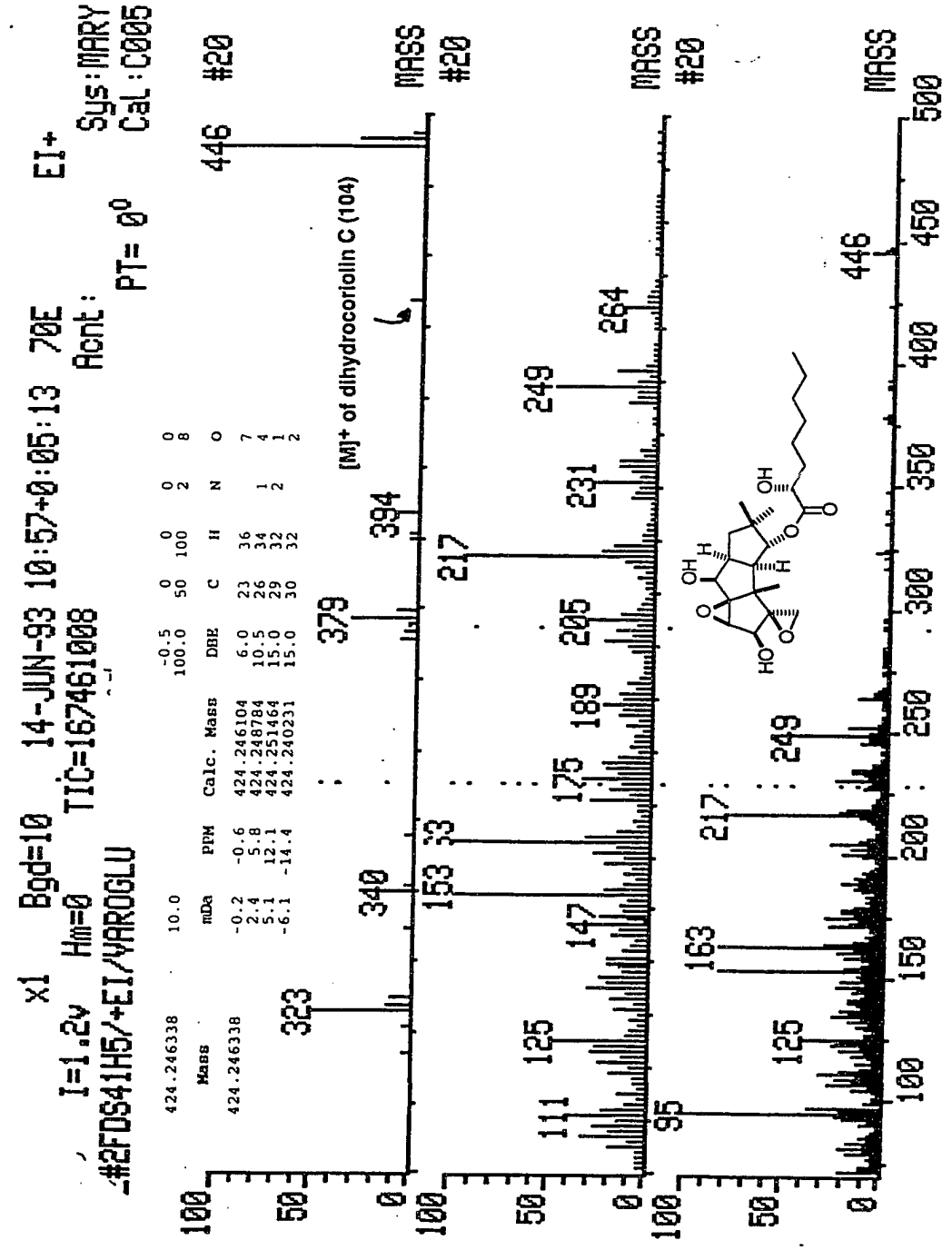
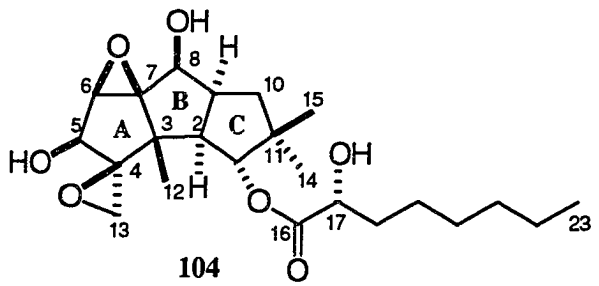


Figure 4.15. Low and high resolution electron impact mass spectra of dihydrocoriolin C (**104**).





104
dihydrocoriolin C

Structure elucidation of chloriolin A-C (105-107)

In a cooperative group effort, Xing Cheng, myself and Phil Crews analyzed the data for chloriolin A (**105**). The previously assigned ^{1}H NMR, Figure 4.9, and ^{13}C NMR Figure 4.7, spectra for coriolin B (**103**) were used as models for the structure elucidation of the novel chloriolins (**105-107**). All the resonances of a mutually coupled spin system consisting of H1-H2-H9-H10/10'-H8 present in coriolin B (**103**) could also be located in the ^{1}H NMR spectrum of chloriolin A (**105**), Table 4.2, Figure 4.16. This allowed the assignment of a bicyclic [3.3.0] ring. A partial formula, $C_{14}H_{19}$ established from ^{13}C NMR and the APT spectra, Figures 4.17 and 4.18, of chloriolin A (**105**) agreed with the intense HRFABMS $[M+H]^{+}$ = 273.1254 corresponding to a molecular formula of $C_{14}H_{21}O_3Cl$, Figure 4.19. The remaining two unsaturations consisted of a trisubstituted double bond (δ 147.0, s, C7; 130.3, d, C8) and a ketone carbonyl (δ 202.5, s, C4).

Figure 4.16. ^1H NMR spectrum of chloriolin A (**105**), 250 MHz, in $CDCl_3$, benzene-d_6 and dioxane-d_8.

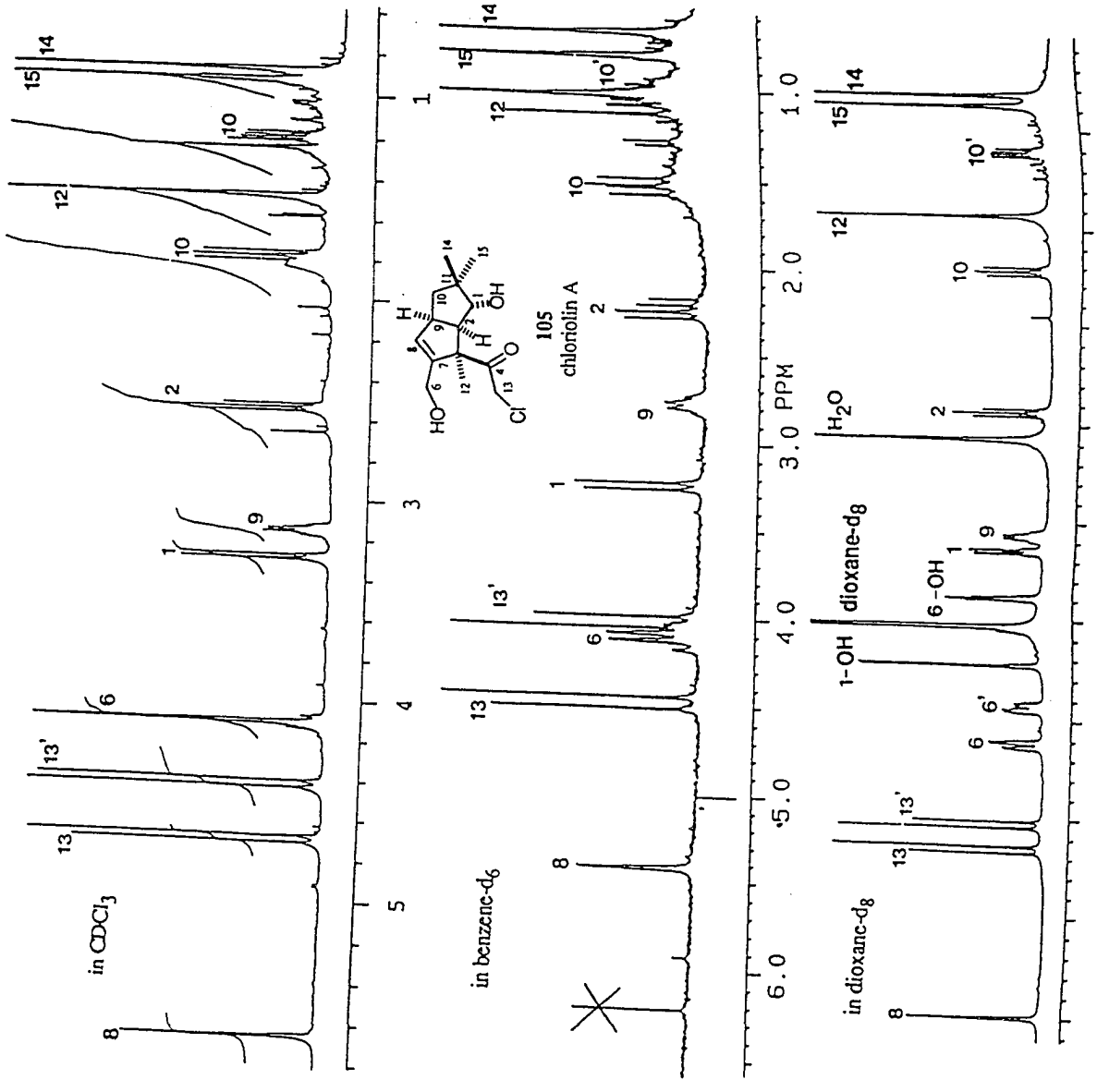


Figure 4.17. ^{13}C NMR of chloriolin A (**105**), 125 MHz, dioxane-d_8.

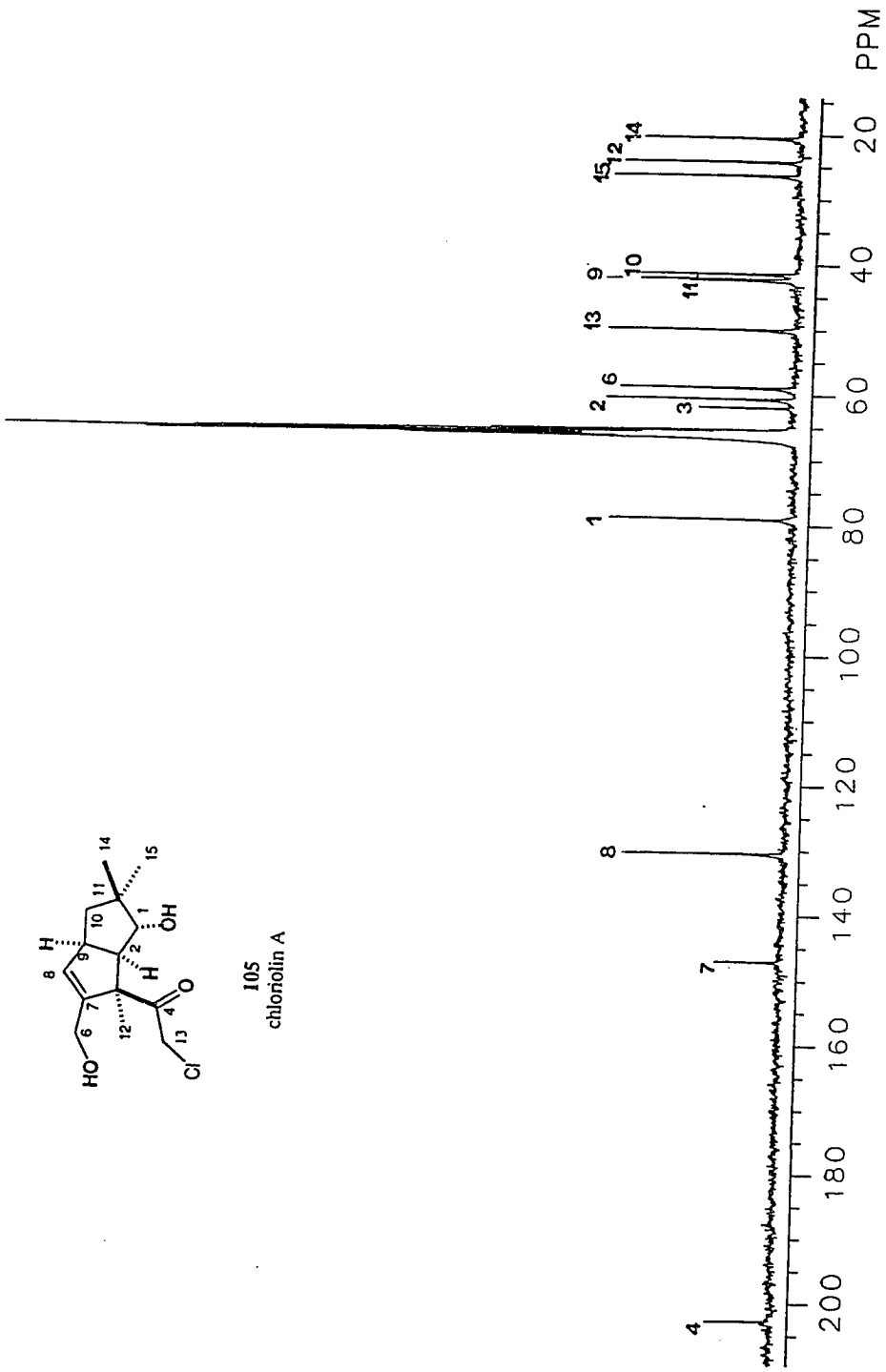


Figure 4.18. APT spectrum of chloriolin A (**105**), 62.9 MHz, $CDCl_3$.

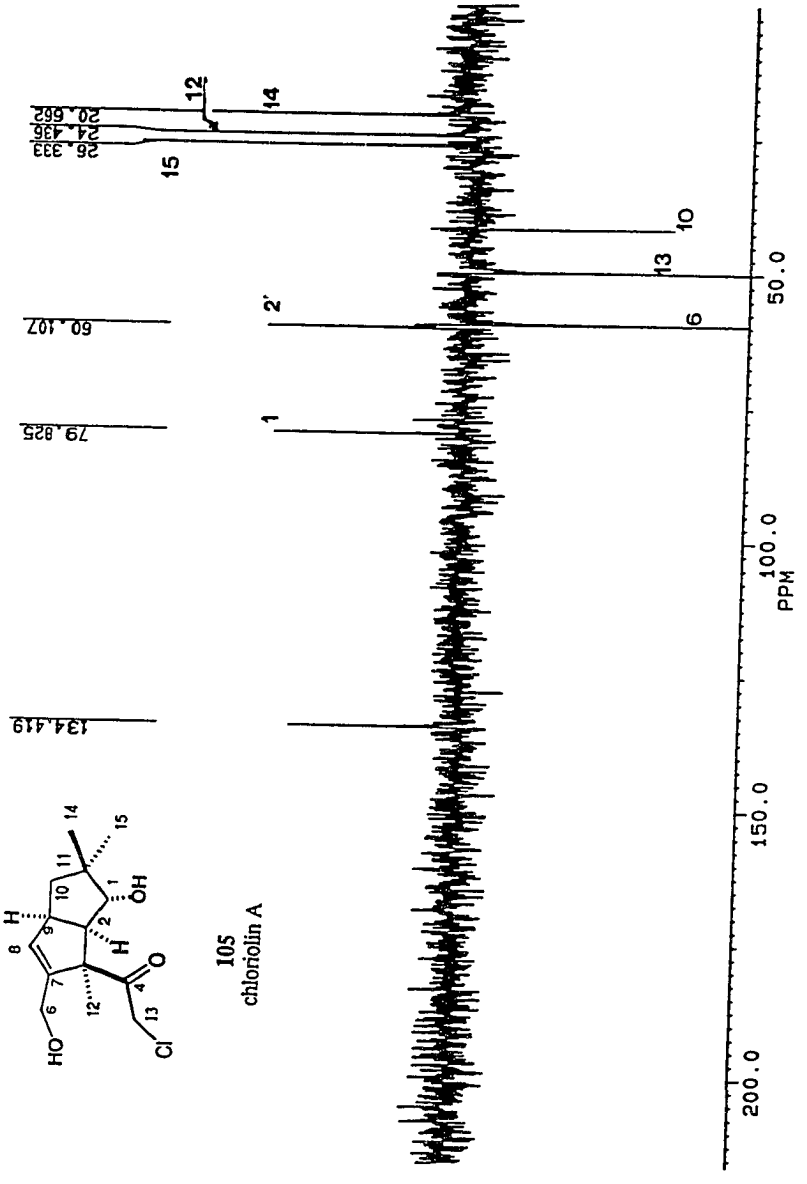


Figure 4.19. Low and high resolution FABMS of chloriorin A (**105**), dithiothreotol/dithioerythrotol matrix.

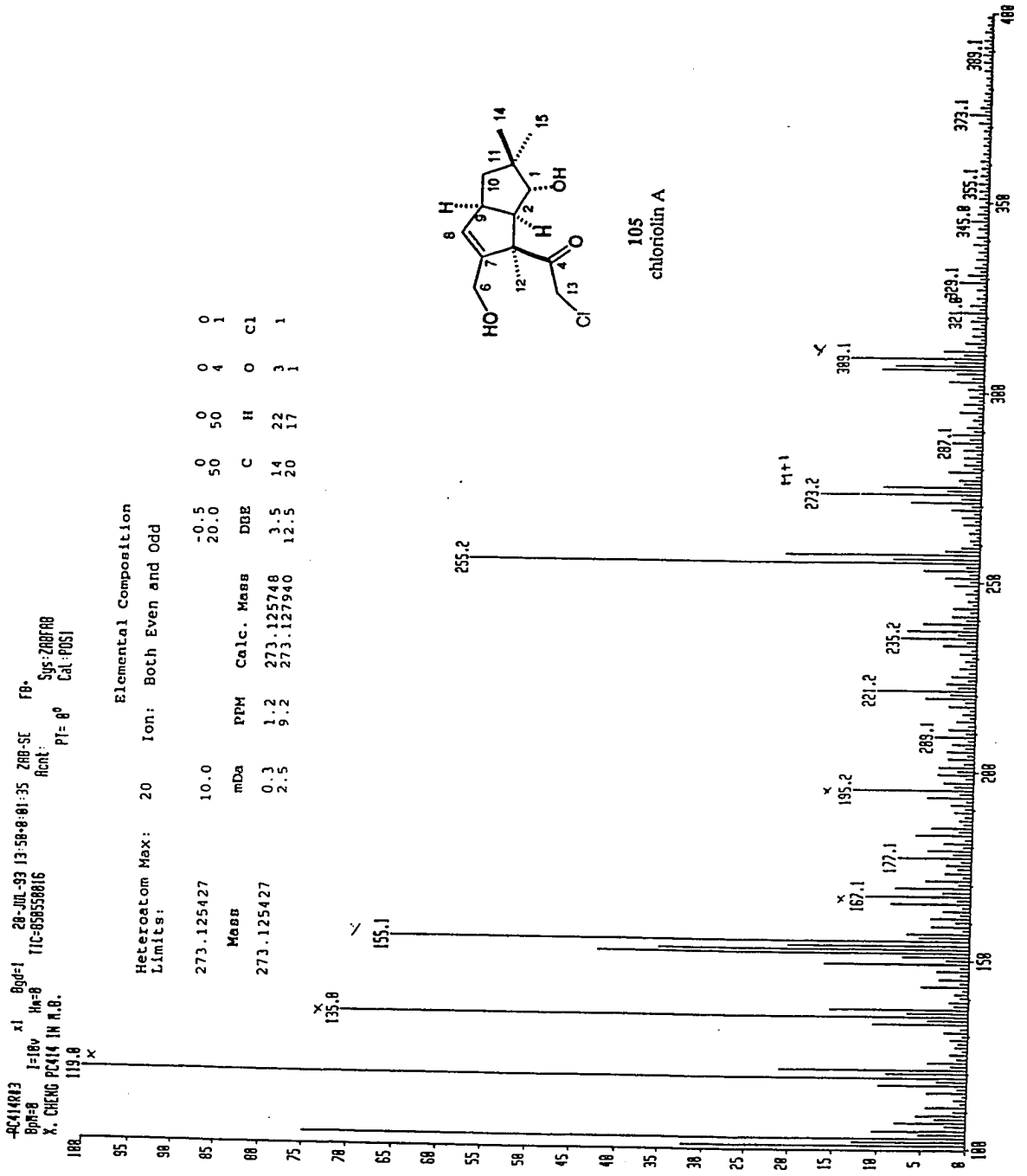


Table 4.2. NMR assignments for chloriolin A (**105**) and comparative assignments of coriolin B (**103**).

	coriolin B (**103**)	chloriolin A (**105**)			
Atom #	^{1}H NMR[a]	^{1}H NMR[b]	^{13}C NMR[c]	^{1}H-^{1}H COSY	^{1}H-^{13}C COSY (J=9)
1	5.3 (d, 8.3)	3.15 (dd, 9.0, 4.5)	78.9 (d)	H2	
2	2.40 (dd, 8.4, 12.0)	2.43 (dd, 12.0, 9.0)	60.5 (d)	H1,H9	C1, C7, C8, C12
3	—	—	61.8 (s)		
4	—	—	202.5 (s)		
5	4.40 (d, 1.8)	not present[d]	not present[d]		
6	3.55 (d, 1.8)	4.10 (dd, 14.0, 3.2)	58.8 (t)	H8	
6'	--	3.94 (dd, 14.0, 6.6)			
7	--	--	147.0 (s)		
8	3.99 (d, 6.4)	5.5 (bs)	130.3 (d)	H6,H6',H9	C3, C10
9	2.92 (m)	3.05 (m)	41.8 (d)	H8,H10,H10'	
10	1.93 (t, 12.4)	1.72 (dd, 9.6, 12.7)	41.7 (t)	H9	C1, C9, C14
10'	1.48 (dd, 12.4, 8.3)	1.10 (dd, 7.2,12.7)		H9	
11	—	—	42.3 (s)		
12	1.08 (s)	1.42 (s)	24.1 (q)		
13	2.59 (d, 4.8)	4.65 (d, 16.0)	49.9 (t)		C4
13'	2.46 (d, 4.8)	4.52 (d, 16.0)			
14	0.98 (s)[e]	0.82 (s)	20.4 (q)		
15	1.04 (s)[e]	0.87 (s)	26.2 (q)		
1-OH		3.72 (d, 4.5)	—		
6-OH		3.35 (t, 5.4)	—		

[a] $CDCl_3$. [b] dioxane-d8, 500 MHz. [c] dioxane-d8, 125 MHz. [d] nucleus 5 was excluded to match the numbering scheme of coriolin B (**103**). [e] may be switched.

Three sets of ^{1}H NMR spectra were eventually examined as changing solvents from $CDCl_3$ to benzene-d_6 to dioxane-d_8 revealed separate resonances for the lowfield diastereotopic protons of C6 and C13, Figure 4.16. The dioxane-d_8 spectrum also revealed the vicinal couplings to the two OH groups at C1 and C6. Proper attachment of the bicyclic ring substituents consisting of a quaternary methyl (δ 1.42), gem dimethyls (δ 0.82, 0.87), a hydroxymethylene and chloroacetyl was guided by 2D NMR correlations contained in the ^{1}H-^{1}H COSY, Figure 4.20, and HMBC, Figure 4.21, (Table 4.2) summarized in Figure 4.22.

Figure 4.20. ^{1}H-^{1}H COSY spectrum of chloriolin A (**105**), 500 MHz, dioxane-d_8.

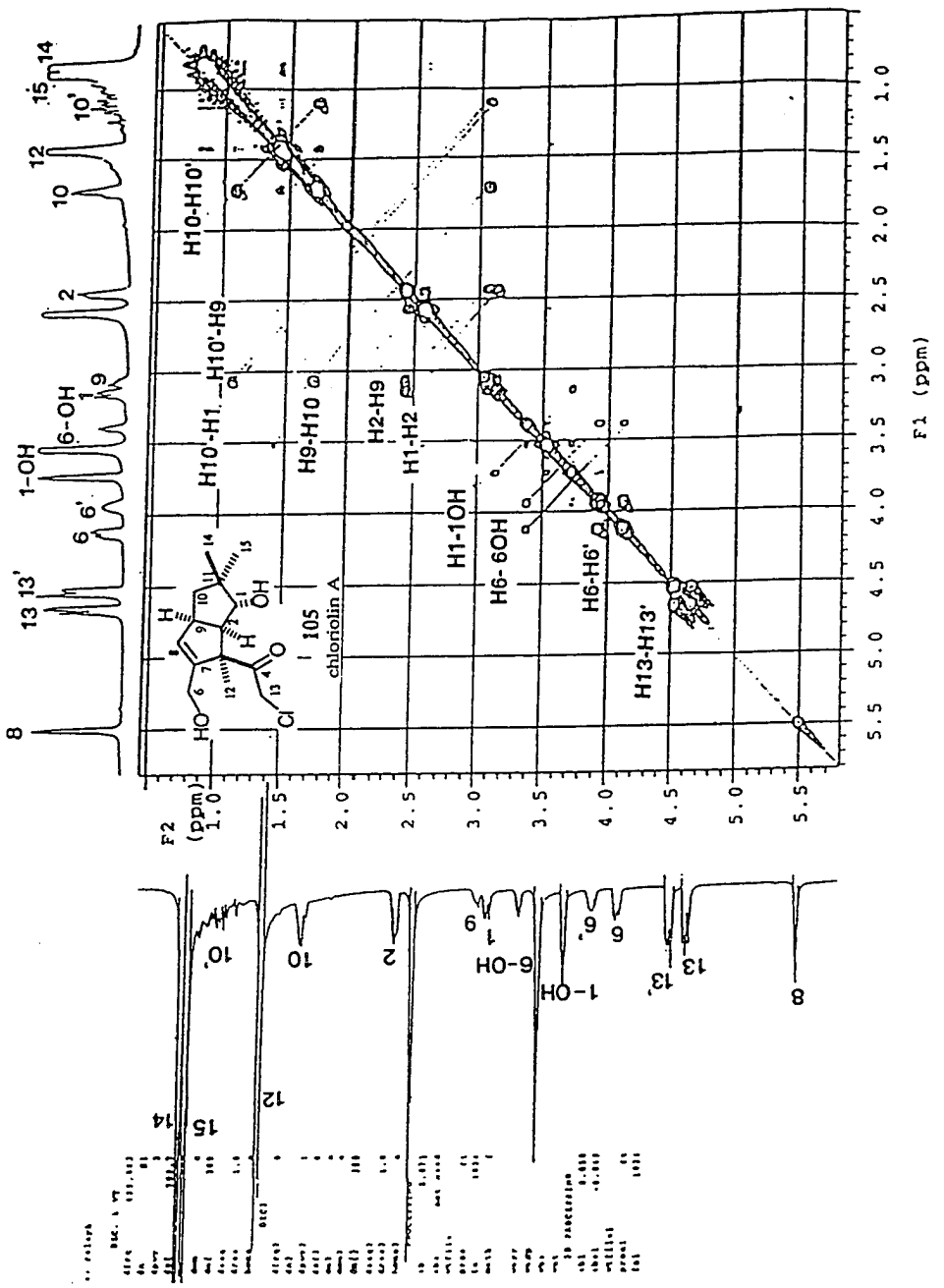
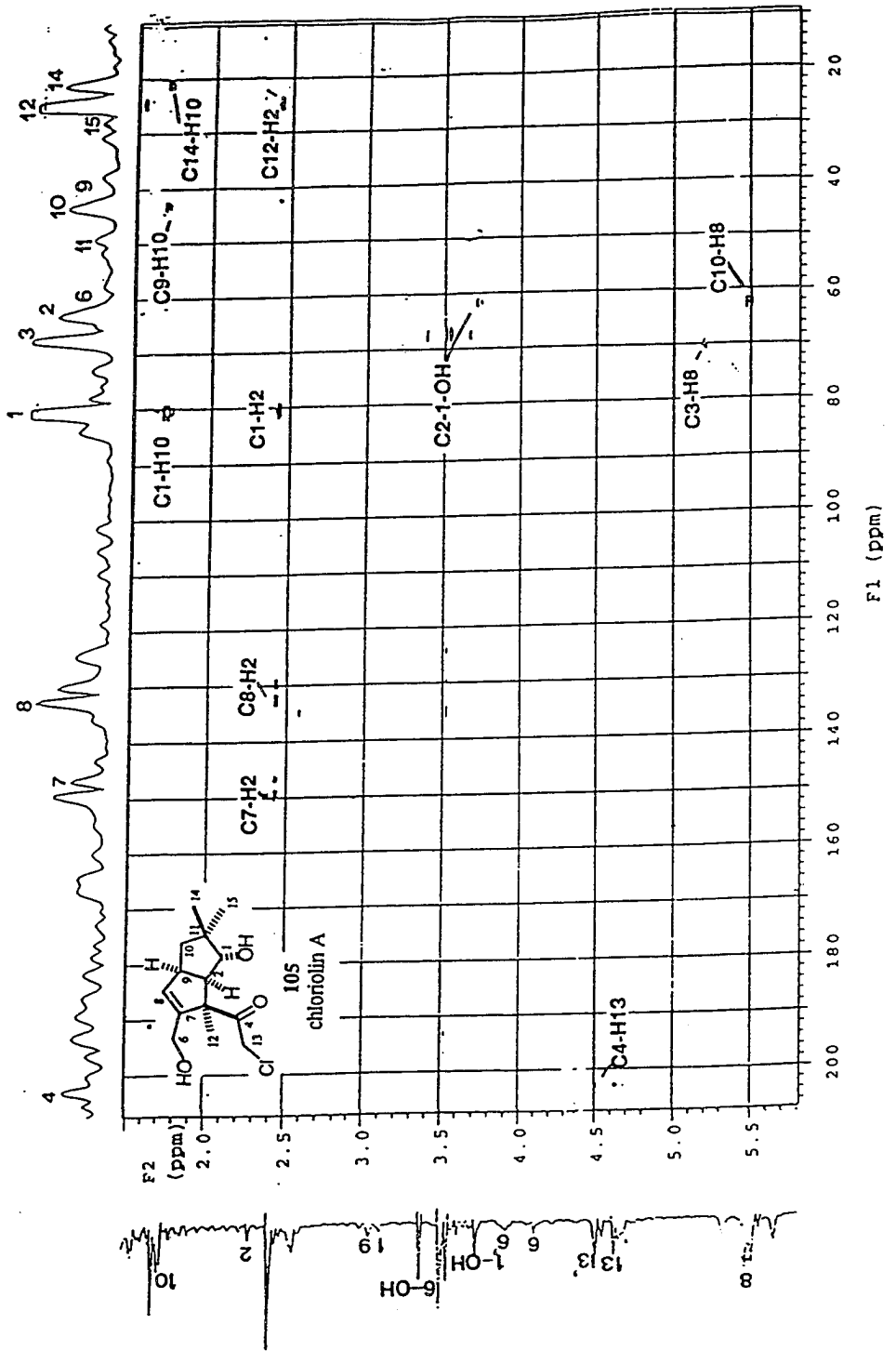


Figure 4.21. HMBC spectrum of chloriolin A (**105**), 500 MHz, dioxane-d_8.



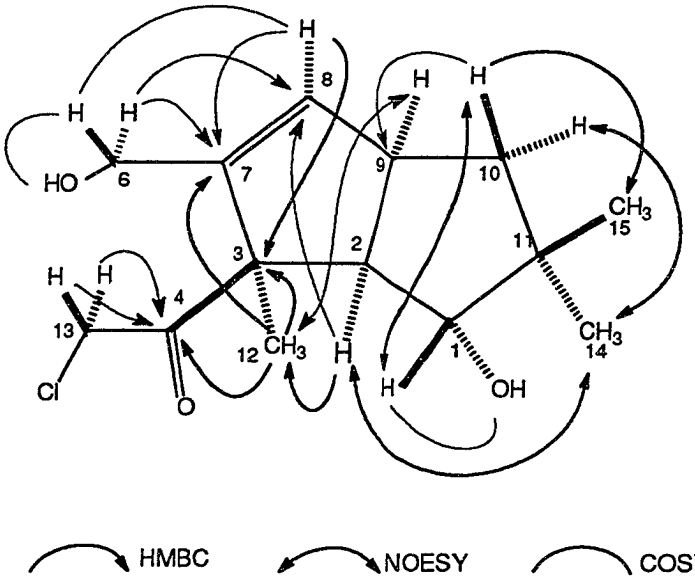
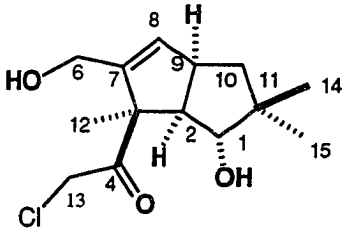


Figure 4.22. Summary of HMBC, NOESY, and ^{1}H-^{1}H COSY correlations for chloriolin A (**105**).

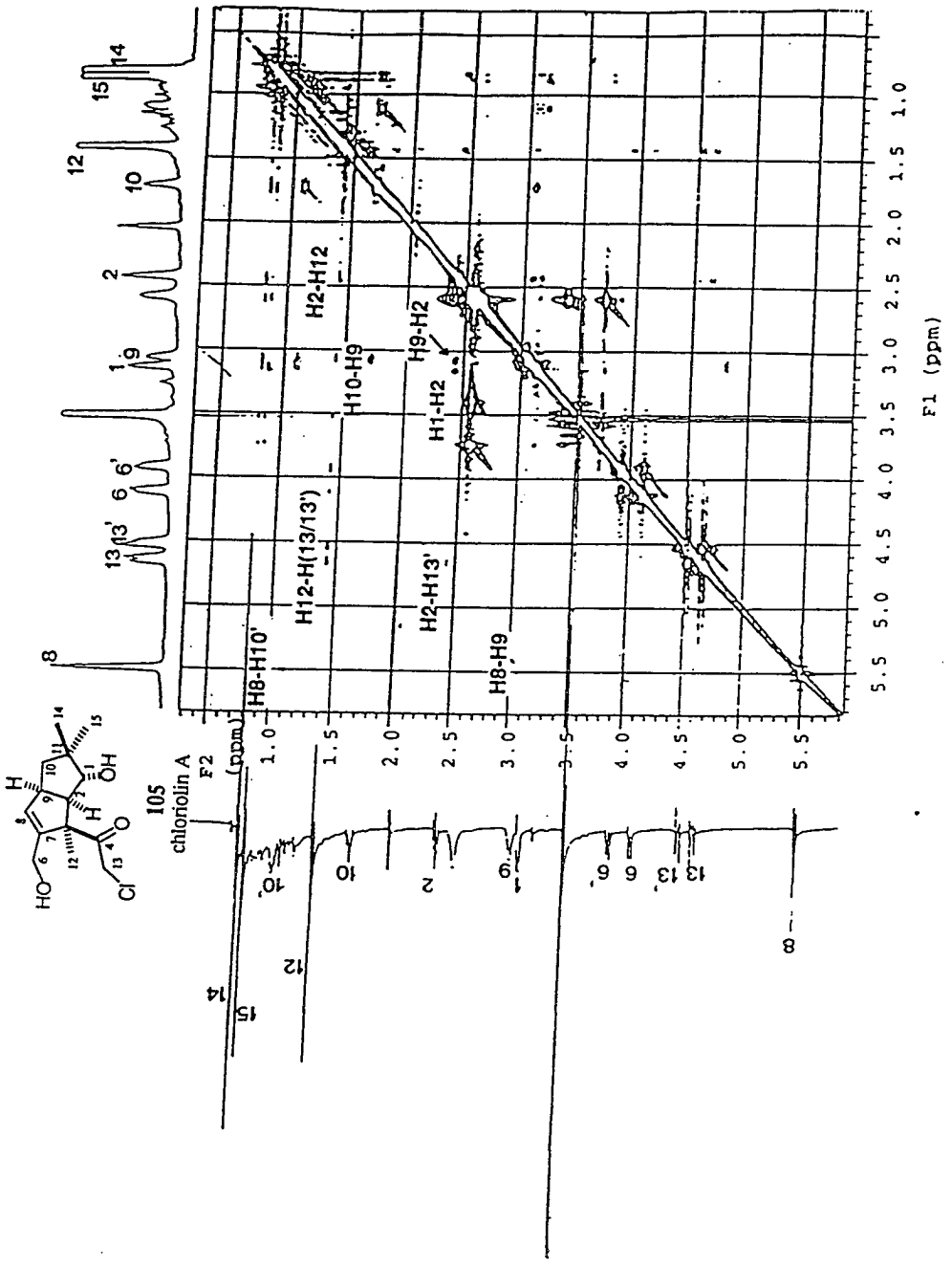
The nOe difference and NOESY ^{1}H NMR correlations, Figure 4.23, observed between the various methine, methylene and methyl protons, as outlined in Figure 4.23, supported the relative stereochemistry shown for chloriolin A (**105**).



105
chloriolin A

In order to gain additional evidence to justify the proposed structure and to determine the absolute stereochemistry of chloriolin A (**105**) an X-ray structure was obtained. Crystals suitable for X-ray diffraction analysis were eventually deposited by slow evaporation of a methanol solution of chloriolin A (**105**). Xing Cheng sent these crystals to Jon Clardy (Cornell

Figure 4.23. NOESY spectrum of chloriolin A (**105**), 500 MHz, dioxane-d_8.



University) who determined that the space group of the crystals was orthorhombic, a = 7.943 (5), b = 11.049(12), c = 16.440 (12)Å and they belonged to space group $P2_12_12_1$. The X-ray structure of chloriolin A (**105**) was solved by direct methods and refined to a final R-factor of 4.7%. The absolute structure parameter of 0.00 (4) indicated that the absolute configuration is correct as shown (the same as in coriolin B (**103**)[104] at C1, C2 and C9). A computer generated perspective drawing of the final X-ray model is shown in Figure 4.24.

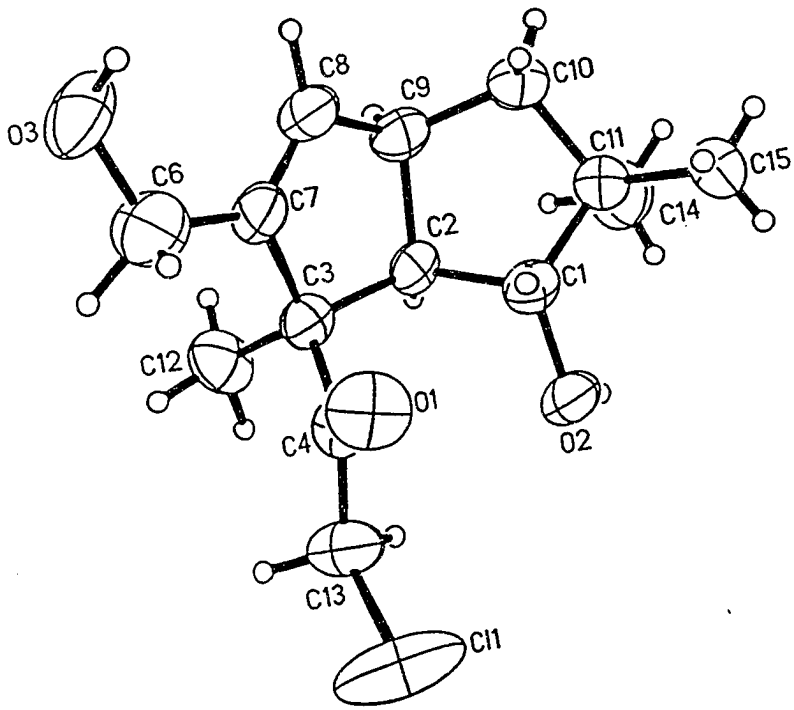


Figure 4.24. Computer generated model of X-ray structure of chloriolin A (**105**).

The structure determination of the chloriolins B (**106**) and C (**107**) isolated by Xing Cheng was next undertaken. This was initially a cooperative group effort between Xing Cheng, myself and Phil Crews, however after Xing Cheng left UCSC the final structures of chloriolin B (**106**) and C (**107**) were determined by myself and Phil Crews.

Side-by-side comparison of the ^{1}H NMR and ^{13}C NMR data of chloriolin B (**106**), Figures 4.25 and 4.26, and chloriolin C (**107**), Figures 4.27 and 4.28, showed that all three chloriolins had the common spin system, H1-H2-H9-H10/10'-H8. The HRFABMS $[M+H]^+$ peak at 459.2164 of chloriolin B (**106**) indicated a molecular formula of $C_{23}H_{36}O_7Cl$, Figure 4.29, while the HRFABMS peak at 443.2192 $[M+H]^+$ of chloriolin C (**107**) gave a molecular formula of $C_{23}H_{36}O_6Cl$, Figure 4.30. Due to the greater availability of chloriolin B (**106**) the structures of both chloriolin B (**106**) and C (**107**) were solved by analysis of the chloriolin B (**106**) spectral data. Five of the six unsaturations of chloriolin B (**106**) were accounted for by the B and C rings, an ester carbonyl (C16, δ 175.1), a tetrasubstituted double bond (C6, δ 124.3; C7, δ 183.4), and a ketone (C5, δ 204.3), Figure 4.26. It was apparent that the octanyl ester was attached at C1 from the relatively lowfield shift of H1 (δ 5.18) plus the various HMBC correlations shown in Figures 4.31-4.33. The remaining unsaturation was ascribed to the presence of ring A fused to ring B analogous to that in coriolin B (**103**). Determining the substitution pattern of ring A required dioxane-d_8 ^{1}H NMR spectral data wherein the coupling to the OH protons could be seen, Figure 4.25. The important anchor points were the ^{1}H NMR resonances of methyl-12 (δ 1.24), the AB spin system of H13/13' (δ 3.39, 3.53), the OH at C4 (δ 4.60) and the OH at C8 (δ 4.55). The HMBC correlations from C4 (δ 86.4), C3 (δ 54.1), and C2 (δ 48.2) to methyl-12 showed that this methyl must be attached to C3, Figures 4.31-4.33. In addition, HMBC correlations involving both methyl-12 and H13/13' defined the C3-C4 connectivity as shown in Figures 4.31 and 4.33. Likewise the correlations from methyl-12 to C3 and C7 defined the C3-C7 connectivity. That both an -OH and a $-CH_2OH$ must be attached to C4 was supported by HMBC correlations from the δ 4.60 OH to C13 (δ 66.3) and to C4, from the δ 3.72 OH to C13, plus the correlation from H13 to C4, Figures 4.31-4.33. An additional correlation from the OH at C4 to C5 revealed that the ketone was adjacent to C4. Finally, the chlorine was assigned to the only remaining atom C6. It was apparent from ^{1}H, ^{13}C, and ^{1}H-^{1}H COSY NMR data and mass spectral data that

Figure 4.25. ^{1}H NMR spectrum of chloriolin B (**106**), 500 MHz, dioxane-d_8 and CD_3OD.

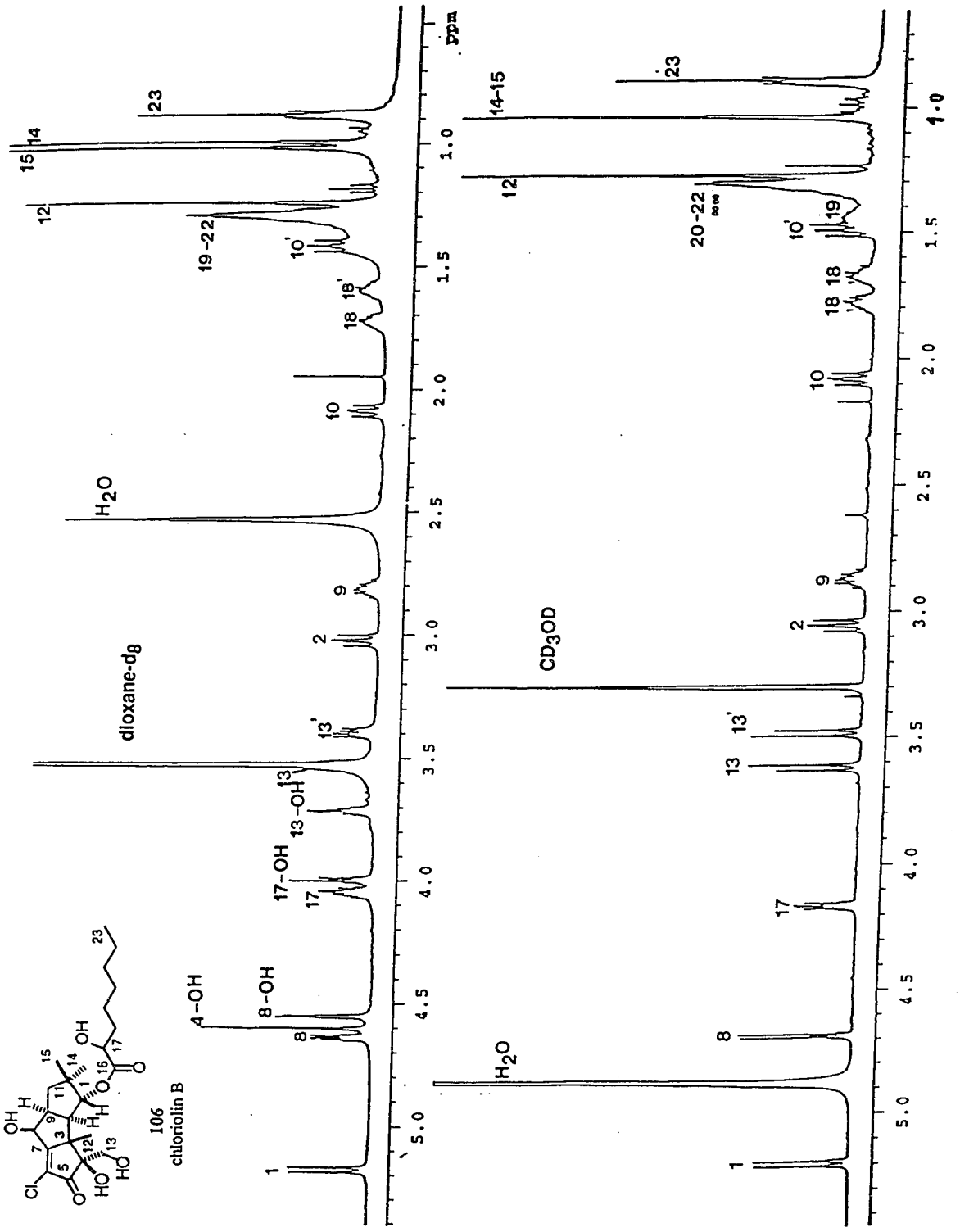


Figure 4.26. ^{13}C NMR spectrum of chloriolin B (**106**), 125 MHz, dioxane-d_8.

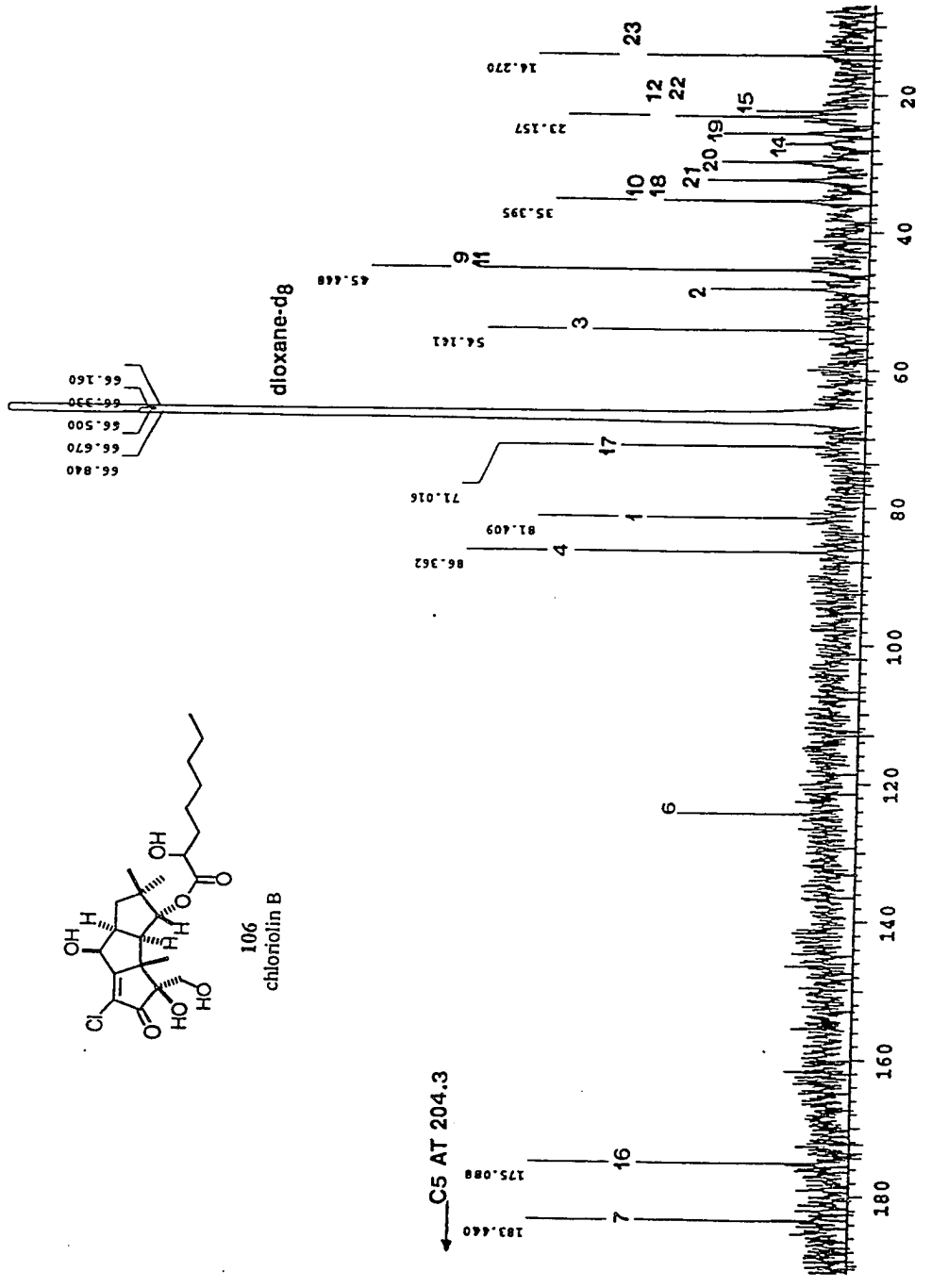


Figure 4.27. ^{1}H NMR spectrum of chloriolin C (**107**), 500 MHz, dioxane-d_8.

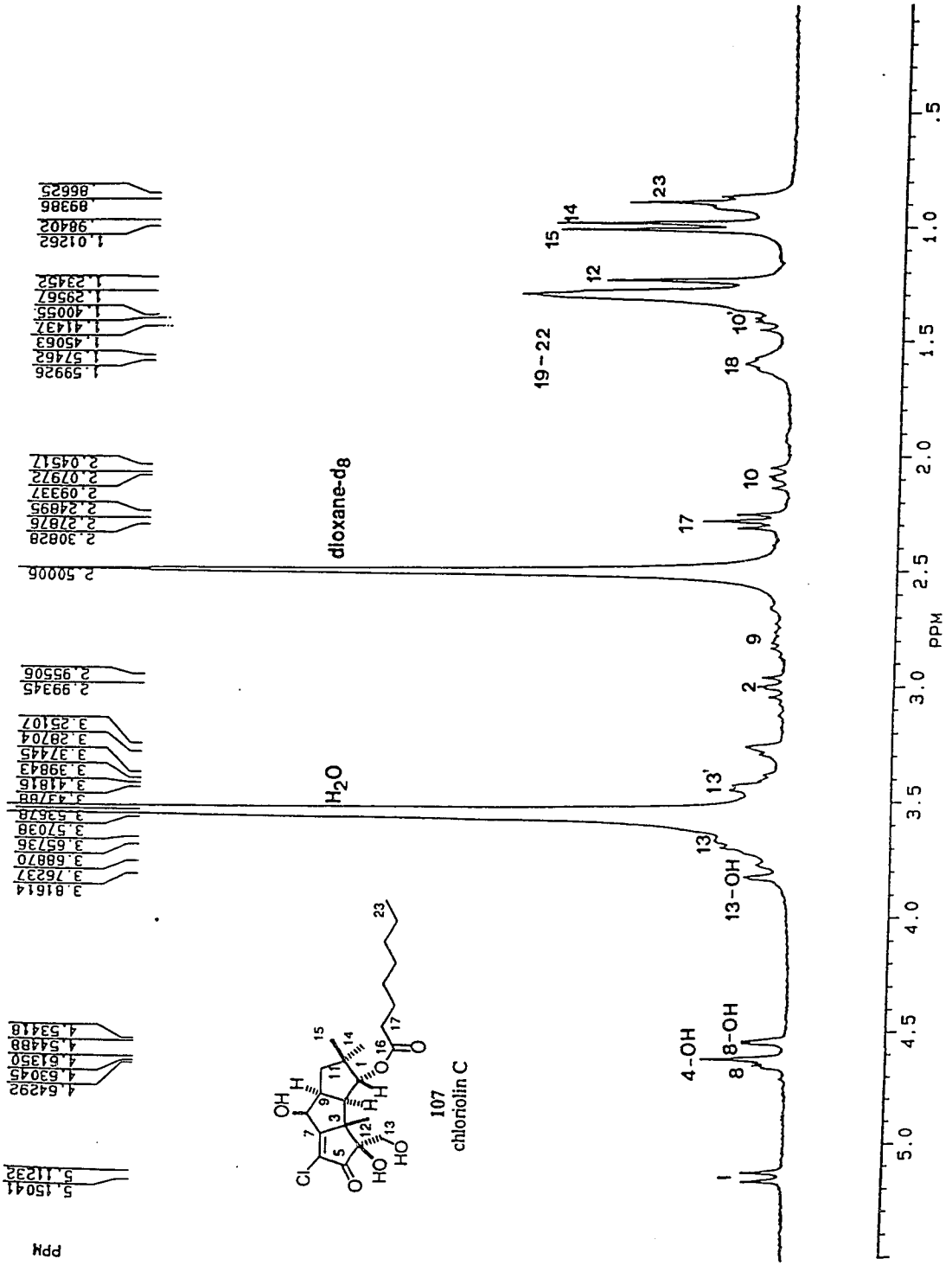


Figure 4.28. ^{13}C NMR spectrum of chloriolin C (**107**), 125 MHz, $CDCl_3$.

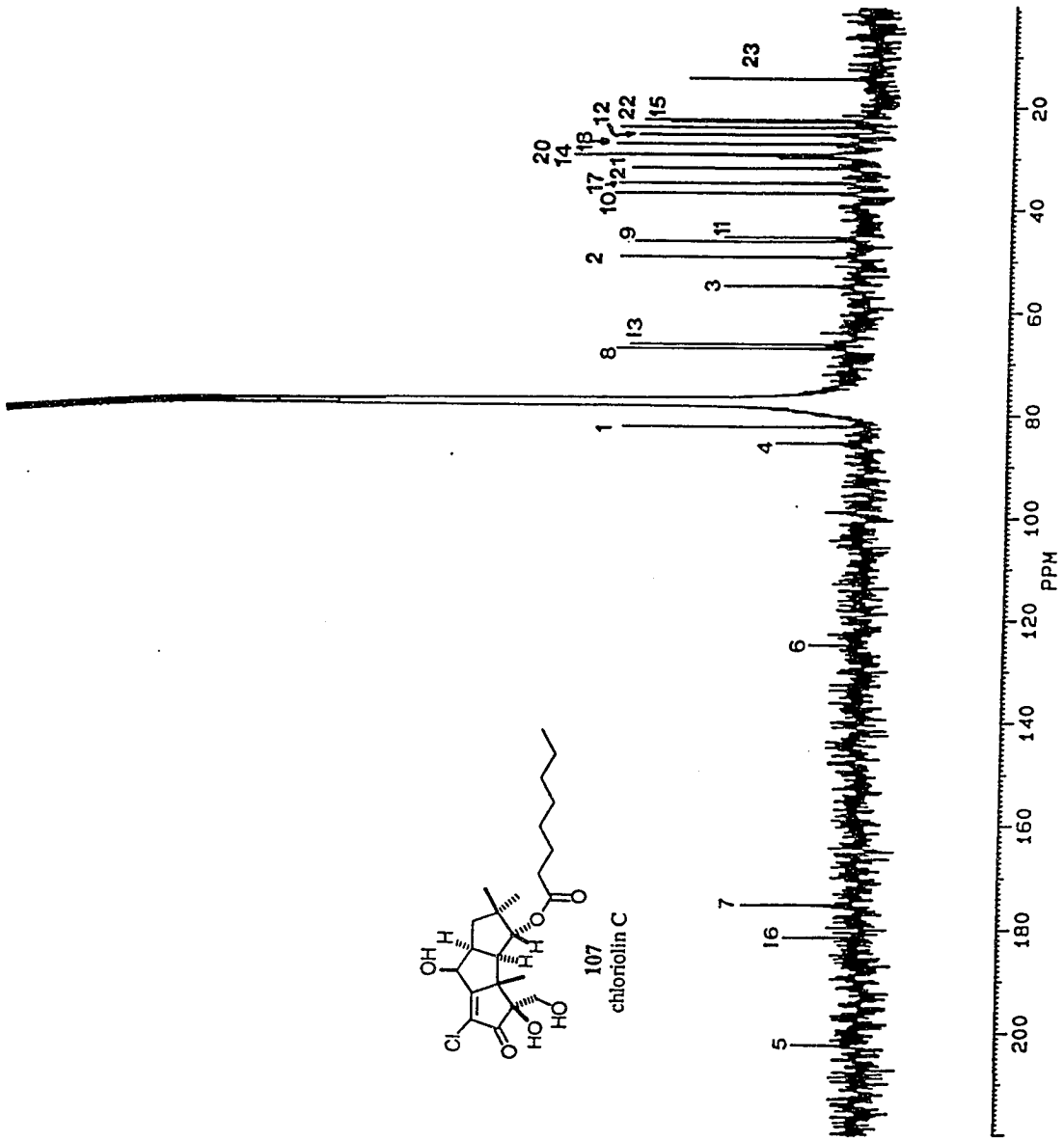


Figure 4.29. Low and high resolution FAB MS of chloriolin B (**106**), matrix dithiothreotol/dithioerythrotol.

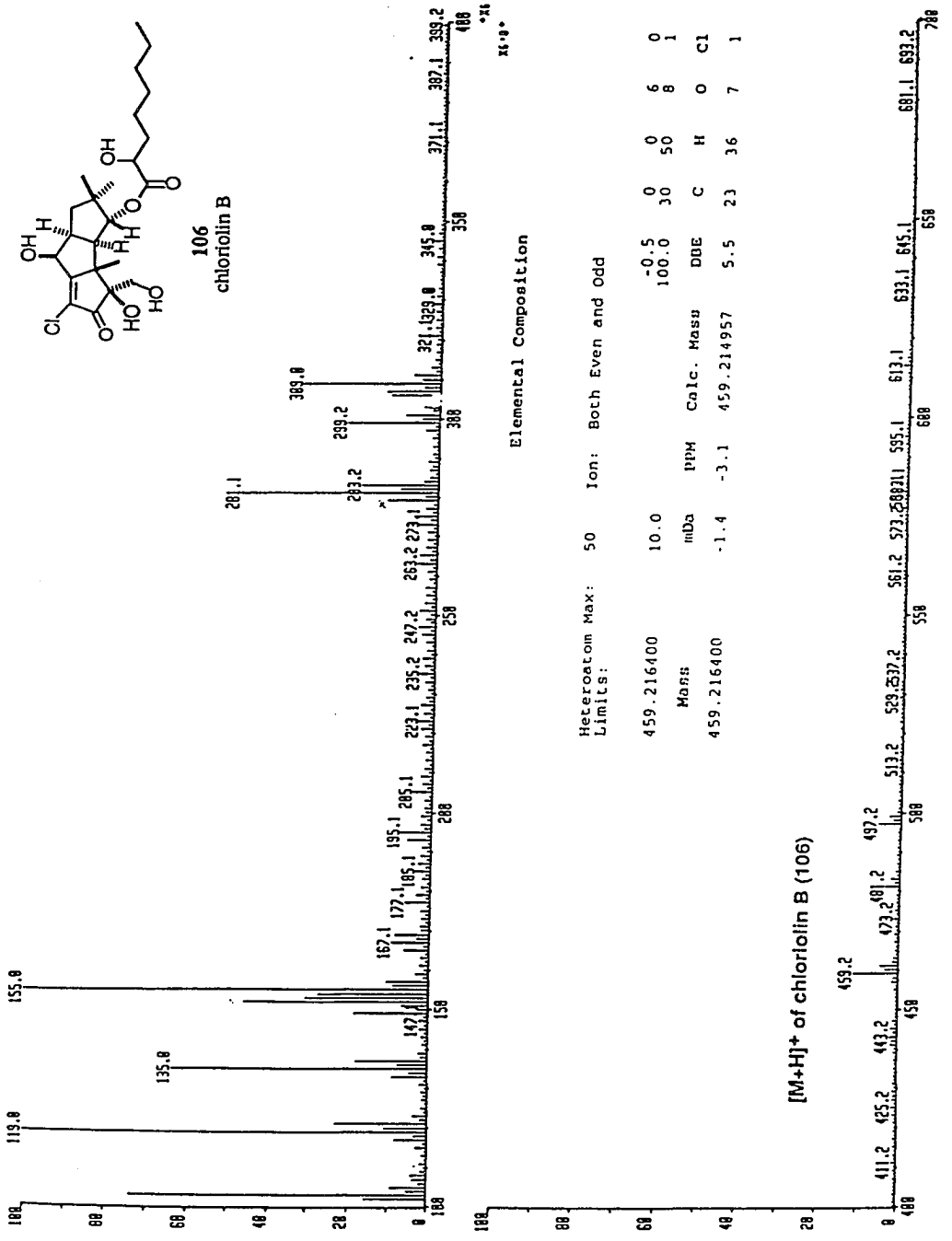


Figure 4.30. Low and high resolution FAB MS of chloriolin C (**107**), matrix dithiothreotol/dithioerythrotol.

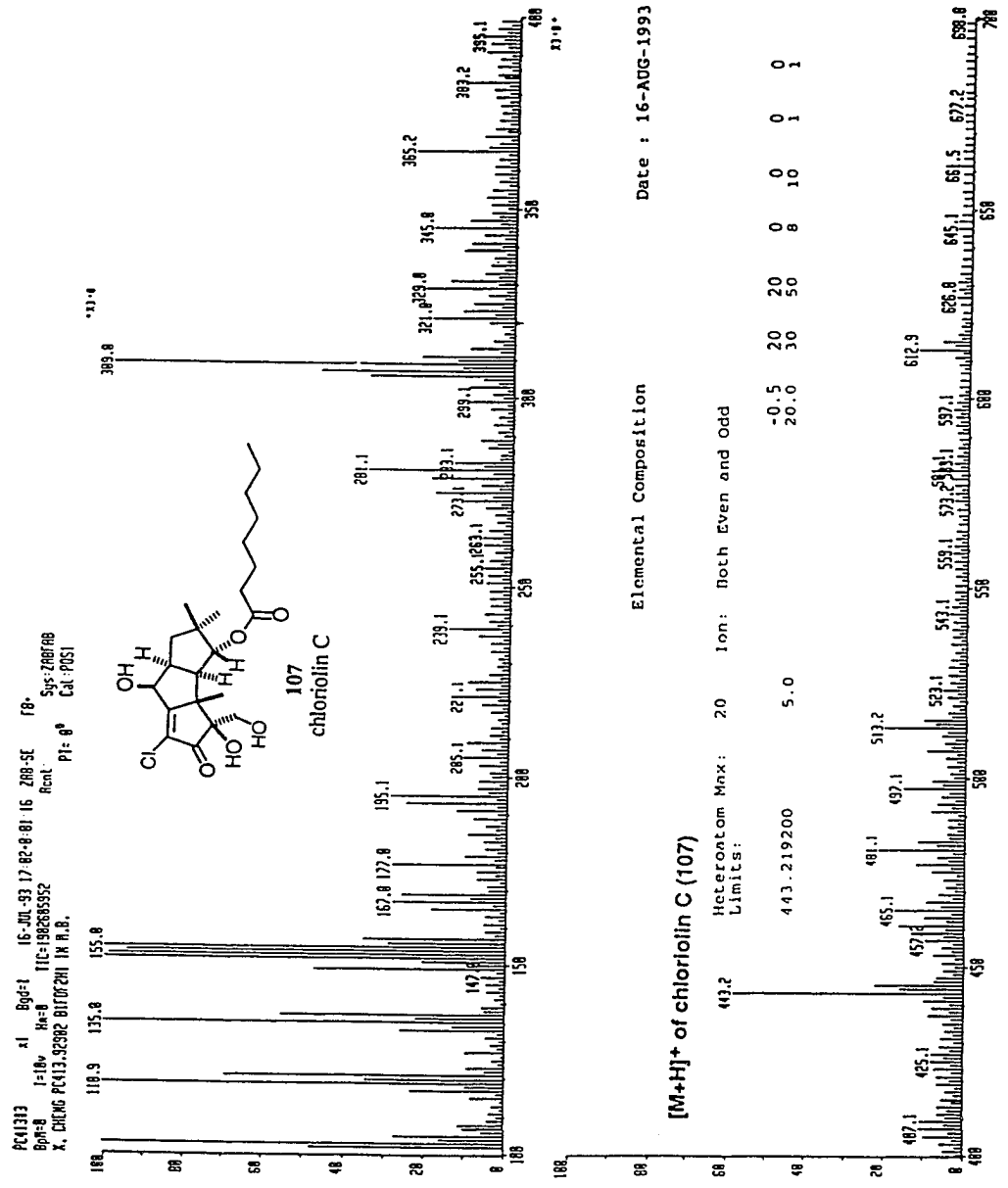


Figure 4.31. HMBC spectrum of chloriolin B (**106**), ^1H region 0 to 2.2 ppm, 500 MHz, dioxane-d_8.

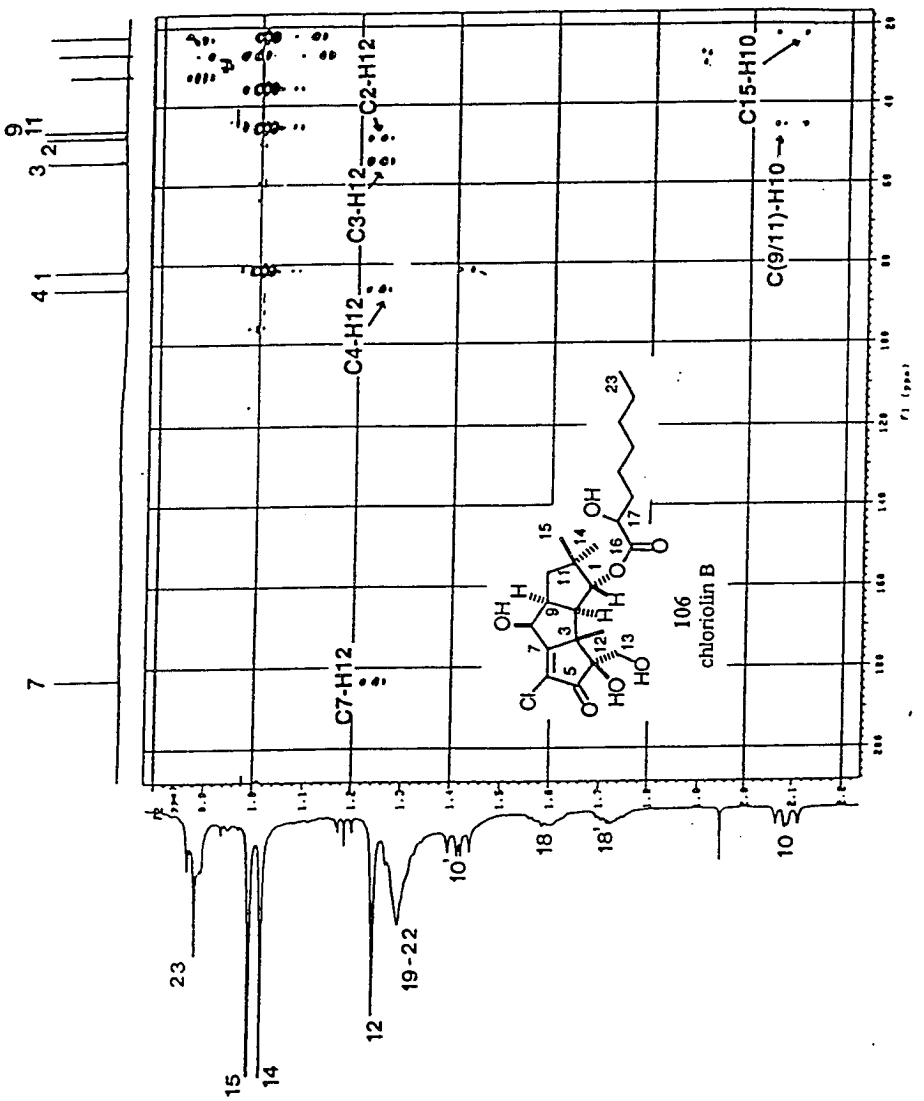
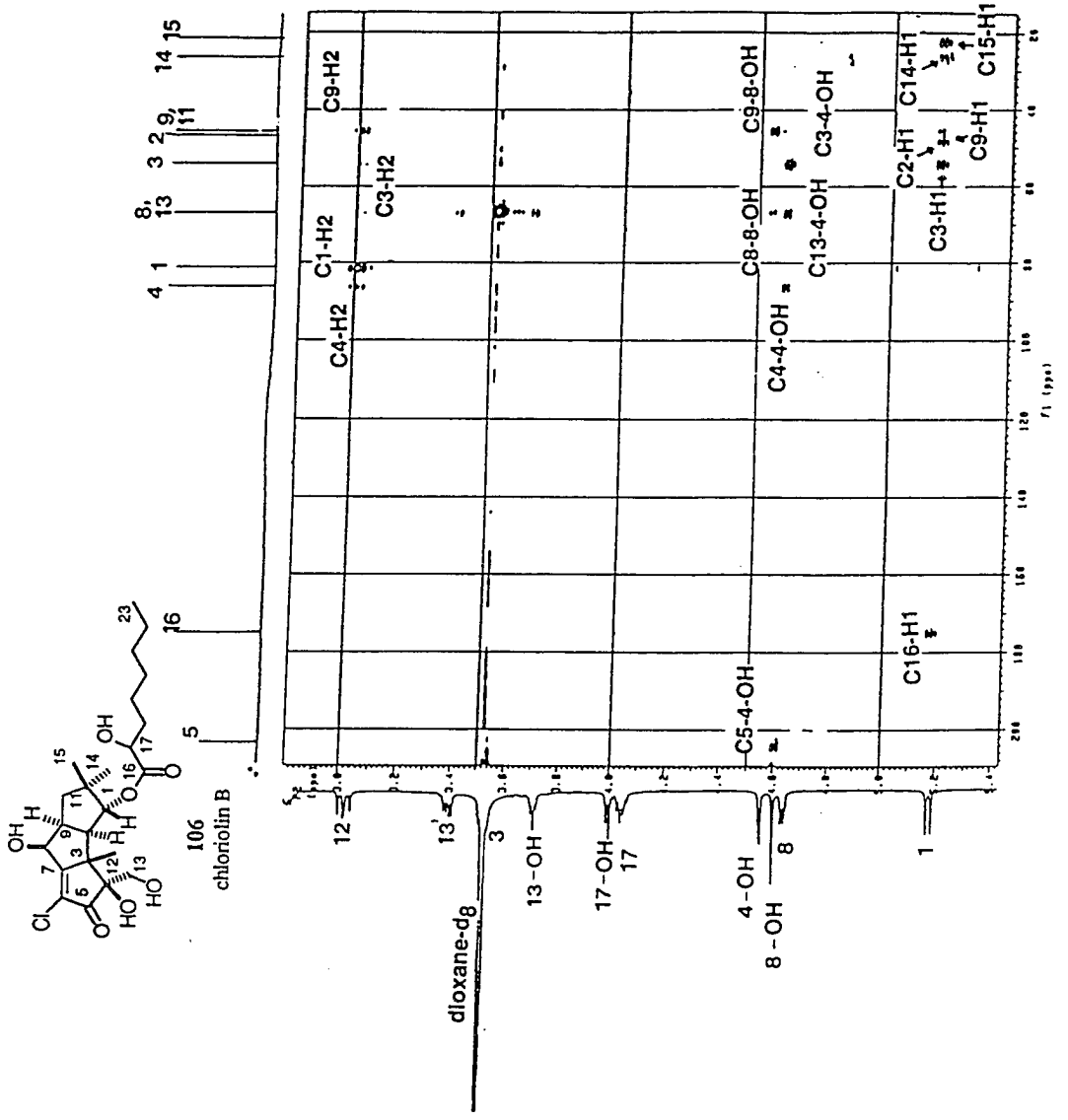


Figure 4.32. HMBC spectrum of chloriolin B (**106**), ^1H region 3.0 to 5.5 ppm, 500 MHz, dioxane-d_8.



chloriolin B (**106**) and C (**107**) differed from one another only by the presence of a hydroxyl group on the octanyl ester.

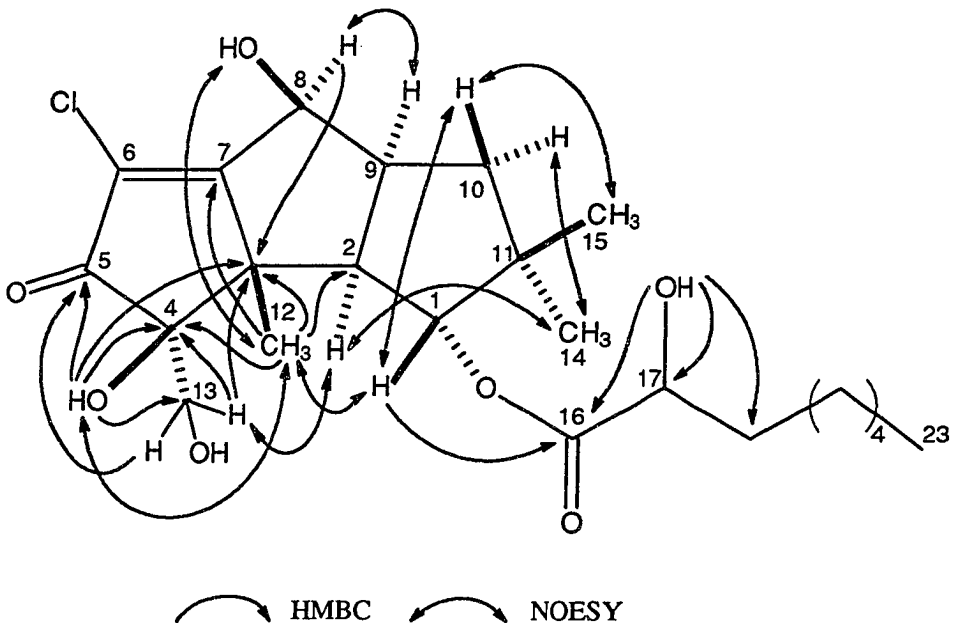
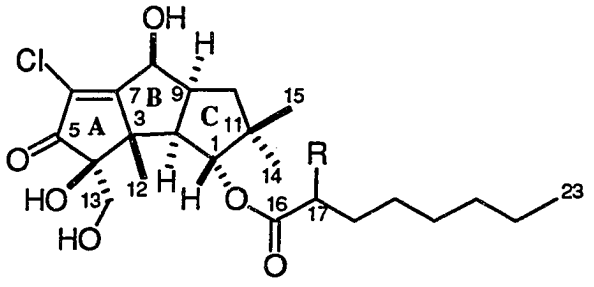


Figure 4.33. Summary of important HMBC and NOESY correlation of chloriolin B (**106**).

NOESY data obtained in dioxane-d_8 was used to assign the relative stereochemistry of chloriolin B (**106**). The configurations of the B and C rings were found to be identical to that of coriolin B (**103**), Figure 4.33. The methyl-12 was determined to be on the same face as H1 from the NOESY correlations between H1 (δ 5.18) and methyl-12 and between the OH at C8 and methyl-12. The stereochemistry at C4 was set by a correlation from methyl-12 to the OH at C4. An important correlation between H2 (δ 3.02) and H13' established the syn stereochemistry between the hydroxymethylene at C4 and H2, Figure 4.33. A parallel stereochemistry was envisioned for chloriolins B and C because their ^{13}C and ^{1}H spectra were quite similar. It is noteworthy that the stereochemistry of methyl-12 in chloriolin B (**106**) and C (**107**) is the same as that found in coriolin B (**103**), however it is the opposite of that found in chloriolin A (**105**). Also,

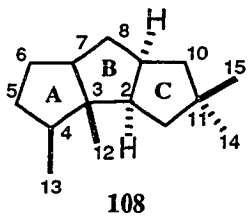
the stereochemistry of the C4 substituents is the same in chloriolin B (**106**) and C (**107**) and coriolin B (**103**).



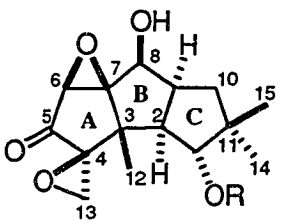
106 chloriolin B R = OH
107 chloriolin C R = H

DISCUSSION

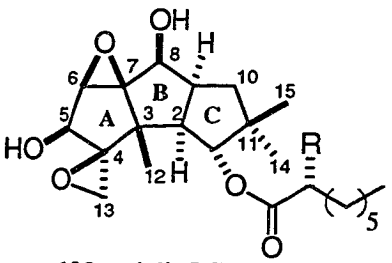
The coriolin family are all based on the common hirsutane (**108**) skeleton. Hirsutene (**109**), the simplest member of this compound class, has been previously isolated from *Coriolus consors*,[105] the original source of the coriolins (**103-104, 110-112**). Much of the structural diversity present in the coriolins is present in the chloriolins (**105-107**). However, the chloriolins also incorporate completely novel structural features not found in the coriolins.



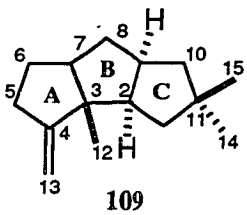
108
hirsutane



110 coriolin A R = H

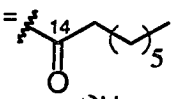


103 coriolin B R = H
104 dihydrocoriolin C R = OH

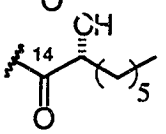


109
hirsutene

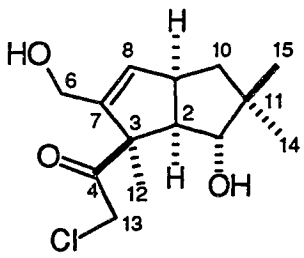
111 5-ketocoriolin B R =



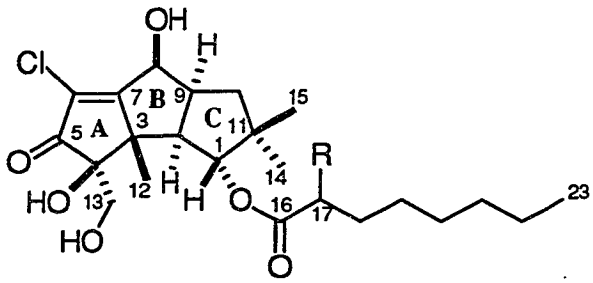
112 coriolin C R =



The structural variations in the coriolins arise from either the ketone or its reduced form at C5 in the A ring and the octanyl chain at C1. The chloriolins (**105-107**) have the same general variations as the coriolins at C1. For all three types of octanyl chains (including the lack thereof) observed in the coriolins there are the equivalent structures in the chloriolins. However, it is in the functionalization of the A ring that the chloriolins display truly unique features.

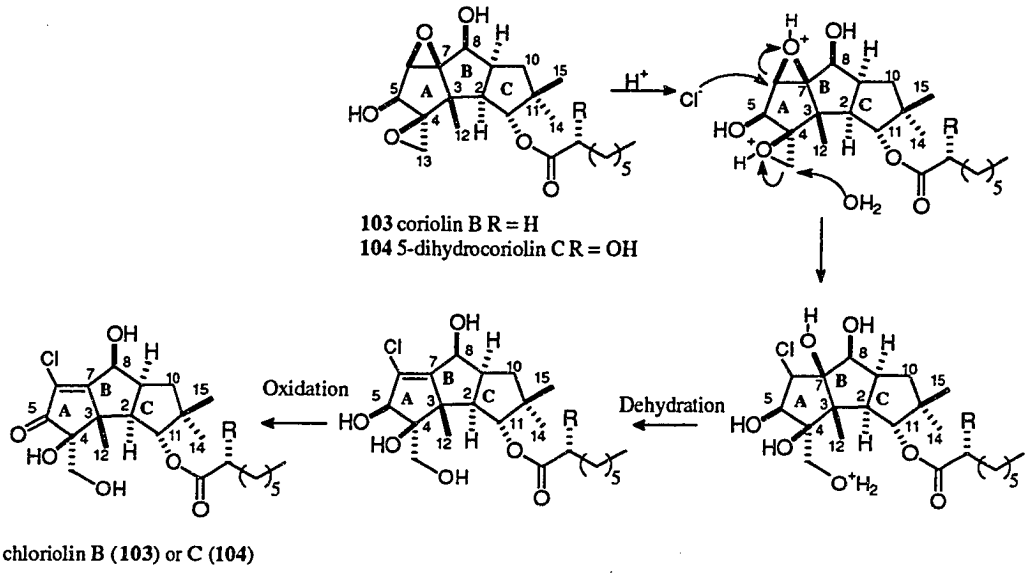


105
chloriolin A



106 chloriolin B, R = OH
107 chloriolin C, R = H

Chloriolins B and C possess an identical ring system with the same variations in ring A compared to the coriolins. The conversion of the 6-7 epoxide to the chloro vinyl unit and addition of water to the 13-4 epoxide characterize the changes in the coriolin core. Chloriolin A (**105**) displays the greatest change from the hirsutane (**108**) skeleton. Ring A is opened by the loss of a CO unit and the addition of chlorine has occurred in the cleavage of the 13-4 epoxide ring. A dehydration of the H8 position also moves the structure further from the coriolin system. A stereochemical change of methyl 12 has also occurred in the production of chloriolin A (**105**). The chloriolins (**105-107**) were isolated from a malt extract broth at pH 4.7. The variations present in chloriolins A-C (**105-107**) may have been caused by the seawater growth media conditions. The acidic broth may be responsible for the chloriolins by conversion of either coriolin B (**103**) or another unisolated coriolin by the mechanisms outlined in Scheme 4.1.



Scheme 4.1. Possible generation of chloriolins from coriolins.

To test this hypothesis pure coriolin B (**103**) was placed in a solution of sea salt buffered to pH 4.1 and the solution was stirred for nine days. At the end of this period there was no formation of chloriolins and only pure coriolin B (**103**) was recovered. This may indicate the structural changes present in the chloriolins are from an enzymatic secondary metabolite pathway rather than from the fungal growth media. Apparently in saltwater culture a different biosynthetic pathway is present as well as the normal coriolin biosynthetic route.

Unfortunately, before the chloriolin producing culture could be grown in freshwater, the coriolin and chloriolin producing capability of the strain was lost. This loss of ability may have been caused by mutation of the strain because of continual reculture of one line without cryopreservation of the original strain. In order to preserve the original producing strain cryopreservation is now performed early in the culture of each strain.

The loss of the epoxide rings and the addition of the chlorine atom in chloriolins B and C rendered these compounds inactive compared to coriolin B (**103**) in the National Cancer Institute's

(NCI) 60 cell line disease oriented screen.[106,107,108] Coriolin B (**103**) was active in the T-47 breast (IC_{50} = 0.7 μM) and in the CNS ((IC_{50} = 0.5 μM) cell lines.

The more extensive changes in the structure of chloriolin A (**105**) also removed the cytotoxic activity of this compound. The α-chloro ketone functionality in chloriolin A (**105**) is the reactive trigger in compounds designed to act as either irreversible inhibitors, affinity labels, or suicide substrates of proteases.[109] The reactive functionality present did not confer activity for chloriolin A (**105**) at 100 μg/mL in the NCI 60 cell line screening program.

How unique are fungal marine products?

There has been a rapid growth in the isolation and characterization of novel marine fungal natural products. While there have been many reviews of this newly emerging field, no analysis of the uniqueness of the natural products isolated from marine fungi has appeared. It is important to understand how different the marine fungal compounds are compared to their terrestrial counterparts in order to decide if the secondary metabolites of marine fungi display the diversity and novelty that is desired in the search for new natural products. In order to place the coriolins and chloriolins in perspective against the other natural products isolated from marine derived fungi it is important to analyze the related natural products research produced previous to and during the course of this work.

The information summarized in Table 4.1 may be used to answer some key questions about the uniqueness of marine fungal natural products. The simplest question is: how unique are marine fungal natural products compared to their terrestrial counterparts? Table 4.3 provides a synopsis of the degree of novelty marine fungal natural products possess. Three different categories are proposed. In the first category, the compounds isolated from a marine-derived culture are identical to compounds previously isolated from terrestrial fungi. The second category contains novel marine fungal compounds that have functional group variations compared to an established

terrestrial compound. These compounds all possess a skeleton previously seen in terrestrial fungal metabolites. The truly unique marine fungal secondary metabolites are in the third category. These structures have either novel carbon skeletons or have unprecedented rearrangements of known skeletons. While not shown as a separate heading in the Table 4.3, terrestrial fungi have produced approximately 9000 compounds of which the great majority of structure types have not been isolated from marine derived fungi.[1-3]

The compounds discussed in the introduction are represented by a single member of their class in each category instead of listing each individual compound. This is to prevent one compound class with a large number (i.e. the leptosins (**10-24**) or dendryphiellins (**63-74**) from dominating a category. However, if the class of compounds has members in more than one category then the important individual compounds are listed individually. Obionin A (**42**), leptosphaerolide (**44**) and leptosphaerodione (**45**) have all been grouped into one class as they differ only in oxidation levels of a common skeleton. Chlorocarolides (**98-99**) and hexylitaconic acid (**100**) are also grouped together because of a postulated common biosynthetic origin. However, individual members of this class are present in different categories. The chloriolins (**105-107**) and coriolins (**103-104**) are counted as one structural class, but they are also distributed through the three categories discussed above.

Table 4.3. Degree of novelty of marine fungal natural products.

Previously Isolated as Terrestrial Compounds	O/F*	Compounds Closely Related to Terrestrial Compounds	O/F*	"Genuinely Unique" Marine Compounds	O/F*
coriolins (**103-104**)	F	chloriolin B (**106**), C (**107**)	F	chloriolin A (**105**)	F
gliovictin (**4**)	O	leptosins (**10-24**)	O	zopfinol (**39**)	O
gancidin W (**5**)	O	fumiquinalozines (**26-32**)	F	zofimarin (**41**)	-
melinicidins (**6-7**)	O	auranticins (**51-52**)	F	communesins (**37-38**)	F
gliotoxin (**25**)	F	fellutamides (**33-34**)	F	trichoharzin (**55**)	F
mellein (**50**)	O	demethylnectriapyrones (**48-49**)	F	halymecin (**60**)	F
7-hydroxyergosterol (**97**)	O	obionin/leptosphaerolide (**42,44-45**)	O	dendryphiellins A-D (**63-67**)	F
culmorin (**88**)	O	phomactins (**79-87**)	F	hymenoscyphin A (**61**)	O
siccayne (**89**)	O	dendryphiellin E-G (**70-74**)	O	stachybotrins (**90-91**)	F
cephalosporin P_1 (**62**)	F	helicascolides (**46-47**)	O	leptosphaerin (**94**)	O
penicillic acid (**101**)	F	ceramide (**96**)	O	phosphorohydrazide	O
hexylitaconic acid (**100**)	F	chlorocarolides (**98-99**)	F	thioate (**95**)	
BK 223 A-C (**57-59**)	O				

* O = obligate marine fungi source; F = facultative marine fungi source

The coriolins (**103-104**) and chloriolins span three different categories. Coriolin B (**103**) and dihydrocoriolin C (**104**) are identical to previously discovered terrestrial compounds. The chloriolins B (**106**) and C (**107**) vary only in the oxidation levels and functional groups of the same hirsutane (**108**) skeleton that the coriolins (**103-104**) are based on, thus they are placed in the second category. Chloriolin A (**105**) is the truly unique compound isolated from this project. The opening of the A ring and the loss of a carbon atom suggest a completely novel biosynthetic pathway. This is also accompanied by an inversion of the stereochemistry at C4 which is also unprecedented in hirsutane (**108**) derived compounds. For these reasons chloriolin A (**105**) is placed in the third category.

Of the 31 structural families isolated from marine fungi, 13 of the compound classes are identical to compounds previously isolated from terrestrial fungi. However, this number may be low because the isolation of a terrestrial fungal metabolites may not be reported if the goal is to isolate novel compounds. One of the hypotheses at the beginning of the chapter was that the ability

to adapt and to grow in a marine environment would require adaptations in metabolism that would extend to the secondary metabolism. However 61% of the known compounds isolated from marine-derived fungi were from obligate marine fungi. This category also contains the largest proportion and absolute number of compounds isolated from obligate marine fungi. It appears that the obligate marine fungi do not have a special ability to produce novel fungal metabolites.

The second category contains the novel marine fungal compounds that share a common skeleton with terrestrial fungal compounds. Some of the compounds in this category (the chlorocarolides (**98-99**), dendryphiellins E-E_2 and G (**70-74**), and chloriolins B and C (**106-107**)), are members of structural classes that have other representatives in the other categories. The phomactins (**79-87**) are also in this group because phomactin A (**79**) was found from a crab shell-derived fungi and a similar compound Sch 47918 (phomactin C) (**84**) was isolated from a terrestrial *Phoma* species isolated from wood. The fungal sources for this category are primarily facultative marine fungi which produced 55% of the compounds isolated in this category. This indicates promise for facultative marine fungi to provide novel secondary metabolites.

The most interesting category contains the "genuinely novel compounds." Some of these compounds possess completely novel skeletons not encountered previously, i.e., communesins (**37-38**), while others possess novel modifications to known skeletons, i.e., chloriolin A (**105**) and dendryphiellins A-D (**63-67**). Of the compounds found in this category 67% are produced by facultative marine fungi. Again the ability of fungi to adapt to a marine habitat does not necessarily lead to the formation of novel secondary metabolic pathways.

These results indicate that there is a definite ability of both obligate and terrestrial fungi to produce new structural classes. This summary also highlights that in the search for novel natural products it is not necessary, or even desirable to examine only obligate marine fungi. Facultative marine fungal cultures have an excellent ability to generate novel compounds. The obligate marine fungi may be underrepresented because of the culture and isolation techniques employed by the

chemists. The necessity of observing reproduction in the marine environment poses unique difficulties in the culture of obligate marine fungi. Unfortunately, reproductive structures in the marine environment are very small and are difficult to locate underwater. Therefore it is more efficient to select inoculi from the marine environment to grow cultures. This automatically determines that the fungi will be facultative marine fungi. A facultative marine fungi may be classified as an obligate marine fungi only if the isolated culture can reproduce in the marine environment.

To utilize the marine environment as a source of fungi for new natural products it is necessary to identify effective substrates to initiate the laboratory culture of fungi. At the beginning of this project the sponge *Jaspis johnstonii* was chosen as the fungal source with the hopes that jasplakinolide (**1**) would be isolated from the culture. As stated in the results section there was no trace of jasplakinolide (**1**) observed in the culture extracts. However, the production of novel compounds raises the question of effective inoculi for marine fungi. The marine source of fungi that produce novel secondary metabolites are presented in Table 4.4. This summary is not fully complete as there is no source information available for BK 223 (**57-59**), halymecin (**60**), hymenoscyphin A (**61**), leptosphaerin (**94**) nor zofimarin (**41**). The categories defined in Table 4.3 are employed to organize the compounds by type of source material.

Table 4.4. Marine fungal natural products classified according to source.

Plant/Algae/Wood	O/F	Sediment/Sand	O/F	Macroorganisms	O/F
UNIQUE COMPOUNDS		UNIQUE COMPOUNDS		UNIQUE COMPOUNDS	
stachybotrins (**90-91**)	F	zopfinol (**39**)	O	chloriolin A (**105**)	F
communesins (**37-38**)	F			trichoharzin (**55**)	F
dendryphiellins (**63-67**)	O				
phosphorohydrazide thioate (**95**)	O				
RELATED COMPOUNDS		RELATED COMPOUNDS		RELATED COMPOUNDS	
leptosins (**10-24**)	O	none		chloriolins B,C (**106-107**)	F
obionin A (**42**)	O			chlorocarolides (**98-99**)	F
leptosphaerolide (**44**)					
helicascolides (**46-47**)	O			phomactins (**79-87**)	F
ceramide (**96**)	O			fumiquinalozines (**26-32**)	F
auranticins (**51-52**)	O			fellutamides (**33-34**)	F
dendryphiellin E-E_2, G (**70,72-74**)	O			demethylnectriapyrone (**46-47**)	F
TERRESTRIAL COMPOUNDS		TERRESTRIAL COMPOUNDS		TERRESTRIAL COMPOUNDS	
gliovictin (**4**)	O	mellinicidins (**6-7**)	O	hexylitaconic acid (**100**)	F
7-hydroxyergosterol (**97**)	F	gancidin W (**5**)	O	penicillic acid (**101**)	F
culmorin (**88**)	O	gliotoxin (**25**)	F	mellein (**50**)	F
siccayne (**89**)	O				
cephalosporin P_1 (**62**)	F				

O = obligate marine fungi source; F = facultative marine fungi source

Fungi cultured from either macroorganism or cellulose containing substrates are the most studied and have produced the greatest number of structure classes. It is interesting to note that only one novel compound was isolated from sediment and sand-derived fungi. However, there have only been three examples of cultures isolated from sediment so it is difficult to determine a trend from such a small sample. To differentiate whether a macroorganism or a plant, algae, or piece of wood is a better culture source is difficult considering the few examples present. It appears that essentially an equal number of new structure classes will become available from culture from either macroorganisms or plants, algae or dead wood.

The chloriolins (**105-107**), coriolins (**103-104**), chlorocarolides (**98-99**), demethyl nectriapyrones (**48-49**) and trichoharzin (**55**) were derived from sponges. The discovery of further novel compounds from fungi derived from sponges seems ensured. This raises the question of what type of habitat do sponges provide for fungi? There are no observations of fungal mycelia reported in electron micrographs in sponges while cyanobacteria and non-photosynthetic bacteria have been observed.[110] While fungi may not live within the tissues, the filter feeding sponges may concentrate either large numbers of spores or hyphal pieces of fungi growing in the vicinity. The fungal sources may also be terrestrial fungi washed to sea from nearby land masses. Until further study is done on the relationship between sponges and fungi it will be difficult to answer these questions.

CONCLUSIONS

The isolation of the chloriolins (**105-107**) from a facultative marine fungi derived from a *Jaspis* cf. *johnstonii* sponge correlates well with the information summarized in Tables 4.3 and 4.4. From the single culture there were compounds that would fit in all three structure classes defined in Table 4.3. The coriolins (**103-104**) isolated from the fungal mycelium have been previously isolated from the terrestrial fungus *Coriolus consors*. The chloriolins B (**106**) and C (**107**) have the same sesquiterpene skeleton as the coriolins (**103-104**) with differing functional groups, in particular the presence of chlorine atoms. It is chloriolin A (**105**) that is the most different compared to any of the related terrestrial coriolins (**103-104**). This is the first example of a norhirsutane skeleton. This parallels the case of the dendryphiellins (**63-67**) which was the first and only report to date of a tri-nor eremophilane skeleton from a fungus.

The chloriolins (**105-107**) represented only the second example of a chlorinated marine fungal product after the isolation of zopfinol (**39**).[53] Salynamide A from a marine bacterium

represents the second case of a chlorinated natural product from a nonphotosynthetic marine microorganism.[111]

It is interesting to note that the chlorine atom on the hirsutane (**108**) skeleton reduces the cytotoxicity of the chloriolins. While coriolin B (**103**) is active in specific cancer cell lines, chloriolins A-C were inactive.

Completely novel, terrestrially related, and known compounds were isolated from a culture derived from a *Jaspis johnstonii* sponge. However, there was no jasplakinolide detected. This indicates that there is a productive future in the examination of fungi separated from sponges and it also provides a warning that marine fungal cultures have the ability to produce known terrestrial compounds.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES—The NMR spectra were recorded at 250 or 500 MHz for ^{1}H and 62.9 and 125.7 MHz for ^{13}C. The assignments of the ^{13}C and ^{1}H NMR data reported for compounds chloriolins A and B were made by using HMQC[112,113] data to determine one bond H–C connectivities, HMBC[113] data to determine two and three bond H–C connectivities and NOESY[114] data to interrelate protons with close spatial proximity. Unequivocal ^{13}C NMR assignments were published by Tanabe[100] for dihydrocoriolin C (**104**) and ^{1}H-^{1}H COSY data was used to decipher the ^{1}H resonances. The ^{13}C NMR assignments for compound coriolin B (**103**) were based in part on ^{1}H-^{13}C COSY data. The NMR assignments for chloriolin C (**107**) were derived by analogy to those of similar protons and carbons of chloriolin B (**106**), coriolin B (**103**), and dihydrocoriolin C (**104**). Low and high resolution fast atom bombardment experiments were performed on a reverse geometry mass spectrometer or an extended geometry four sector mass spectrometer, respectively. High performance liquid chromatography (HPLC) was done using columns of 10 μm silica gel and ODS. All solvents were distilled and dried for HPLC use and were spectral grade for NMR spectroscopy.

EXTRACTION AND ISOLATION OF CORIOLINS (**103-104**) AND CHLORIOLINS (**105-107**)—The mycelium and broth were separated by filtration and each was extracted independently. A representative extraction for each work-up is as follows: The broth (4.5 L) was extracted three times with EtOAc. The EtOAc was evaporated to yield 775 mg of yellow oil which was further partitioned between 10% aqueous MeOH and hexanes. The aqueous soluble portion was partitioned between CH_2Cl_2 and 50/50 MeOH/H_2O. The CH_2Cl_2 portion (714 mg) was applied to a silica gel flash column eluted with hexanes/EtOAc (the solvent was varied from 2:1 hexanes:EtOAc to 1:2 hexanes: EtOAc). One of the flash fractions was further purified by normal phase HPLC (hexanes:EtOAc 3:7) to yield chloriolin A (**105**) (18.0 mg). Other flash fractions were

also purified by HPLC (hexanes:EtOAc 1:1) to yield chloriolin B (**106**) (16.0 mg) and chloriolin C (**107**) (3.0 mg).

The mycelium (56 g) was extracted three times (24 hours each time) with methanol. The methanol was evaporated and the resulting gum was partitioned in an analogous manner to the EtOAc extract to yield a CH_2Cl_2 fraction of 227 mg which was applied to a Sephadex-LH20 column eluted with methanol. The Sephadex fractions with unusual NMR shifts were purified further with reverse phase HPLC (eluted with 20% aqueous methanol) to yield coriolin B (**103**) (17.0 mg) and dihydrocoriolin C (**104**) (16.5 mg).

COLLECTION AND GROWTH OF FUNGI— A small piece of *Jaspis* aff. *johnstonii* sponge from Benga Harbor Fiji was aseptically removed underwater (at -20m) from a large colony and placed in a sterile whirl pack bag. At the surface the sealed bag was opened under sterile conditions and a small portion of the sponge was placed on solid media made from 1g of yeast extract (DIFCO), 10 g of α–cellulose (SIGMA), 15 g of agar (DIFCO) in 1 L of filtered (0.2 μm) Monterey Bay seawater spiked with 100 mg/L each of streptomycin and penicillin G. A plug of the agar containing mycelium (no. 92902) was transferred to a liquid media of 15 g/L of malt extract (DIFCO) dissolved in filtered Monterey Bay seawater. The culture broths (75 mL in a 125 mL Erlenmeyer flask) were placed at 25 °C on a gyro-rotary shaker at 180 rpm. These 75 mL broths were used to start three larger 500 mL malt extract broths in 1 L Erlenmeyer flasks. These cultures were subsequently grown for approximately 21 days at 27 °C in a gyro-rotary shaker at 120 rpm and then transferred to another set of three 500 mL broths, Plate 4.1. This was repeated three more times to yield 56 g of mycelium from 4.5 L of malt extract broth.

TAXONOMIC IDENTIFICATION—Attempts to obtain fruiting bodies from this fungus have been unsuccessful. Prof. J. Kohlmeyer (U. of No. Carolina) has kindly provided the following information about the voucher culture. The fungus is composed of a non-descript mycelium which

fragments into short segments that may be arthrospores. It may be in the form-class hyphomycete in the form-subdivision Deuteromycotina.

CHLORIOLIN A (**105**)— white orthorhombic crystals (18.0 mg); [α] = -35.0° (c, 0.01, $CHCl_3$); IR (film) 1737, 1447, 1368, 1250, 1022 cm^{-1}; LRFABMS, positive ion, *m/z* (relative intensity) 273 ($[M+H]^+$, 18), 255 (65), 221 (11), 152 (42), 119 (100); HRFABMS 273.1254 $[M+H]^+ = C_{14}H_{22}O_3Cl$ (Δ 0.3 mmu of calcd); NMR data are presented in Table 4.2.

CHLORIOLIN B (**106**)— white solid (16.0 mg); [α] = + 31.5° (c = 0.005, MeOH); UV (MeOH) λ_{max} 235, 252 nm; IR (film) 2965, 1741, 1373, 1250 cm^{-1}; LRFABMS, positive ion, *m/z* (relative intensity) 459 $[M+H]^+$ (23), 299 (47), 281 (100); HRFABMS 459.2164 $[M+H]^+ = C_{23}H_{36}O_7Cl$ (Δ -1.4 mmu of calcd); ^1H NMR (250 MHz, dioxane-d_8) δ 5.18 (d, *J* = [illegible].5 Hz, H1), 4.63 (dd, *J* = 10.0, 2.5 Hz, H8), 4.60 (s, OH4), 4.55 (d, *J* = 2.5 Hz, OH8), 4.05 (m, H17), 4.00 (d, *J* = 6.5 Hz, OH17), 3.72 (t, *J* = 5.5 Hz, OH13), 3.53 (dd, *J* = 5.5, 11.5 Hz, H13), 3.39 (dd, *J* = 5.5, 11.5 Hz, H13'), 3.02 (dd, *J* = 9.5, 11.5 Hz, H2), 2.81 (m, H9), 2.09 (dd, *J* = 9.5, 13 Hz, H10), 1.71 (m, H18), 1.60 (m, H18'), 1.40 (dd, *J* = 9.5, 13 Hz, H10'), 1.29 (m, $H_2$19, $H_2$20, $H_2$21, $H_2$22), 1.24 (s, Me12), 1.02 (s, Me14), 0.95 (s, Me15), 0.88 (t, *J* = 6.7 Hz, Me23); ^1H NMR (250 MHz, CD_3OD) δ 5.20 (d, *J* = 9.5 Hz, H1), 4.69 (d, *J* = 6.0 Hz, H8), 4.17 (dd, *J* = 4.5, 7.5 Hz, H17), 3.62 (d, *J* = 10.5 Hz, H13), 3.46 (d, *J* = 10.5 Hz, H13'), 3.06 (dd, *J* = 9.5, 11.5 Hz, H2), 2.89 (m, H9), 2.08 (dd, *J* = 12.5 Hz, 9.5 Hz, H10), 1.78 (m, H18), 1.67 (m, H18'), 1.50 (dd, *J* = 9.5, 12.5 Hz, H10'), 1.45 (m, $H_2$22), 1.33 (br, m, $H_2$19, $H_2$20, $H_2$21), 1.28 (s, Me12), 1.05 (s, Me14, Me15), 0.90 (t, *J* = 7.0 Hz, Me23); ^{13}C NMR (62.9 MHz, dioxane-d_8) δ 204.3 (C5), 183.4 (C7), 175.1 (C16), 124.3 (C6), 86.4 (C4), 81.4 (C1), 71.0 (C17), 66.3 (C8, C13), 54.1 (C3), 48.2 (C2), 45.4 (C9, C11), 35.4 (C10, C18), 32.3 (C21), 29.7 (C20), 27.1 (C14), 25.6 (C19), 23.2 (C12, C22), 22.4 (C15), 14.3 (C23); ^{13}C NMR (62.9 MHz, CD_3OD) δ 205.6 (C5), 184.3 (C7), 175.8 (C16), 125.3 (C6), 87.2 (C4), 82.6 (C1), 71.7 (C17), 67.2 (C8), 67.1 (C13),

54.9 (C3), 48.9 (C2), 46.3 (C9), 45.9 (C11), 36.1 (C10), 35.4 (C18), 32.8 (C21), 30.0 (C20), 27.3 (C14), 26.0 (C19), 24.0 (C12), 23.5 (C22), 22.4 (C15), 14.3 (C23).

CHLORIOLIN C (**107**)— white solid (3.0 mg); [α] = +27.5° (c = 0.002, MeOH); white solid; UV (MeOH) λ_{max} 235, 253 nm; LRFABMS, positive ion, *m/z* (relative intensity) 443 $[M+H]^+$ (100), 299 (19), 281 (62); HRFABMS 443.2192 $[M+H]^+ = C_{23}H_{36}O_6Cl$ (Δ -0.8 mmu of calcd); ^1H NMR (250 MHz, $CDCl_3$) δ 4.90 (d, *J* = 7.3 Hz, H1), 4.83 (d, *J* = 7.6 Hz, H8), 3.57 (d, *J* = 11.1 Hz, H13), 3.42 (d, *J* = 11.1 Hz, H13'), 3.03 (m, H9), 2.69 (dd, *J* = 7.6, 12.0 Hz, H2), 2.32 (dd, *J* = 7.4, 8.0 Hz, H17,17'), 2.00 (dd, *J* = 11.8, 12.5 Hz, H10), 1.61 (m, H18,18'), 1.53 (m, H10'), 1.45 (s, Me12), 1.28 (m, $H_2$19, $H_2$20, $H_2$21, $H_2$22), 1.11 (3H, s), 1.05 (3H, s), 0.88 (t, *J* = 6.9, Me23); ^1H NMR (250 MHz, dioxane-d_8) δ 5.13 (d, *J* = 9.5 Hz, H1), 4.63 (dd, *J* = 3.1, 6.0 Hz, H8), 4.62 (s, OH4), 4.54 (d, *J* = 2.7 Hz, OH8), 3.82 (bs, OH13), 3.68 (m, H13), 3.43 (dd, *J* = 4.9, 9.9 Hz, H13'), 3.00 (dd, *J* = 9.6, 11.4 Hz, H2), 2.83 (m, H9), 2.28 (t, *J* = 7.4, $H_2$17), 2.09 (dd, *J* = 9.5, 12.5 Hz, H10), 1.6 (m, $H_2$18), 1.41 (dd, *J* = 9.6, 12.5 Hz, H10'), 1.29 (m, $H_2$19, $H_2$20, $H_2$21, $H_2$22), 1.23 (s, Me12), 1.01 (s, Me14), 0.98 (s, Me15), 0.89 (t, *J* = 6.9 Hz, Me23); ^{13}C NMR (62.9 MHz, $CDCl_3$) δ 202.1 (C5), 181.2 (C7), 174.8 (C16), 124.4 (C6), 85.1 (C4), 81.9 (C1), 66.7 (C8), 65.9 (C13), 54.5 (C3), 48.8 (C2), 45.9 (C9), 45.1 (C11), 36.6 (C10), 34.6 (C17), 31.7 (C21), 29.2 (C20), 28.9 (C19), 26.9 (C14), 25.1 (C18), 23.6 (C12), 22.6 (C22), 22.1 (C15), 14.1 (C23).

CORIOLIN B (**103**)— white solid; mp 198 - 202 °C; [α] = +105.2° (c = 0.26, CH_2Cl_2) with ^1H NMR properties in accord with the literature[99,115] and ^{13}C NMR (62.9 MHz, $CDCl_3$) δ 172.0 (C16), 80.0 (C1), 76.1 (C7), 70.6 (C5), 70.3 (C8), 65.7 (C4), 63.1 (C6), 50.7 (C2), 45.8 (C3), 44.2 (C13), 43.8 (C11), 41.8 (C9), 35.9 (C10), 34.6 (C17), 31.7 (C21), 29.7 (C19), 29.2 (C20), 26.6 (C15), 25.2 (C18), 22.6 (C22), 21.6 (C14), 14.1 (C23), 13.7 (C12).

DIHYDROCORIOLIN C (**104**)— white solid; mp 152 - 155 °C; [α] = +49.8° (c = 0.27, CH_3OH) with ^{13}C NMR properties in accord with the literature[100] and ^1H NMR (250 MHz,

$CDCl_3$) 5.19 (d, J = 8.4 Hz, H1), 4.38 (d, J = 1.4 Hz, H5), 4.12 (dd, J = 6.8, 4.2 Hz, H17), 3.98 (d, J = 6.3 Hz, H8), 3.54 (d, J = 1.4 Hz, H6), 2.92 (m, H9), 2.57 (d, J = 4.7 Hz, H13) 2.44 (dd, J = 12.1, 8.5 Hz, H2), 2.40 (d, J = 4.6 Hz, H13'), 1.96 (t, J = 12.2 Hz, H10), 1.80 (m, H18), 1.64 (m, H18') 1.49 (dd, J = 13.1, 8.5 Hz, H10') 1.28 (s, 6H), 1.06 (s, 3H) 1.04 (s, 3H), 0.98 (s, 3H) 0.87 (t, Me23).

ATTEMPTED CONVERSION OF CORIOLIN B (**103**) TO CHLORIOLIN B (**106**)— To a 75 mL solution of water buffered to pH 4.1 with ammonium formate was added 0.22g NaCl. This solution matched the salinity and surpassed the acidity of the original pH 4.6 malt extract broth used to grow the chloriolin producing fungi. Coriolin B (**103**) (11.8 mg) was added to this solution and the mixture was allowed to stir for nine days. The water solution was extracted three time with ethyl acetate. The dried extract was checked by ^{1}H NMR and was found to contain only the resonances of coriolin B (**103**).n

REFERENCES

(1) Cole, R. J.; Cox, R. H. *Handbook of Toxic Fungal Metabolites*; Academic Press: San Francisco, 1981.

(2) Turner, W. B. *Fungal Metabolites*; Academic Press: New York, 1971.

(3) Turner, W. B.; Aldridge, D. C. *Fungal Metabolites II*; Academic Press: San Francisco, 1983.

(4) Moore-Landecker, E. ; *Fundametals of the Fungi*; Prentice Hall: Englewood Cliffs, 1990, pp. 521-522.

(5) Stanier, R. Y.; Ingraham, J. L.; Wheelis, M. L.; Painter, P. R. *The Microbial World*; 5 ed.; Prentice-Hall: Eaglewood Cliffs, 1986.

(6) Wildman, G. *Can. J. Bot.* **1995**, *73 (Suppl. 1)*, 5907-5916.

(7) Kobayashi, J.; Ishibashi, M. In *The Alkaloids*; Brossi, A., Cordell, G. A., Eds.; Academic Press: San Diego, 1992; Vol. V. 41, pp. 41-124.

(8) Yasumoto, T.; Yasumura, D.; Yotsu, M.; Michishita, T.; Endo, A.; Kotaki, Y. *Agric. Biol. Chem.* **1986**, *50*, 793-795.

(9) Noguchi, T.; Jeon, J.-T.; Arakawa, O.; Sugita, H.; Deguchi, Y.; Shida, Y.; Hashimoto, K. *J. Biochem.* **1986**, *99*, 311-314.

(10) Yotsu, M.; Yamazaki, T.; Meguro, Y.; Endo, A.; Murata, M.; Nooki, H.; Yasumoto, T. *Toxicon.* **1987**, *25*, 225-228.

(11) Tachibana, K.; Scheuer, P. J.; Tsukitani, Y.; Kikuchi, H.; van Engen, D.; Clardy, J.; Gopichard, Y.; Schmitz, F. J. *J. Am. Chem.Soc.* **1981**, *103*, 2469-2471.

(12) Murakami, Y.; Oshima, Y.; Yasumoto, T. *Bull. Jpn. Soc. Sci. Fish.* **1982**, *48*, 69-72.

(13) Unson, M. D.; Holland, N. D.; Faulkner, D. J. *J. Mar. Biol.* **1994**, *119*, 1-11.

(14) Unson, M. D.; Faulkner, D. J. *Experientia* **1993**, *49*, 349-353.

(15) Crews, P.; Manes, L. V.; Boehler, M. *Tetrahedron Lett.* **1986**, *27*, 2797.

(16) Crews, P.; Farias, J. J.; Emrich, R.; Keifer, P. *J. Org. Chem.* **1994**, *59*, 2932-2934.

(17) Zabrieskie, T. M.; Klocke, J. A.; Ireland, C. M.; Marcus, A. H.; Molinski, T. F.; Faulkner, D. J.; Xu, C.; Clardy, J. C. *J. Am. Chem. Soc.* **1986**, *108*, 3123.

(18) Braekman, J. C.; Daloze, D.; Moussiaux, B.; Riccio, R. *J. Nat. Prod.* **1987**, *50*, 994.

(19) Chan, W. R.; Tinto, W. F.; Manchand, P. S.; Todaro, L. J. *J. Org. Chem* **1987**, *52*, 3091.

(20) de Silva, E. D.; Andersen, R. J.; Allen, T. M. *Tetrahedron Lett.* **1990**, *31*, 489.

(21) Coleman, J. E.; de Silva, E. D.; Kong, F.; Andersen, R. J.; Allen, T. M. *Tetrahedron* **1995**, *51*, 10653-10662.

(22) Talpir, R.; Benayahuh, Y.; Kashman, Y.; Pannell, L.; Schleyer, M. *Tetrahedron Lett.* **1994**, *35*, 4453-4456.

(23) Barghoom, E. S.; Linder, D. H. *Farlowia* **1944**, *1*, 397-467.

(24) Kohlmeyer, J.; Kohlmeyer, E. *Marine Mycology, The Higher Fungi*; Academic Press: San Francisco, 1979.

(25) Hyde, K. D.; Jones, E. B. G. *J. Linn. Soc.* **1990**, *100*, 237.

(26) Kohlemeyer, J. *Veroeff. Inst. Meeresforcsh. Bremerhaven, Suppl.* **1974**, *5*, 339-356.

(27) Tyndall, T. G.; Kirk, P. W. J. *Va. J. Sci.* **1973**, *24*, 136.

(28) Shin, J.; Fenical, W. *Phytochemistry* **1987**, *26*, 3347.

(29) Furuya, K.; Okudaira, S.; Shindo, T.; Sato, A. *Sankyo Kenkyusho Nempo* **1985**, *37*, 140-142.

(30) Strunz, G. M.; Heissner, C. J.; Kakushima, M.; Stillwell, M. A. *Can. J. Chem.* **1974**, *52*, 325.

(31) Michel, K. H.; Chaney, M. O.; Jones, N. D.; Hoehn, M. M.; Nagarajan, R. *J. Antibio.* **1974**, *27*, 57.

(32) Dorn, F.; Arigoni, D. *Experientia* **1974**, *30*, 135.

(33) Argoudelis, A. D. *J. Antibio.* **1972**, *25*, 171.

(34) Argoudelis, A. D.; Mizsak, S. A. *J. Antibio.* **1977**, *30*, 468.

(35) Hauser, D.; Loosli, H.-R.; Niklaus, P. *Helv. Chim. Acta* **1972**, *55*, 2182.

(36) Cole, R. J.; Cox, R. H. *Handbook of Toxic Fungal Metabolites*; Academic Press: San Francisco, 1981.

(37) Aiso, K.; Arai, T.; Suzuki, M.; Takamizawa, Y. *J. Antibio.* **1956**, *30*, 468.

(38) Takahashi, C.; Numata, A.; Ito, Y.; Matsumura, E.; Araki, H.; Iwaki, H.; Kushida, K. *J. Chem. Perk. Trans. I.* **1994**, 1859-1864.

(39) Takahashi, C.; Numata, A.; Matsumura, E.; Minoura, K.; Eto, H.; Shingu, T.; Ito, Y.; Hasegawa, T. *J. Antibio.* **1994**, *47*, 1242-1249.

(40) Katagiri, K.; Sato, K.; Hayakawa, S.; Matsuhima, T.; Minato, H. *J. Antibio., Ser. A* **1970**, *23*, 420.

(41) Minato, H.; Matsumoto, M.; Katayama, T. *Chem. Commun.* **1971**, 44.

(42) Turner, W. B.; Aldridge, D. C. *Fungal Metabolites II*; Academic Press: San Francisco, 1983.

(43) Okutani, K. *Bull. Jpn. Soc. Fish.* **1977**, *43*, 995.

(44) Wilkinson, S.; Spilsbury, J. F. *Nature* **1965**, *206*, 619.

(45) Numata, A.; Takahashi, C.; Matsushita, T.; Miyamoto, T.; Kawai, K.; Usami, Y.; Matsumura, E.; Inoue, M.; Ohishi, H.; Shingu, T. *Tetrahedron Lett.* **1992**, *33*, 1621-1624.

(46) Yamazaki, M.; Fujimoto, H.; Okuyama, E. *Tetrahedron Lett.* **1977**, *25*, 2554.

(47) Yamazaki, M.; Okuyama, E.; Maebayashi, Y. *Chem. Pharm. Bull.* **1979**, *27*, 1611.

(48) Shigemori, H.; Wakuri, S.; Yazawa, K.; Nakamura, T.; Sasaki, T.; Kobayashi, J. *Tetrahedron* **1991**, *47*, 8529-8534.

(49) Locci, R.; Merlini, L.; Nasimi, G.; Locci, J. R. *Microbiol.* **1965**, *13*, 271.

(50) Aldridge, D. C.; Carr, D. M. Unpublished results.

(51) Aoyagi, T. *J. Antibio* **1969**, *22*, 283.

(52) Numata, A.; Takahashi, C.; Ito, Y.; Takada, T.; Kawai, K.; Usami, Y.; Matsumura, E.; Imachi, M.; Ito, T.; Hasegawa, T. *Tetrahedron Lett.* **1993**, *34*, 2355-2358.

(53) Kondo, M.; Takayama, T.; Furuya, K.; Okudaira, M.; Hayashi, T.; Kinoshita, M. *Sankyo Kenkyusho Nempo* **1987**, *39*, 45.

(54) Nakagawa, F.; Kodama, K.; Furuya, K.; Naito, A. *Agric. Biol. Chem.* **1979**, *43*, 1597.

(55) Nair, M. S. R.; Carey, T. *Tetrahedron Lett.* **1979**, 3233.

(56) Ogita, T.; Hayashi, T.; Sato, A.; Furuya, K. In *JPN KoKai Tokkyo Koho*; Sankyo Co. Ltd.: Japan, 1987; Vol. 39.

(57) Poch, K. G.; Gloer, J. B. *Tetrahedron Lett.* **1989**, *30*, 3483-3486.

(58) Parisot, D.; Barbier, M. *Phytochem.* **1985**, *24*, 1977.

(59) Poch, G. K.; Gloer, J. B. *J. Nat. Prod.* **1989**, *52*, 257-260.

(60) Abrell, L. M.; Cheng, X.-C.; Crews, P. *Tetrahedron Lett.* **1994**, *35*, 9159-9160.

(61) Nishikawa, E. *Agric. Chem. Soc. Jpn.* **1933**, *9*, 775.

(62) Yubuta, T.; Sumiki, Y. *J. Agric. Chem. Soc. Jpn.* **1933**, *9*, 1264.

(63) Nair, M. S. R.; Carey, T. *Tetrahedron Lett.* **1975**, *19*, 1655.

(64) Poch, G. K.; Gloer, J. B. *J. Nat. Prod.* **1991**, *54*, 213-217.

(65) Kawahara, N.; Nozawa, K.; Nakajima, S.; Kawai, K.; Yamazaki, M. *J. Chem. Soc. Perkin Trans. I* **1988**, 2611.

(66) Sierankiewicz, J.; Gatenbeck, S. *Acta Chim. Scand.* **1972**, *26*, 455.

(67) Weber, H. A.; Baenziger, N. C.; Gloer, J. B. *J. Am. Chem. Soc.* **1990**, *112*, 6718-6719.

(68) Weber, H. A.; Gloer, J. B. *J. Org. Chem.* **1991**, *56*, 4355-4360.

(69) Kobayashi, M.; Uehara, H.; Matsunami, K.; Aoki, S.; Kitagawa, I. *Tetrahedron Lett.* **1993**, *34*, 7925-7928.

(70) Ichihara, A.; Oikawa, H.; Hayashi, K.; Sakamura, S.; Furusaki, A.; Matsumoto, T. *J. Am. Chem. Soc.* **1983**, *105*, 2907.

(71) Rinehart, K. L.; Tachibana, K. *J. Nat. Prod.* **1995**, *58*, 344-358.

(72) Breinholt, J.; Jensen, G. W.; Nielsen, R. I.; Olsen, C. E.;. *J. Antibio.* **1993**, *46*, 1101-1108.

(73) Fenical, W.; Jensen, P. R. In *Marine Biotechnology, Volume 1 Pharmaceutical and Bioactive Natural Products*; 1st ed.; Attaway, D. H., Zaborsky, O. R., Eds.; Plenum Press: New York, 1993; Vol. 1, pp. 419-474.

(74) Burton, H. S.; Abraham, E. P. *Biochemical J.* **1951**, *50*, 168.

(75) Guerriero, A.; D'Ambrosio, M.; Cuomo, V.; Vanzanella, F.; Pietra, F. *Helv. Chim. Acta* **1988**, *71*, 57-61.

(76) Hikino, H.; Hikino, Y.; Koakutsu, S.; Takemoto, T. *Phytochemistry* **1972**, *11*, 2097.

(77) Bohlman, F.; Kramp, W.; Robinson, H.; King, R. M. *Phytochemistry* **1981**, *20*, 1739.

(78) Guerriero, A.; D'Ambrosio, M.; Cuomo, V.; Vanzanella, F.; Pietra, F. *Helv. Chim. Acta* **1989**, *72*, 438-446.

(79) Guerriero, A.; Cuomo, V.; Vanzanella, F.; Pietra, F. *Helv. Chim. Acta* **1990**, *73*, 2090-2096.

(80) Tanaka, M.; Ohra, J.; Tsujinoy, F. U.; Jimori, T.; *Zeitschrift Fur Naturforschung C-A Journal of Biosciences* **1995**, *50*, 751-756.

(81) Sugano, M.; Sato, A.; Iijima, Y.; Furuya, K.; Hiramitsu, K.; Hata, T. *J. Antibio* **1995**, 1188-1190.

(82) Sugano, M.; Sato, A.; Iijima, Y.; Furuya, K.; Hurayama, H.; Hata, T. *J. Org. Chem.* **1994**, *59*, 564-569.

(83) Sugano, M.; Sato, A.; Iijima, Y.; Oshima, T.; Furuya, K.; Kuwano, H.; Hata, T.; Hanzawa, H. *J. Am. Chem. Soc.* **1991**, *113*, 5463-5464.

(84) Burke, B. A.; Chan, W. R.; Honkan, V. A.; Blount, J. F.; Manchard, P. S. *Tetrahedron* **1980**, *36*, 3489-3493.

(85) Strongman, D. B.; Miller, J. D.; Calhoun, L.; Findlay, J. A.; Whitney, N. J. *Bot. Mar* **1987**, *30*, 21-26.

(86) Greenhalgh, R.; Meier, R. M.; Blackwell, B. A.; Miller, J. D.; Taylor, A.; ApSimon, J. W. *J. Agric. Food. Chem.* **1984**, *2*, 1261-1264.

(87) Ishibashi, K.; Nose, K.; Shindo, M.; Arai, M.; Mishima, H. *Ann. Sankyo Res. Lab.* **1968**, *20*, 76-79.

(88) Kupka, J.; Anke, T.; Streglich, W.; Zechlin, L. *J. Antibio.* **1981**, *34*, 298-304.

(89) Xu, X.; Guzman, F.; Gloer, J. B. *J. Org. Chem.* **1992**, *57*, 6700-6703.

(90) Rissom, T.; Jakobsen, H. J.; Rastrup-Andersen, N.; Lark, H. *Acta Chem. Scand.* **1978**, *32*, 499.

(91) Kaise, H.; Shinohara, M.; Miyazaki, W.; Izawa, T.; Nakano, Y.; Sugawara, M.; Sugiura, K.; Sasaki, K. *J. Chem. Soc. Chem. Commun.* **1979**, 726.

(92) Schienser, G. A.; White, J. D.; Matsumoto, G.; Pezzanite, J. O.; Clardy, J. *Tetrahedron Lett.* **1986**, *27*, 5587-5590.

(94) Abraham, S. P.; Hoang, T. D.; Alam, M.; Jones, E. B. G. *Pure & Appl. Chem.* **1994**, *66*, 2391-2394.

(95) Adamczeski, M.; Quiñoa, E.; Crews, P. *J. Org. Chem.* **1990**, *56*, 2034.

(96) Rodriguez, J.; Nieto, R.; Crews, P. *J. Nat. Prod.* **1993**, *56*, 2034.

(97) Reffstrup, T.; Boll, P. M. *Acta. Chem. Scand. B* **1980**, *B34*, 653.

(98) McCorkindale, N. J.; Blackstock, W. P.; Johnston, G. A.; Roy, T. P.; Troke, J. A. In *11th IUPAC Int. Symp. Chem. Nat. Prod.* 1978; Vol. 1, p. 151.

(99) Takahashi, S.; Naganawa, H.; IInuma, H.; Takita, T.; Maeda, K.; Umezawa, H. *Tetrahedron Lett.* **1970**, 1637-1640.

(100) Tanabe, M.; Suzuki, K. T.; Jankowski, W. C. *Tetrahedron Lett.* **1974**, 2271-2274.

(101) Pretsch, E.; Simon, W.; Seibl, J.; Clerc, T. *Tables of Spectral Data for Structure Determination of Organic Compounds.*; 2nd ed.; Springer-Verlag: New York, 1989.

(102) Korzybski, T.; Kowszyk-Gindifer, Z.; Kurylowicz, W. *Antibiotics : Origin, nature and properties.*; trans. Paryski, E.; American Society for Microbiology: Washington, 1978, pp. 2041-2043.

(103) Takahashi, S.; Naganawa, H.; IInuma, H.; Takita, T.; Maeda, K.; Umezawa, H. *Tetrahedron Lett.* **1971**, 1955-1958.

(104) Nakamura, H.; Takita, T.; Umezawa, H.; Kunishima, M.; Nakamaya, Y.; Iitakaka, Y. *J. Antibio.* **1974**, *27*, 301-302.

(105) Nozoe, S.; Furukawa, J.; Sankawa, U.; Shibata, S. *Tetrahedron Lett.* **1976**, 195.

(106) Grever, M. R.; Schepartz, S. A.; Chabner, B. A. *Seminars in Oncology* **1992**, *19*, 622-638.

(107) Ishizaka, M.; Inuma, H.; Takeuchi, T.; Umezawa, H. *J. Antibiot.* **1972**, *25*, 320.

(108) Kunimota, T.; Umezawa, H. *Biochim. Biophys. Acta* **1974**, *298*, 513.

(109) Reed, P. E.; Katzenellenbogen, J. A. *J. Biol. Chem.* **1991**, *266*, 13-21.

(110) Fusetani, N.; Matsunaga, S. *Chem. Rev.* **1993**, *93*, 1793-1806.

(111) Trischman, J. A.; Tapiolas, D. M.; Jensen, P. R.; Dwight, R.; Fenical, W.; McKee, T. C.; Ireland, C. M.; Stout, T.; Clardy, J. *J. Am. Chem. Soc.* **1994**, *116*, 757.

(112) Bax, A.; Subramanian, S. *J. Magn. Res.* **1986**, *67*, 565.

(113) Bax, A.; Sparks, S. W.; Torchin, D. A. *J. Am. Chem. Soc.* **1988**, *110*, 7926.

(114) Hull, W. E. In *Two Dimensional NMR Spectroscopy for Chemists and Biochemists.*; Croasmun, W. R., Carlson, R. M., Eds.; VCH Publishers, Inc.: New York, 1987, p. 155.

(115) Danishefsky, S.; Zamboni, R.; Kahn, M.; Etheredge, S. J. *J. Am. Chem. Soc.* **1981**, *103*, 3460.

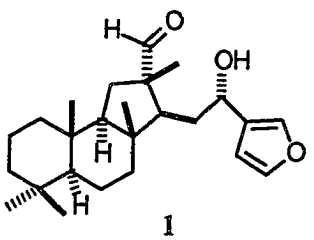
CHAPTER FIVE

Novel and Known Secondary Metabolites from a *Hyrtios proteus* Sponge Derived *Aspergillus niger* Fungal Culture

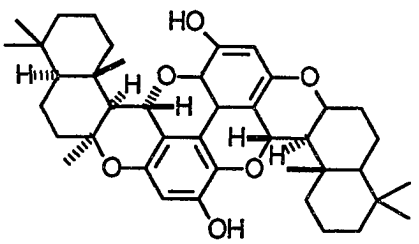
INTRODUCTION

The isolation and discovery of novel and known compounds from an *Aspergillus niger* fungus separated from a *Hyrtios proteus* sponge is examined in this chapter. The development of a new screening and dereplication method was made in part during the course of this work. Observations on the chemistry of a marine-derived *Aspergillus niger* fungi adds to the trends described in Chapter Four. The source of *Hyrtios proteus* sponge-derived compounds is also discussed and compared with the compounds derived from the *Aspergillus niger* culture.

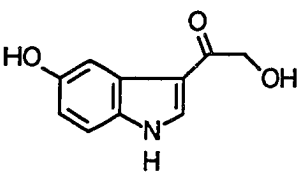
Hyrtios proteus is a member of the family Thorectidae in the order Dictyoceratida. Dictyoceratida sponges are known to produce mainly terpenoid natural products. The genera *Hyrtios*, *Smenospongia*, and *Fascaplysinopsis* (family Thorectidae) produce both the expected isoprenoid secondary metabolites[1,2] as well as a group of tryptophan-derived indole containing alkaloids.[3] Examples of *Hyrtios* sp. isoprene-derived compounds include hyrtiosal (**1**) and puupehenone dimer (**2**) while the hyrtiosins A (**3**) and B (**4**) represent the indole containing compounds.



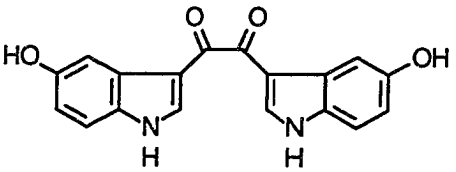
1
hyrtiosal



2
puupehenone dimer



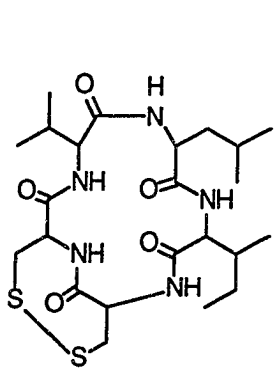
3
hyrtiosin A



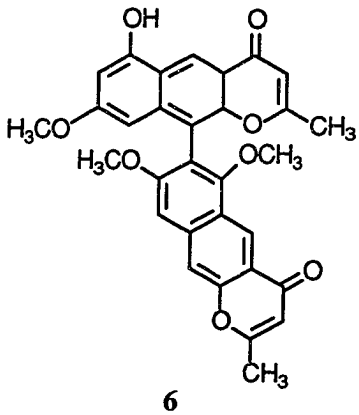
4
hyrtiosin B

The presence of indole compounds in the order Dictyocertida is unexpected, and may imply that there is a non-sponge source for these natural products. The presence of these types of compounds in all members of the family Thorectidae may also mean this family shares a common biosynthetic ability or there may be common microorganisms among these sponges. The examination of a fungal culture from *Hyrtios proteus* was used to probe a possible source of these indole compounds.

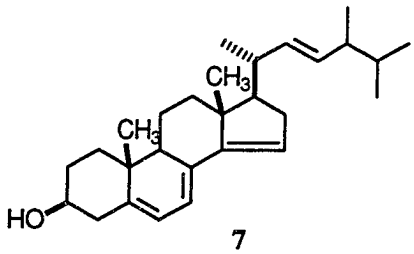
The genus *Aspergillus* is one of the most prolific producers of terrestrial secondary metabolites. Only the genus *Penicillium* approaches the ability of *Aspergillus* in the production of such a biosynthetically diverse number of natural products.[4] In particular, *Aspergillus niger* is one of the most biosynthetically capable members of the genus *Aspergillus*. This species of *Aspergillus* is able to produce compounds of many biosynthetic pathways including those whose biogenesis is difficult to identify. The following compounds represent the different biosynthetic types that terrestrial *A. niger* have produced in culture. Malformin A (**5**),[5] a peptide, aurasperone A (**6**),[6] a polyketide, and 14-dehydroergosterol,[7] a terpenoid compound characterize the major biosynthetic classes. In addition, *Aspergillus niger* produces compounds from the tricarboxylic acid (TCA) cycle, such as glutaconic acid (**8**).[8] Natural products from all of the above biosynthetic pathways have been identified in marine fungi, Chapter Four.



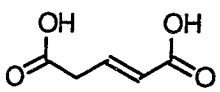
5
malformin A



6
aurasperone A



7
14 - dehydroergosterol

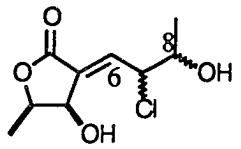


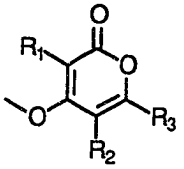
8
glutaconic Acid

A creativity index has been defined in an attempt to quantify the biosynthetic ability of a wide variety of fungi.[9] The genus *Aspergillus* has been found to have the highest value in this index indicating that they have been able to produce the most natural products per species. Approximately 200 *Aspergillus* species have been found to produce over 500 natural products by 1994. In addition, it has been shown that different habitats will lead to the biosynthesis of different natural products.[10] Coupling the high creativity of *Aspergillus* with the relatively unexplored marine environment promises a good chance of discovery of new natural products from this genus.

At the initiation of this project many other fungal cultures had been examined. It was found that while there were active extracts, on separation the extracts yielded many low molecular weight (100-250 Da) compounds such as the chlorocarolides A (**9**) and B (**10**)[11]and the demethyl nectriapyrones A (**11**) and B (**12**).[12] While these compounds were novel, a method was desired to indicate the presence of more sophisticated secondary metabolites in the crude extract partitions

before the effort of separation was undertaken. Electrospray ionization mass spectrometry (ESIMS) was chosen as the analytical tool to screen for the presence of larger compounds. This mass spectrometry method has the advantage that it provides molecular ions with little to no fragmentation without the use of a matrix to complicate the spectrum.

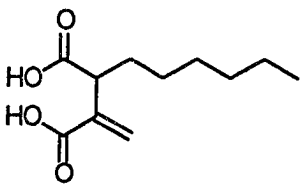




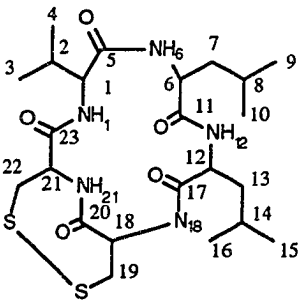
9 chlorocarolide A $7R^*$ $8R^*$ **11** demethylnectriapyrone A, R_1 = H, R_2=H, R_3 = 

10 chlorocarolide B $7S^*$ $8S^*$ **12** demethylnectriapyrone B, R_1 = H, R_2=CH_3, R_3 = CH_2CH_3

This project was among the first fungal cultures to which the ESIMS screening method was applied. After the isolation of the low molecular weight hexylitaconic acid (**13**)[13] from a culture of *Aspergillus niger* there was a need to determine if there were any higher molecular weight compounds present. Examination of the ESIMS spectrum of the remaining chromatography fractions of the crude extract did reveal the presence of a higher molecular weight compound. This led to the isolation of malformin C (**14**) from this culture of *Aspergillus niger*. The incorporation of ESIMS as a new screening method required a new prioritization scheme to be created, Figure 5.1.



13
hexylitaconic acid



14
malformin C

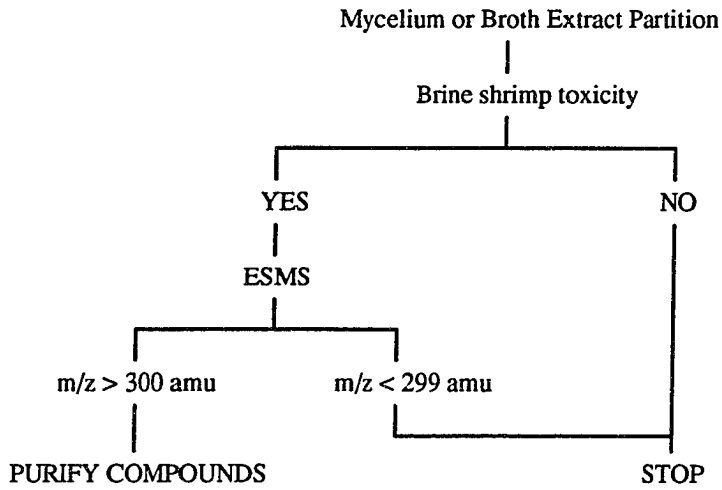


Figure 5.1. Prioritization scheme incorporating ESIMS as screening aid.

In this prioritization scheme the potential of a culture for high molecular weight (> 300 Da) compounds is determined quickly. The molecular weight of 300 Da was chosen as most steroids and triterpenes are below this value and it was hoped that this would lead to molecules that were larger and more interesting than the ubiquitous, relatively large steroids. Coupling the ESIMS data with natural products databases allows the rapid detection of known compounds. While this project was underway a similar screening method employing bioactivity assays, HPLC, UV spectroscopy, and ESIMS was described in the literature.[14]

The *Hyrtios proteus*-derived fungal culture of *Aspergillus niger* was initially examined based on brine shrimp toxicity. As partial results were obtained, new large scale liquid cultures were begun and extraction of each large scale growth until the final growth of the culture provided different compounds. The distribution of the isolated compounds correlated with the starting point of each large culture and demonstrated the continued ability of *A. niger* to produce novel compounds.

RESULTS

Culture and isolation of compounds from culture 94-1212

The sponge inoculum used to grow this culture was collected in Dry Tortugas, Florida by Mr. Leif Abrell, a Ph. D. candidate in the Crews' lab. Under sterile conditions a small piece of the sponge was surface sterilized and placed on four different culture plates. Growth of fungi emanating from the sponge was observed on a corn meal agar plate. Ms. Beth Borgeson, a staff technician, subcultured this fungi to two malt extract agar (MEA) plates MEA 2.1 and 2.2. The numeric notation of 2.1 represents the second generation (2) and the (.1) indicates the first plate of this generation. Beth Borgeson used these to create the series of plate, frozen and liquid cultures summarized in Figure 5.2. There were three large scale liquid broths created and each large scale liquid broth was abbreviated as the culture number followed by a number in brackets (i.e., 94-1212 (3) for the third large scale liquid broths). As the project progressed further large scale liquid cultures were made and examined, Figure 5.2.

The broth was filtered from the mycelium and extracted with ethyl acetate and methanol, respectively. The ethyl acetate broth extract exhibited brine shrimp toxicity at a level of 2 μg/mL while the mycelium did not exhibit any toxicity. The positive brine shrimp toxicity was the justification for the growth of this culture on larger scale. Towards the end of the project, taxonomy of this culture was determined by fatty acid analysis and growth characteristics on a variety of media.

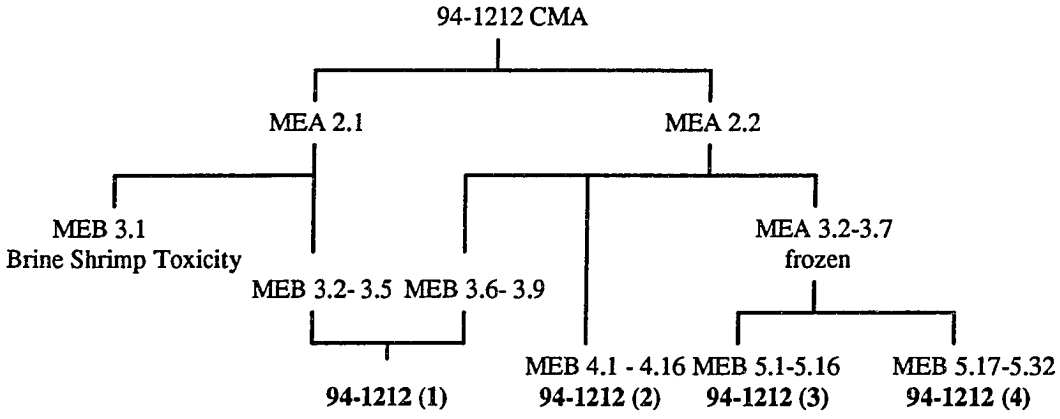


Figure 5.2. Culture tree for culture 94-1212. Text in bold denotes large scale liquid cultures.

Isolation of compounds from 94-1212 (1)

The mycelium and broth of the first large scale liquid culture, 94-1212 (1), were separated by filtration and the broth was extracted with ethyl acetate and partitioned as shown in Figure 5.3. Examination of the ^{1}H and ^{13}C NMR spectra, Figures 5.4 and 5.5, implied a major compound of low molecular weight. This FD partition was separated, as shown in Figure 5.6 with reverse phase flash chromatography and HPLC purification of the minor fractions. From flash chromatography the major compound isolated was determined to be hexylitaconic acid (**13**).[13] Examination of the ^{1}H and ^{13}C NMR of flash fraction F6 indicated the presence of an interesting compound. The electrospray ionization mass spectrum of this fraction, Figure 5.7, had a prominent peak at m/z = 530. HPLC of fraction F6 led to the isolation of malformin C (**14**).[15]

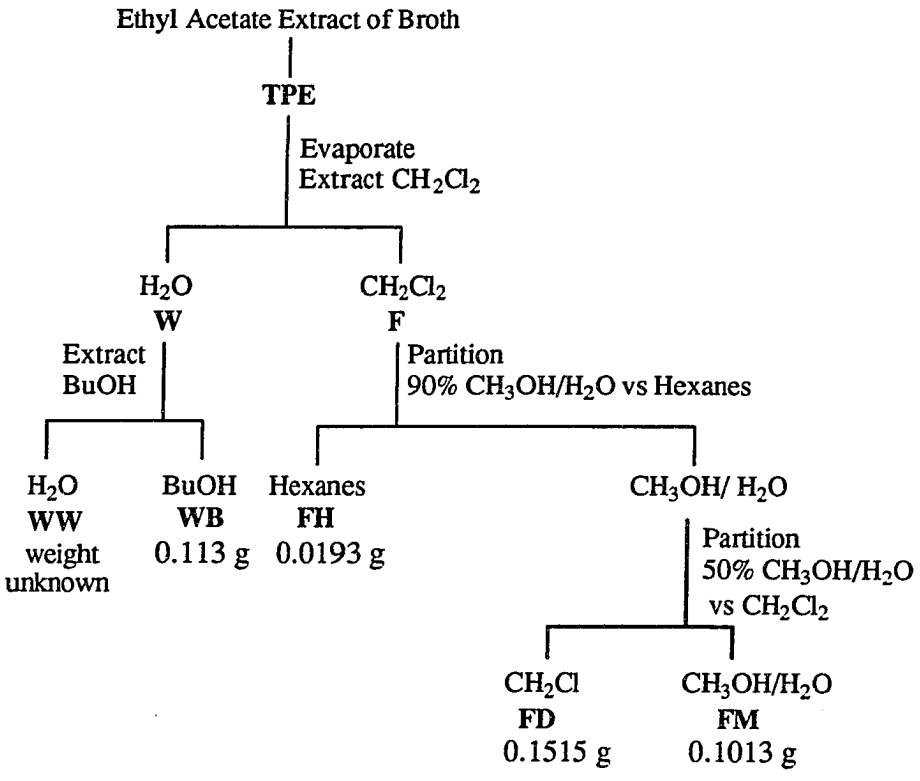


Figure 5.3. Solvent-solvent partition scheme for 94-1212 (1) broth.

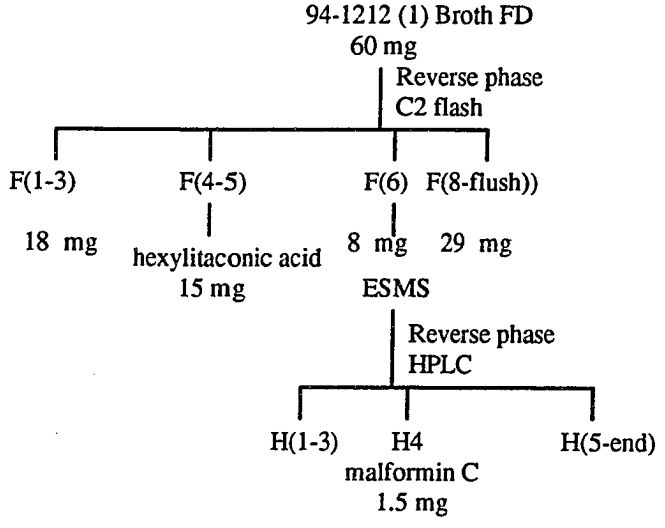


Figure 5.6. Chromatographic separation of 94-1212 (1).

Figure 5.4. ^{1}H NMR of FD partition of 94-1212 (1) broth extract, 250 MHz, $CDCl_3$.

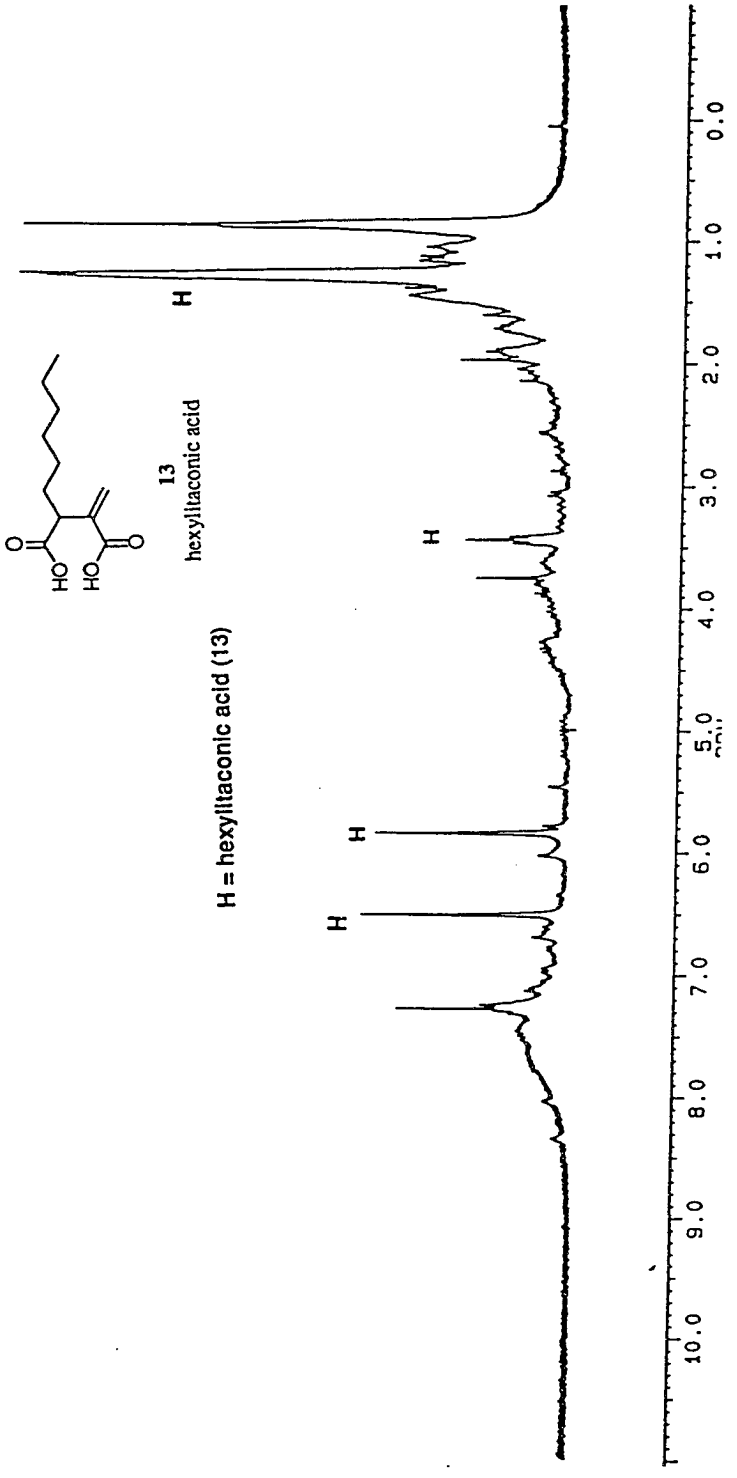


Figure 5.5. ^{13}C NMR of FD partition of 94-1212 (1) broth extract, 62.9 MHz, $CDCl_3$.

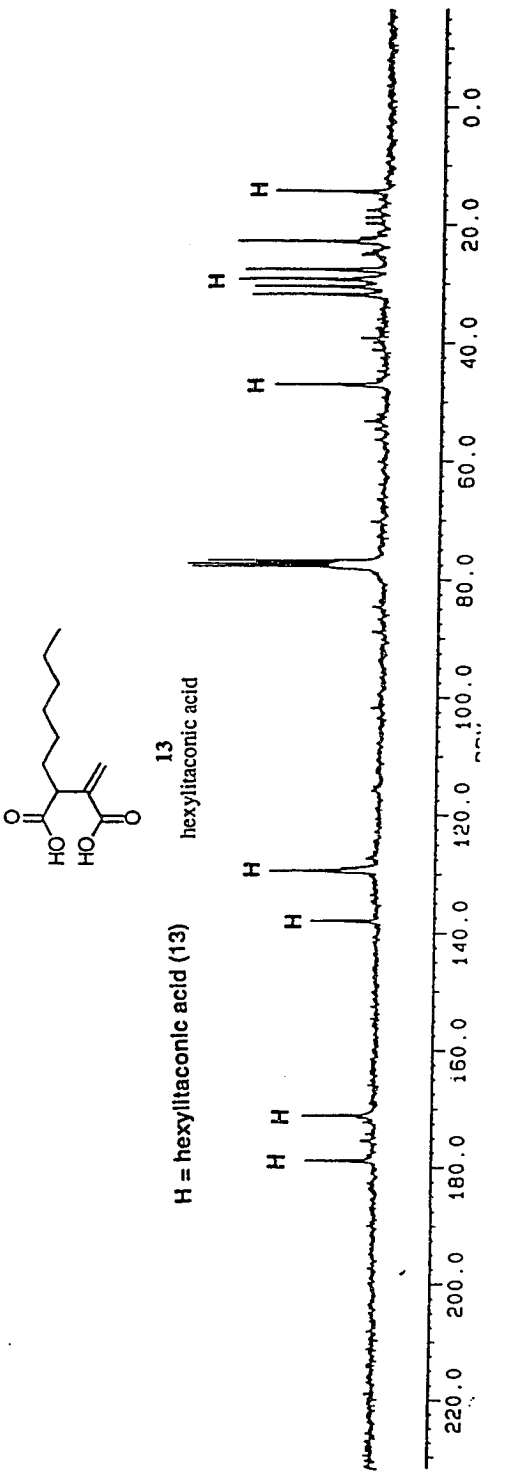
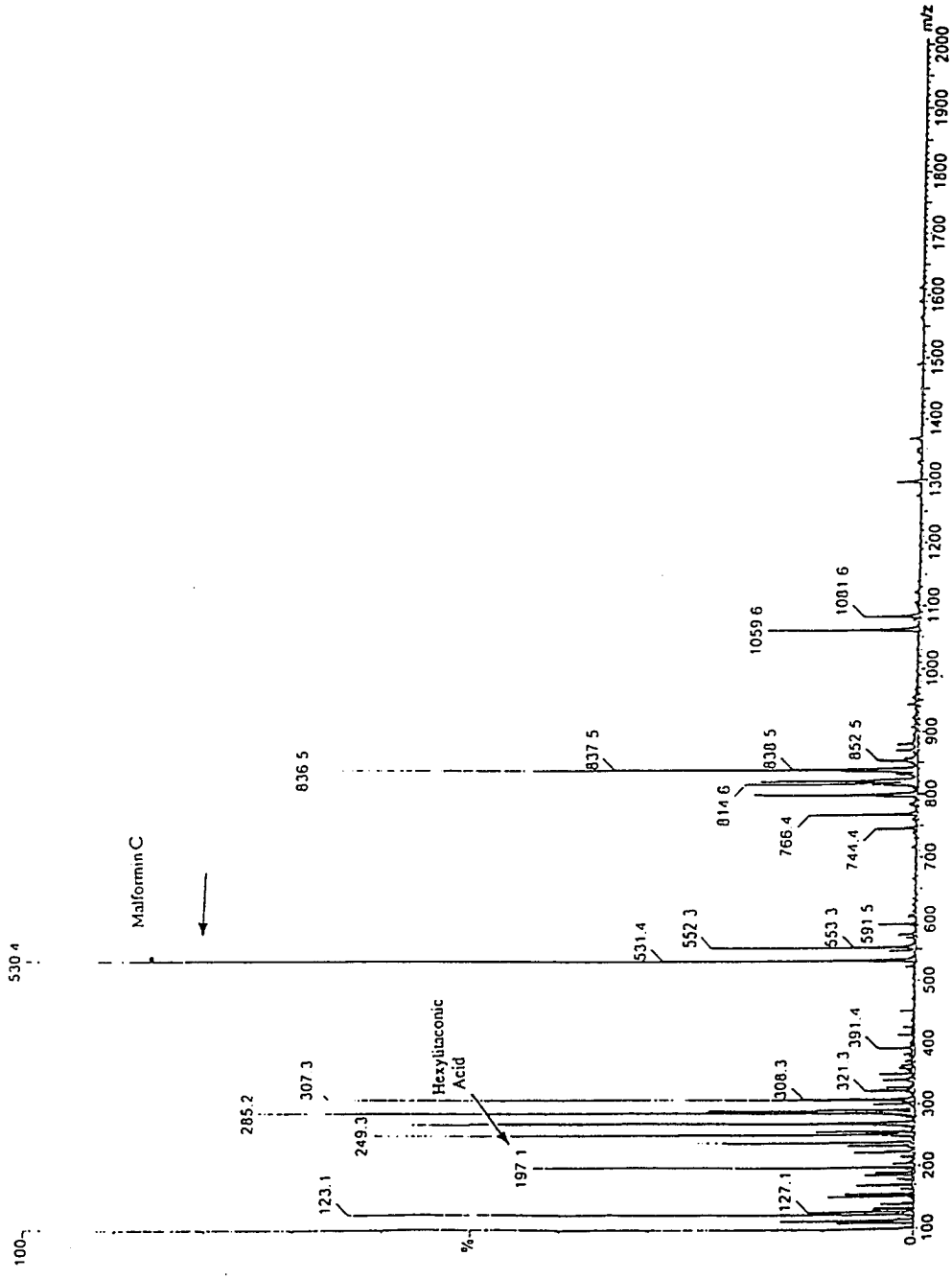
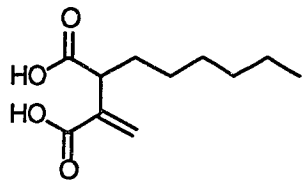
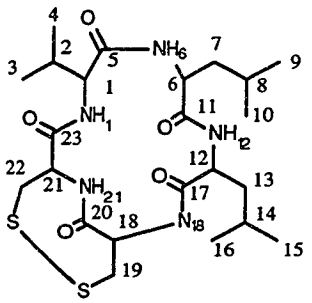


Figure 5.7. ESI mass spectrum of 94-1212(1) FD F(6).





13
hexylitaconic acid



14
malformin C

Between the isolation of malformin C (**14**) and confirmation of its structure another set of 8x 500 mL of liquid culture in malt extract broth was started. As shown in Figure 5.2 only plate MEA 2.2 was used to start cultures MEB 4.1-4.16 (94-1212 (2)) instead of both the MEA 2.1 and 2.2 plates used originally for 94-1212 (1). This culture grew with the same morphology as the first culture for 21 days. At the end of this large growth, 94-1212 (2), the mycelium and broth were again separated by filtration. The broth was extracted with ethyl acetate and this crude extract underwent the same solvent/solvent partition scheme previously described for 94-1212 (1), Figure 5.3, to yield the crude fractions shown in Table 5.1.

Table 5.1. Yield of solvent/solvent partitions of 94-1212 (2).

94-1212 (2) Broth Partitions	WB	FH	FD	FM
Weight of fraction (mg)	228.0	37.8	77.7	19.0

The ^{1}H and ^{13}C NMR of the FD fraction is shown in Figures 5.8 and 5.9. These spectra are different from the spectra of the first large scale growth. Specifically, the hexylitaconic acid (**13**) peaks observed in Figure 5.4 are not present and the malformin C (**14**) peaks (indicated with an M in Figure 5.8) are much more prominent. This crude partition was separated by gradient reverse phase HPLC (water/methanol), Figure 5.10.

Figure 5.8. ^{1}H NMR of FD partition of 94-1212 (2) broth extract, 250 MHz, $CDCl_3$.

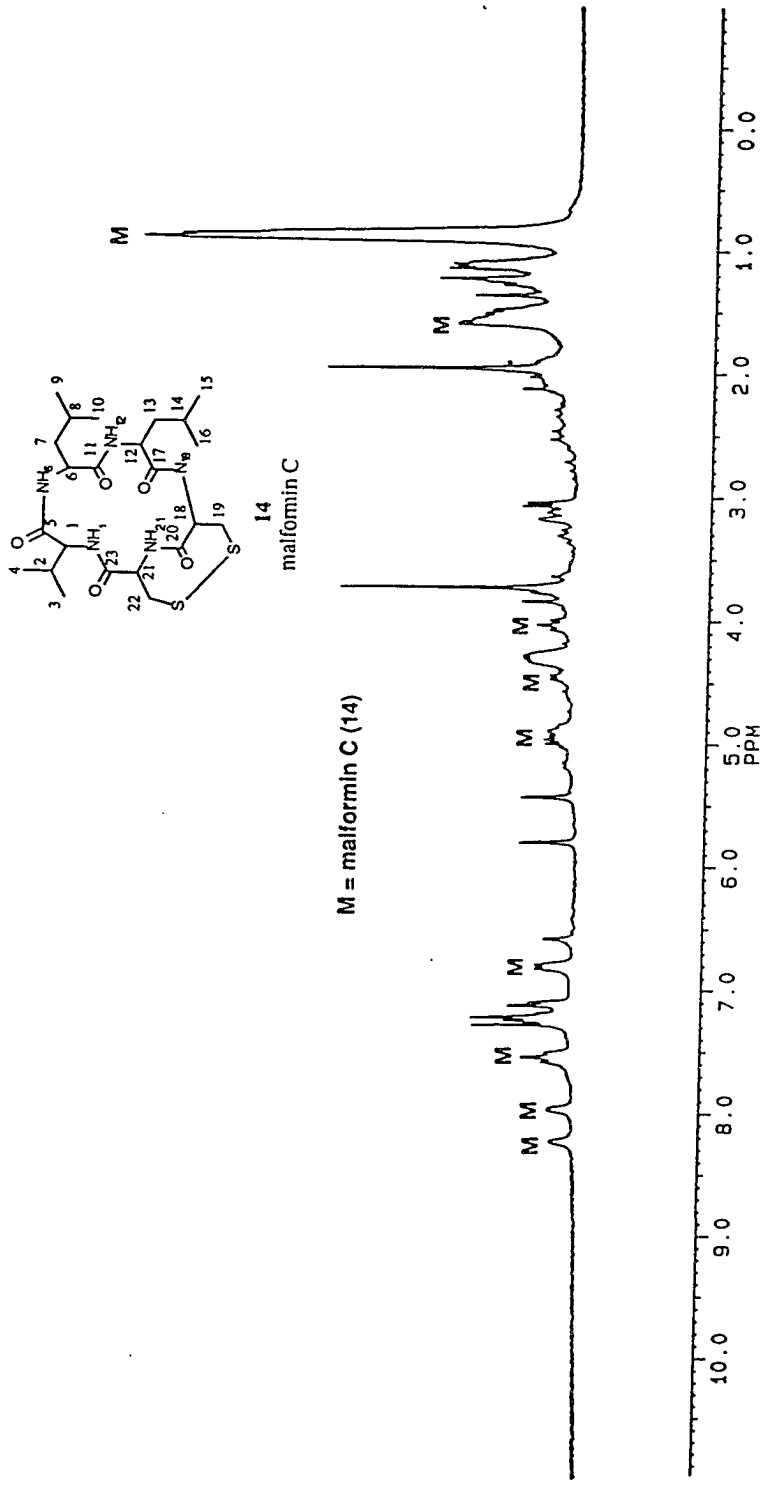
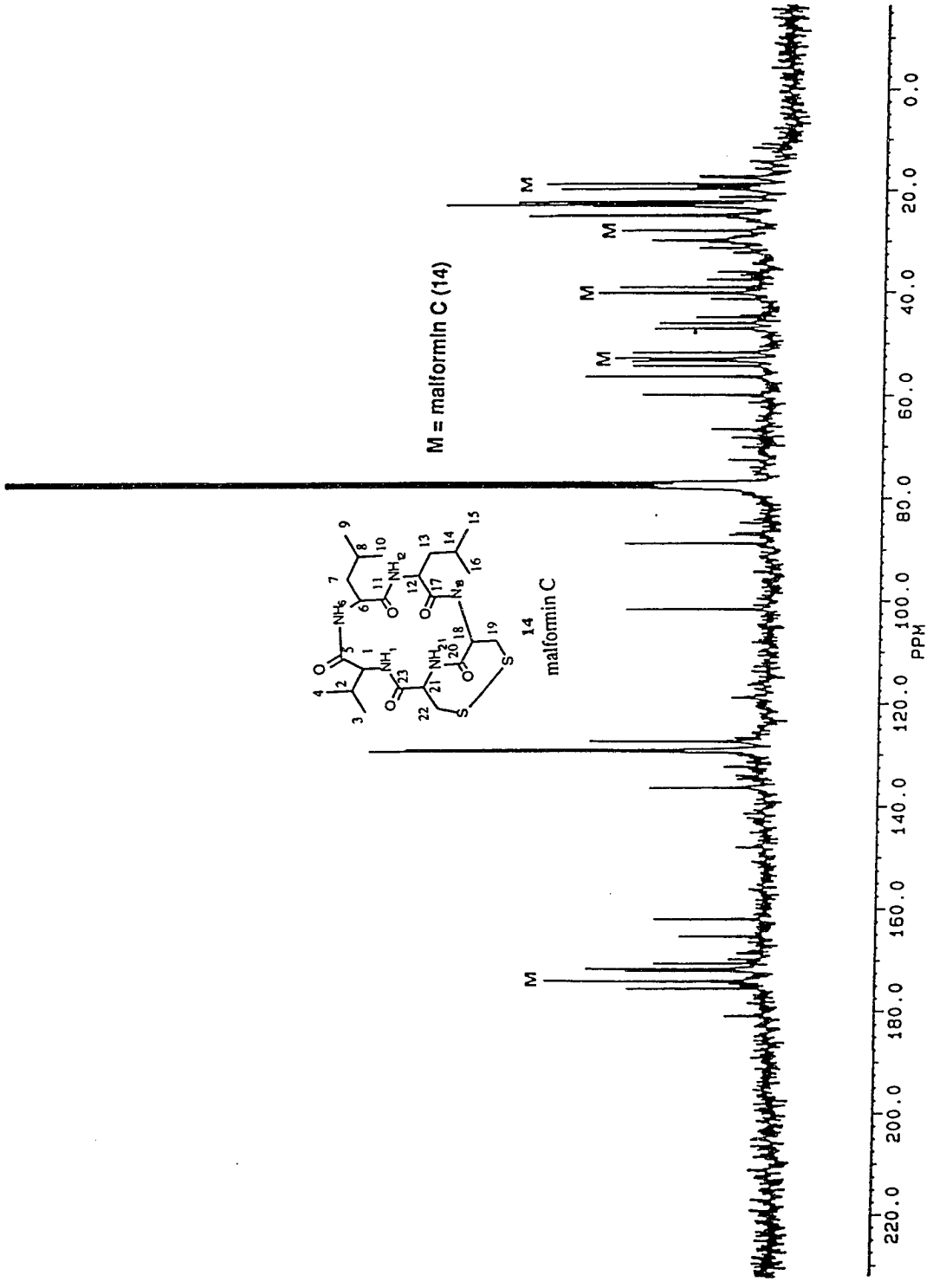


Figure 5.9. ^{13}C NMR of FD partition of 94-1212 (2) broth extract, 62.9 MHz, $CDCl_3$.



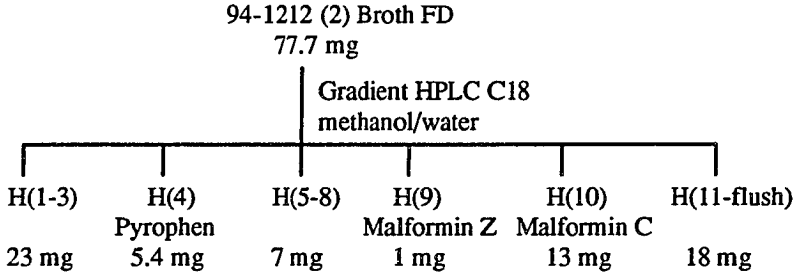
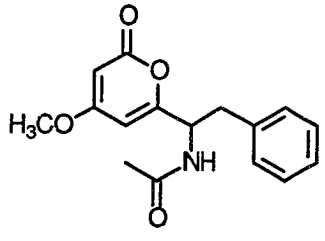
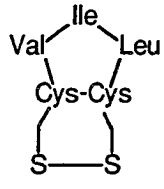


Figure 5.10. Separation scheme for 94-1212 (2).

Using this method the compounds, pyrophen (**15**), malformin Z (**16**) and malformin C (**14**) were obtained in pure form as the concentration of methanol increased. No hexylitaconic acid (**13**) was found in this culture. However, two new compounds, pyrophen (**15**) and malformin Z (**16**), were isolated. After the structure of pyrophen (**15**) was solved it was discovered to be known.[16]



15
pyrophen



16
malformin Z

Only the ^{1}H NMR, Figure 5.11, and ^{13}C NMR, Figure 5.12, and low resolution FAB mass spectrum, Figure 5.13, of malformin Z (**16**) were obtained before the sample was claimed by gravity. To replace the lost malformin Z (**16**) another eight 500 mL malt extract broth cultures, MEB 5.1-5.16, were started from MEA 3.2, a sample of MEA 2.1 that was cryopreserved to maintain the culture, Figure 5.2. The large growth consisting of MEB 5.1-5.16 was called 94-1212 (3). The broth of this culture was extracted with ethyl acetate and the extract was partitioned as before to yield the solvent partitions shown in Table 5.2.

Table 5.2. Yield of solvent/solvent partitions of 94-1212 (3).

94-1212 (3) Broth Partitions	WB	FH	FD	FM
Weight of fraction (mg)	not weighed	143.0	62.9	11.3

Figure 5.11. ^{1}H NMR of malformin Z (**16**), 500 MHz, $CDCl_3$.

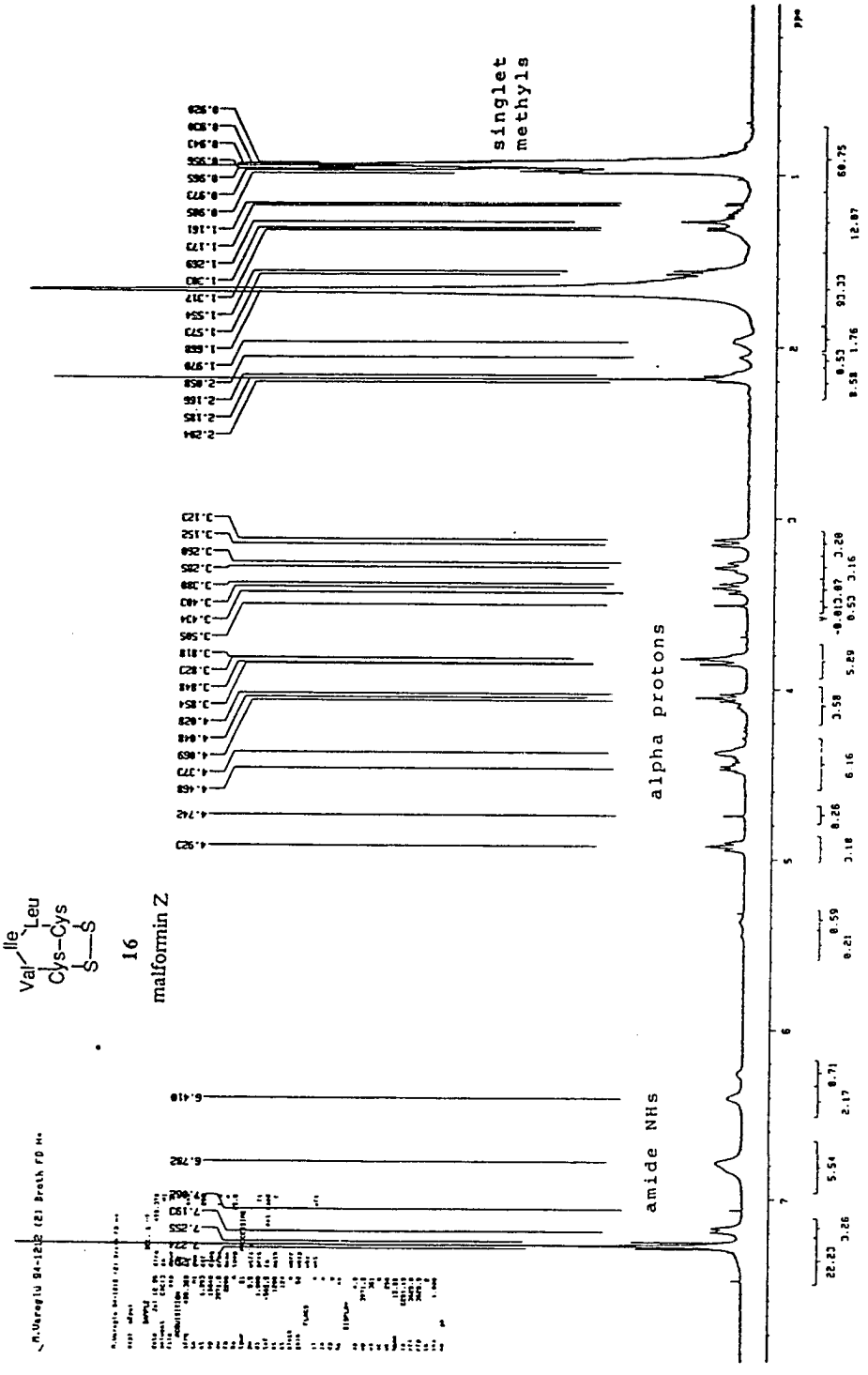


Figure 5.12. ^{13}C NMR of malformin Z (**16**), 125 MHz, $CDCl_3$.

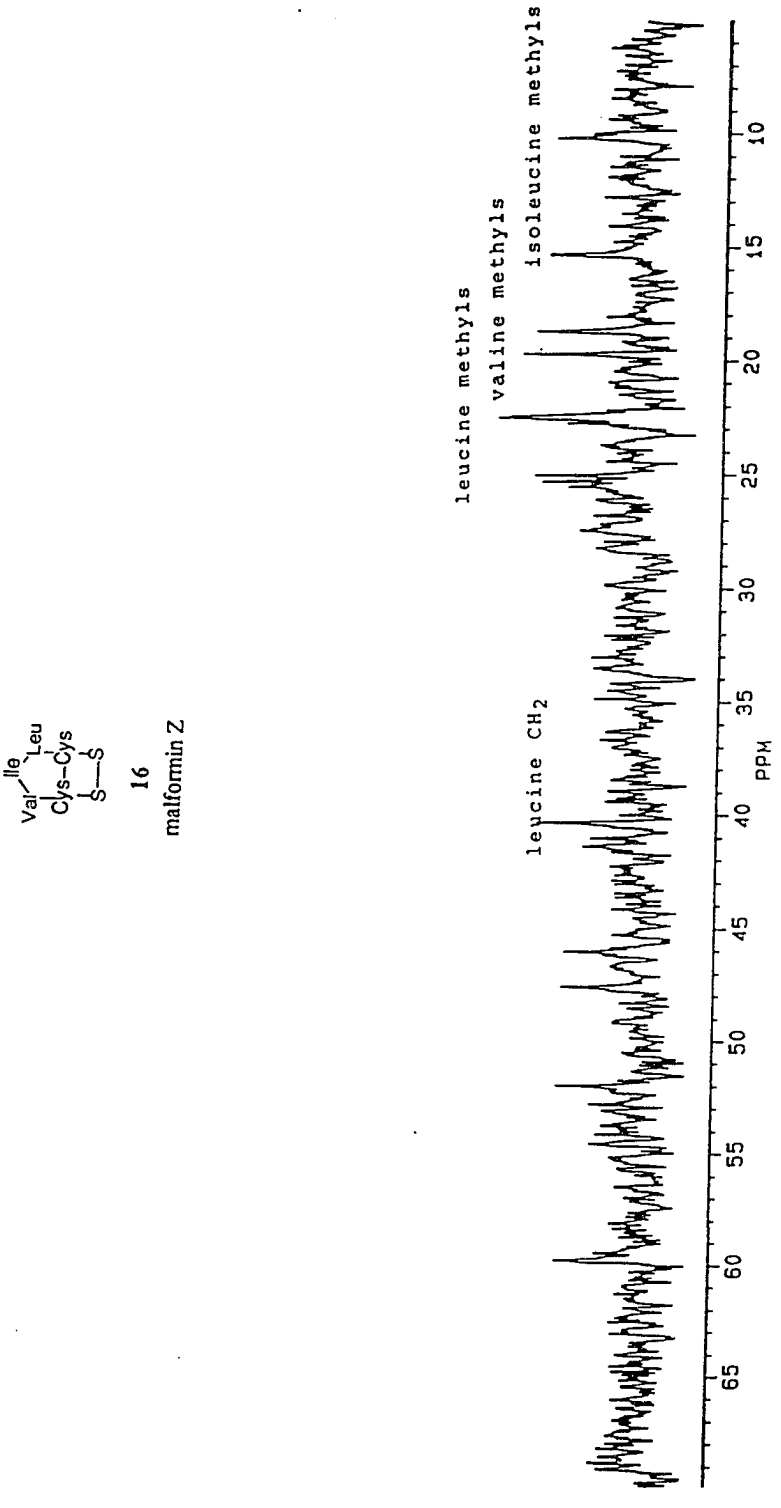
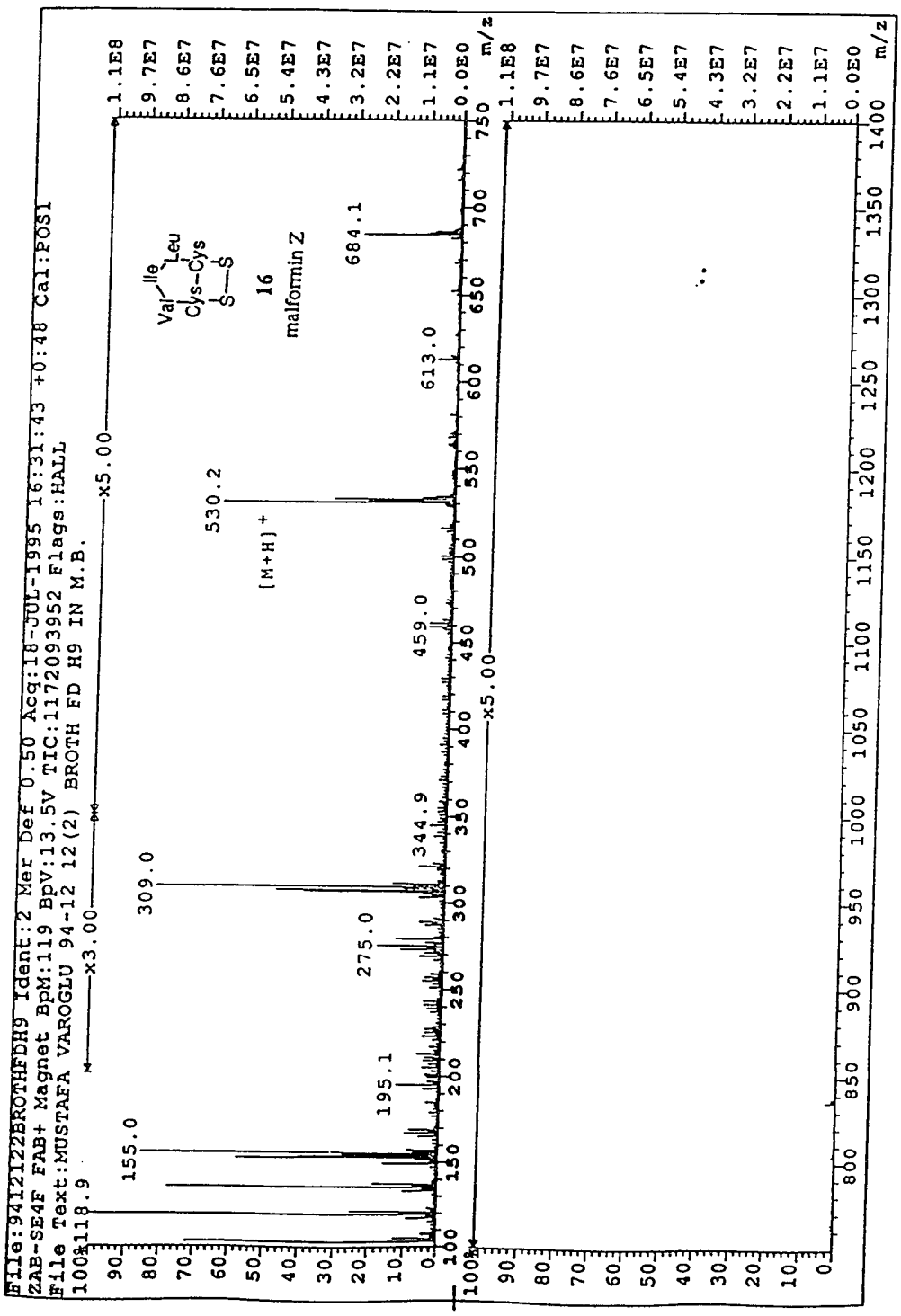


Figure 5.13. Low resolution FAB mass spectrum of malformin Z (**16**). dithiothreotol/ dithithioerythrotol matrix.



The crude ^{1}H and ^{13}C NMR spectra of this culture again indicated the presence of malformin C (**14**) and pyrophen (**15**). Gradient HPLC was used to separate the crude FD partition in the hope that more malformin Z (**16**) could be isolated. Malformin Z (**16**) was previously eluted immediately before malformin C (**14**) by gradient HPLC. On running the same HPLC conditions a new compound, asperic acid (**17**), was eluted immediately before malformin C (**14**), Figure 5.14. Examination of the other HPLC fraction indicated the presence of pyrophen (**15**) again and an impure form of another compound was noted. Fraction H12 appeared interesting from its ^{1}H and ^{13}C NMR spectra. This fraction was further purified by isocratic reverse phase HPLC using 24% aqueous methanol. The compound isolated from H12 proved to be a novel compound and was named asperizine (**18**).

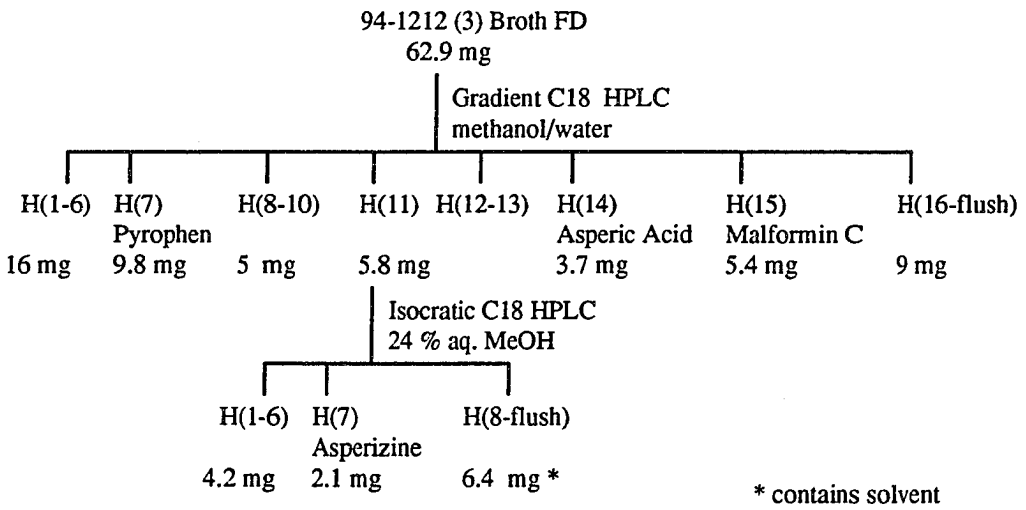
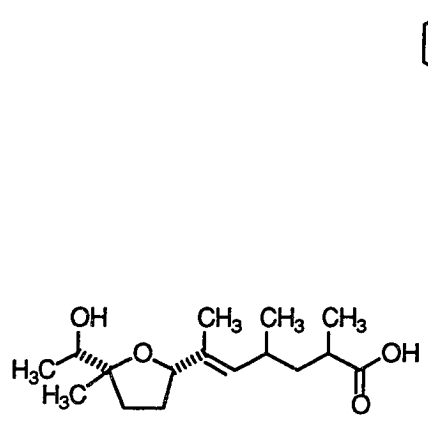
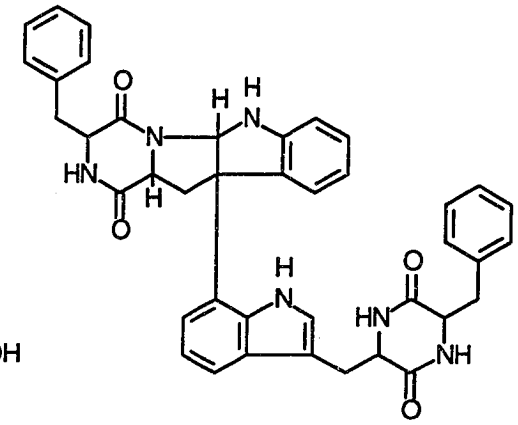


Figure 5.14. Separation scheme for 94-1212 (3).



17
asperic acid



18
asperizine

In order to generate more asperizine (**18**) a fourth culture of 8 x 500 mL malt extract broth was grown using the same inoculum MEA 3.2 as for 94-1212(3). This created the cultures MEB 5.17-5.32 and the large growth was named 94-1212 (4). Identical separation conditions as were used in 94-1212 (3) were applied to 94-1212 (4). The same compounds were found in 94-1212 (4) as were found in the third large growth, 94-1212 (3), Figure 5.15. The distribution and quantity of the compounds isolated in the four separate growths are summarized in Table 5.3.

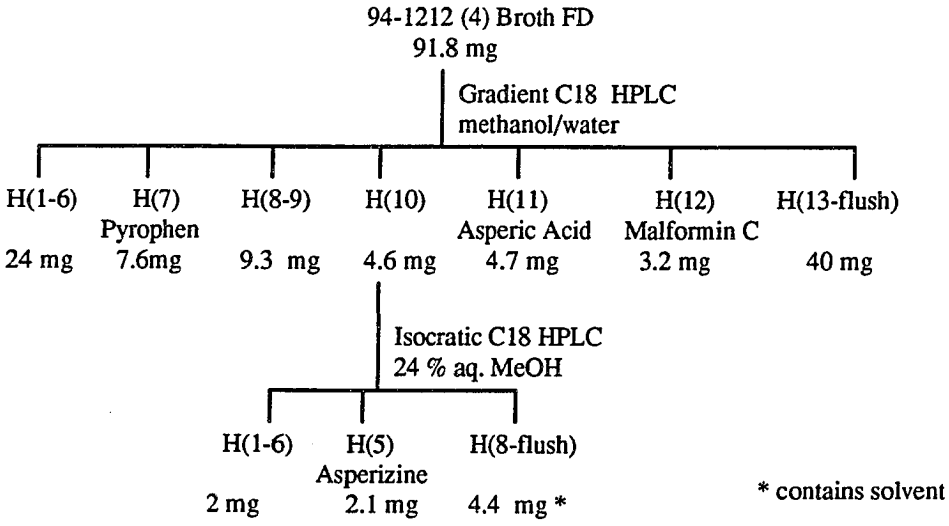


Figure 5.15. Separation scheme for 94-1212 (4).

Table 5.3. Summary of distribution of compounds in large scale liquid cultures.

Growth	hexylitaconic acid (**13**)	pyrophen (**15**)	malformin Z (**16**)	asperic acid (**17**)	asperizine (**18**)	malformin C (**14**)
94-1212 (1)	13	0	0	0	0	1.5
94-1212 (2)	0	5.4	1	0	0	13.0
94-1212 (3)	0	9.8	0	4.0	2.0	5.4
94-1212 (4)	0	7.6	0	4.6	1.0	3.2

Structure elucidation of asperizine (18)

Upon isolation of asperizine (**18**), a sample was immediately submitted for low and high resolution fast atom bombardment (FAB) mass spectrometry. While waiting for the mass spectrometry results attempts were made to determine a working formula and to identify substructures using NMR techniques.

When initially examined, the carbon spectrum indicated 35 separate peaks, Figure 5.16, but, the significantly higher intensity of some of the peaks implied more than one carbon was responsible for some of the observed resonances. The 35 different carbon resonances were determined to be caused by at least 39 different carbons. In particular, it was determined that the resonances at 137.72, 131.63, 131.46, and 130.15 were due to two carbons each. This led to a working carbon count of C_{39}. A combination of DEPT, Figure 5.17, ^{1}H NMR, Figure 5.18, and HMQC experiments, Figure 5.19 determined that 31 protons were attached to carbons. There were no methyl, four methylene, and 23 methine carbons present from the DEPT spectrum, Figure 5.17. The ^{1}H NMR spectrum also had five additional proton signals at 8.81 (H30), 6.49 (H38), 6.18 (H14), 6.16 (H35), and 6.14 ppm (H1) that did not correlate to any carbons in an HMQC, experiment, Figure 5.19, indicating that these five protons were on heteroatoms. The presence of four carbonyl peaks from 168-170 implied at least four oxygens. The ^{13}C NMR shifts of the carbonyls and the presence of four methine carbons typical of the α carbons of peptides in the 50-60 ppm range suggested that the carbonyls were part of an amide functionality. A preliminary formula of $C_{39}H_{36}N_4O_4$ could be determined from the above data.

The presence of peaks in the ^{1}H NMR spectrum, Figure 5.18, between 3.2 and 4.2 ppm and the ^{13}C NMR resonances from 50-60 ppm reinforced the suggestion of a peptide natural product. The large number of aromatic peaks in the ^{13}C NMR spectrum, Figure 5.16, (26 of 40 carbons) and the lack of methyl peaks from a DEPT experiment, Figure 5.17, implied the presence of aromatic amino acids. The phenylalanine moieties were inferred by comparison to the literature

Figure 5.16. ^{13}C NMR spectrum of asperizine (**18**), 125 MHz, CD_3CN.

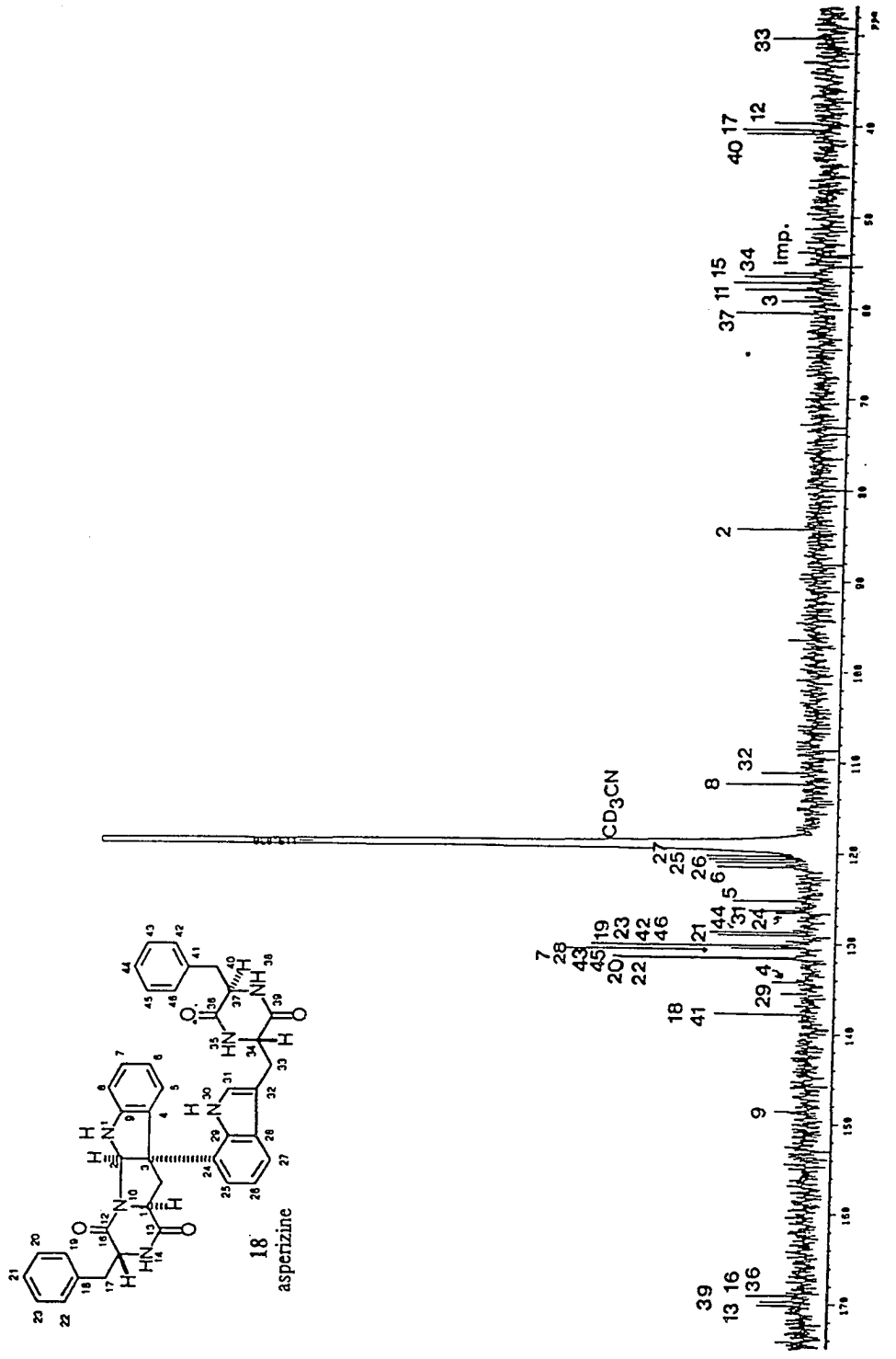


Figure 5.17. DEPT 135 spectrum of asperizine (**18**), 62.9 MHz, CD_3CN.

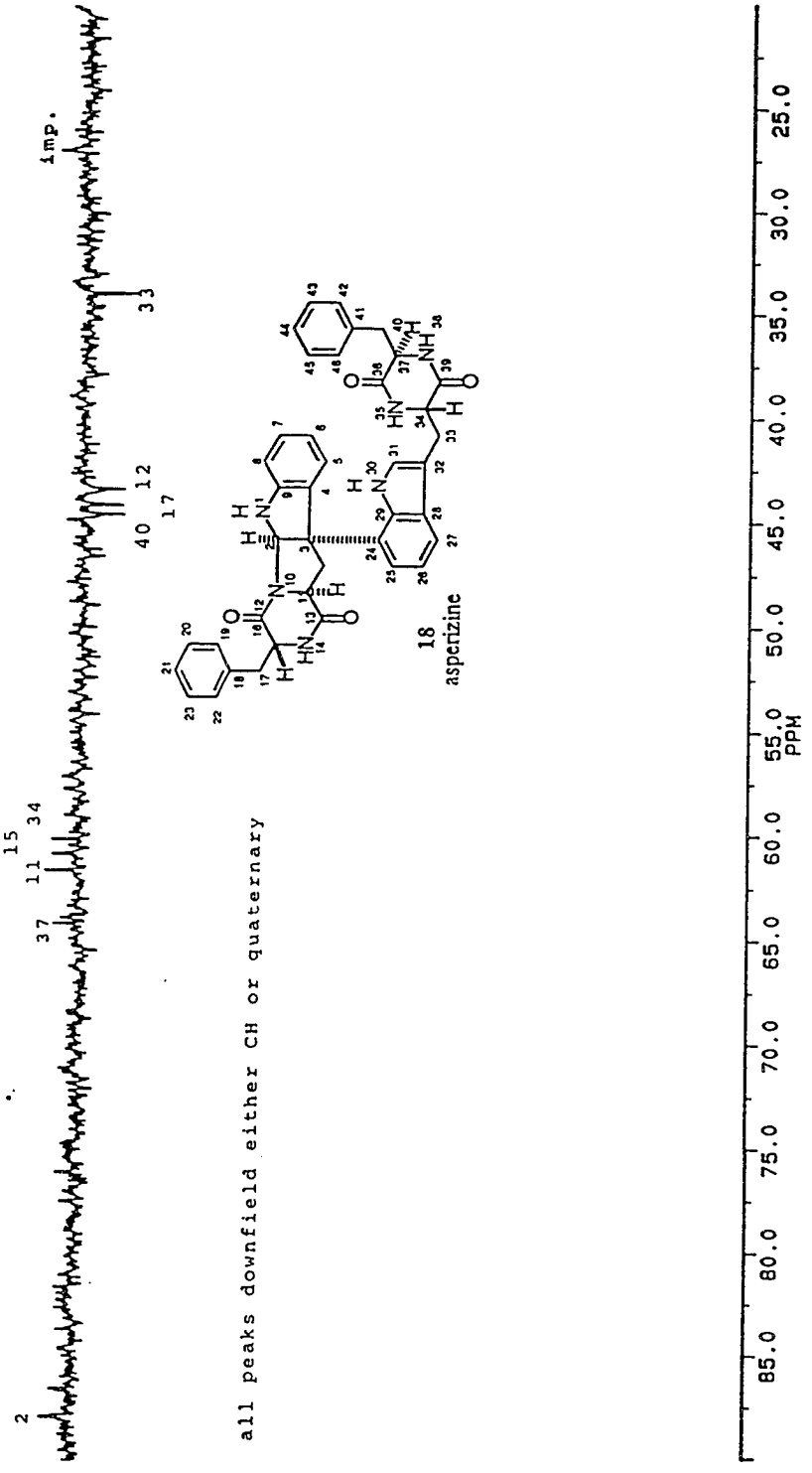


Figure 5.18. ^{1}H NMR spectrum of asperizine (**18**), 500 MHz, CD_3CN.

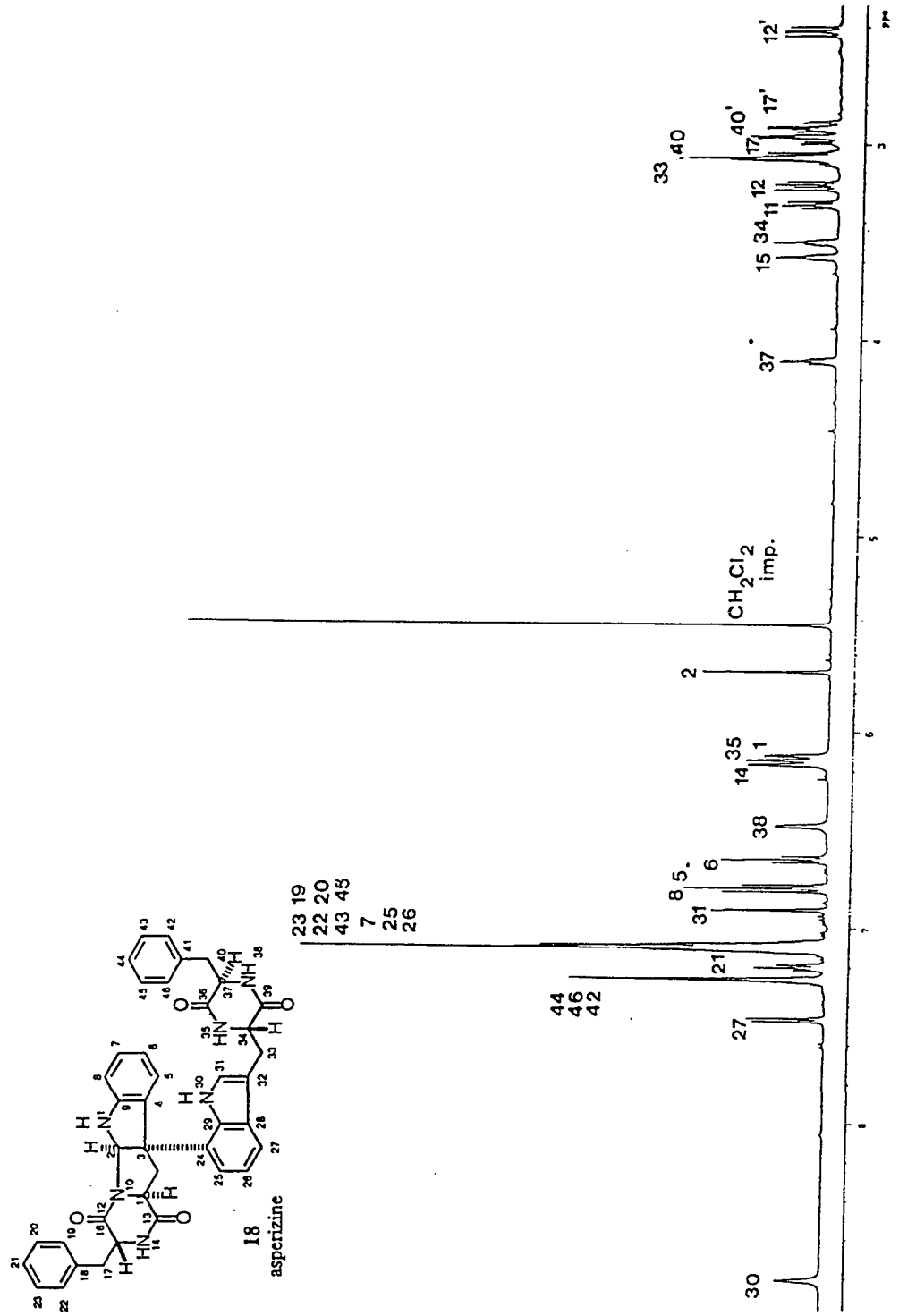
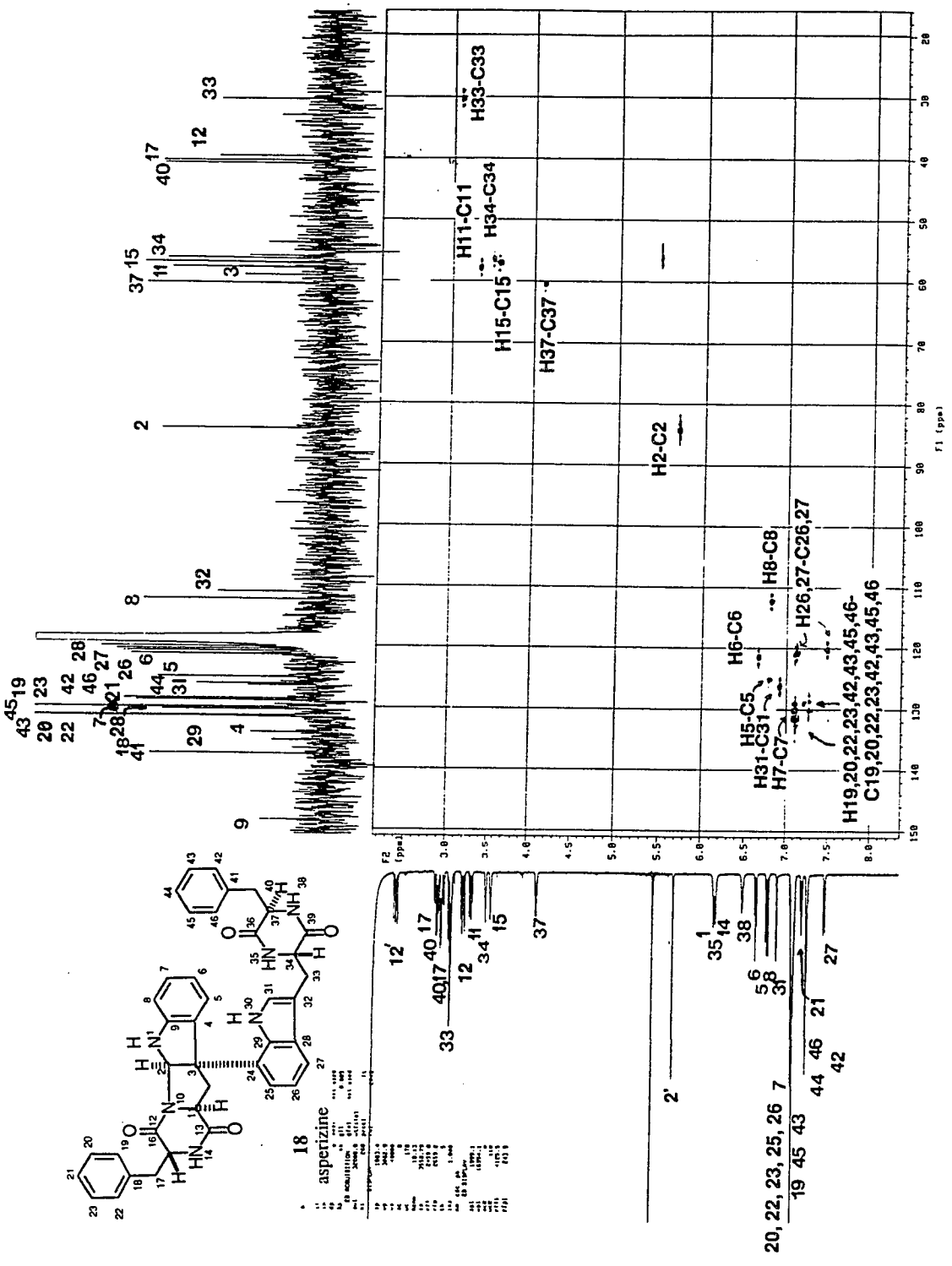


Figure 5.19. HMQC spectrum of asperizine (18), 500 MHz, CD_3CN.



shifts for phenylalanine.[17] Matching the literature shifts of phenylalanine (P denotes phenylalanine in Figure 5.20) was complicated by the overlap in the 128-131 ppm range of the ^{13}C NMR spectra. The ^{1}H resonances of the phenylalanine subunits were assigned by an HMQC experiment. However, it was apparent that the phenylalanine did not account for all of the peaks present in the lowfield region of the ^{1}H NMR, Figure 5.21.

At this point a great deal of difficulty was encountered in trying to assign the other resonances. Some of the remaining shifts suggested a tryptophan unit. However, when an HMBC experiment, Figure 5.22, was used to try and confirm the presence of a tryptophan unit it was found that there were too many HMBC correlations to the ^{13}C resonance of the methine at 130.46 ppm (marked with an *, Figures 5.20 and 5.22). It was not possible to place all of the atoms with an HMBC correlation to 130.46 ppm within three bonds of this atom without violating other three bond HMBC correlations.

In order to overcome this difficulty the ^{1}H NMR and ^{1}H-^{1}H COSY spectra were reexamined and the newly arrived mass spectral data was analyzed, Figure 5.23. Even though there was a great deal of overlap present in the ^{1}H NMR spectra it was determined that H5, H6, H7, and H8 were mutually coupled, Figure 5.21. Both the H5 (dd) and H6 (dt) were coupled to H7 ($^{3}J_{5\text{-}7}$ = 1Hz, $^{3}J_{6\text{-}7}$= 7Hz) as well as to each other ($^{3}J_{5\text{-}7}$ =7Hz). These couplings were confirmed by a ^{1}H-^{1}H COSY experiment, Figure 5.24. Unfortunately, the signal for H7 overlapped with the nine protons signals at 7.12 ppm. By HMQC C7 was correlated to be the signal at 130.46. HMBC correlations observed from H6 to C8 and C4 and from H5 to C9 and C7 confirmed the presence ofthe quaternary carbons C4 and C9, Figure 5.25. As is typically observed for HMBC correlations, the two bond C-H correlations in aryl rings were very weak while the three bond correlations were very strong.[18] The identification of the H5-H8 spin system led to the construction of an ortho disubstituted benzene ring, Figure 5.26.

Figure 5.20. ^{13}C NMR spectrum of asperizine (**18**), 110 - 150 ppm expansion, 125 MHz, CD_3CN.

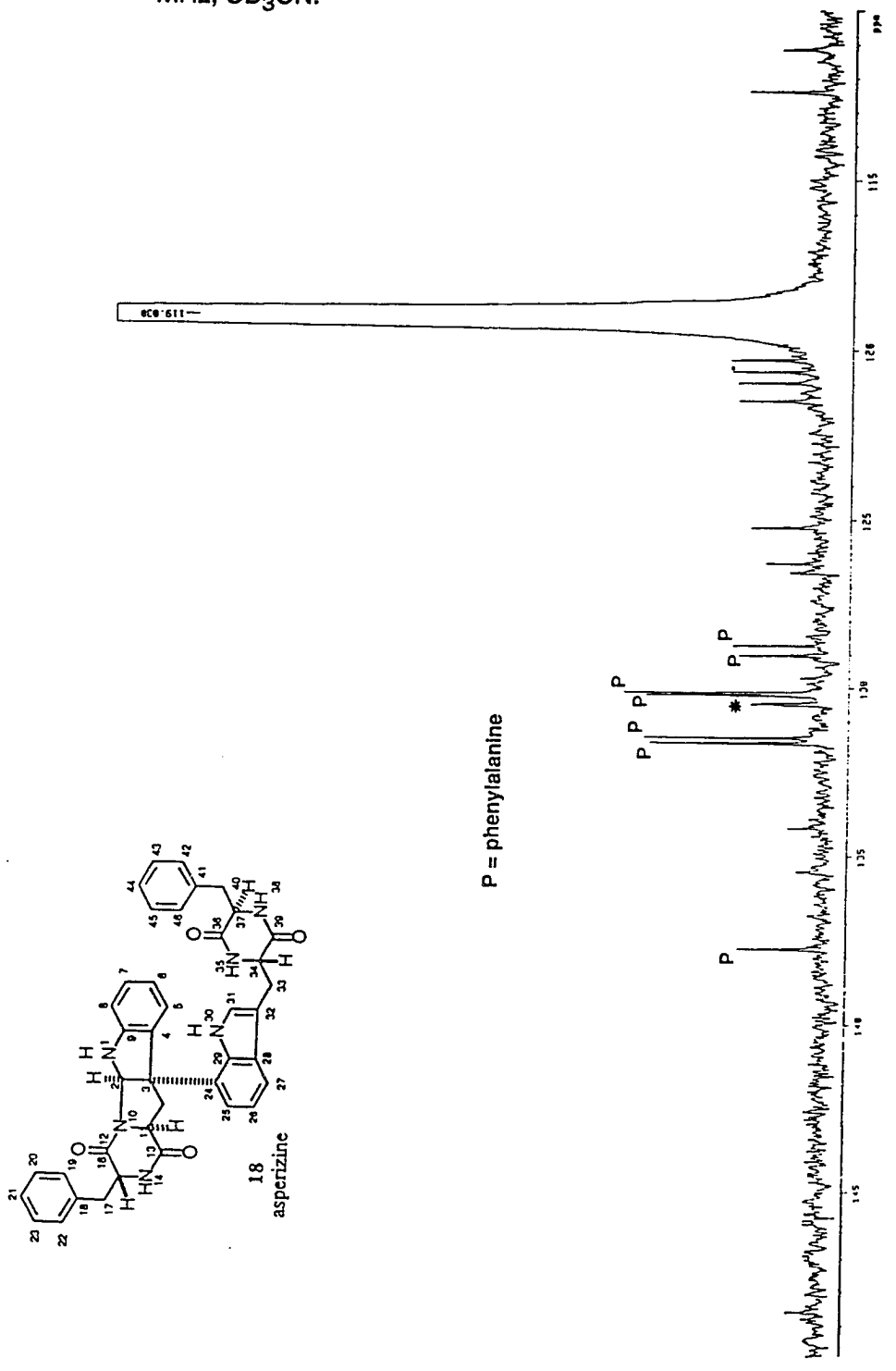


Figure 5.21. ^{1}H NMR spectrum of asperizine (**18**), 5.6 - 8.9 ppm expansion, 500 MHz, CD_3CN.

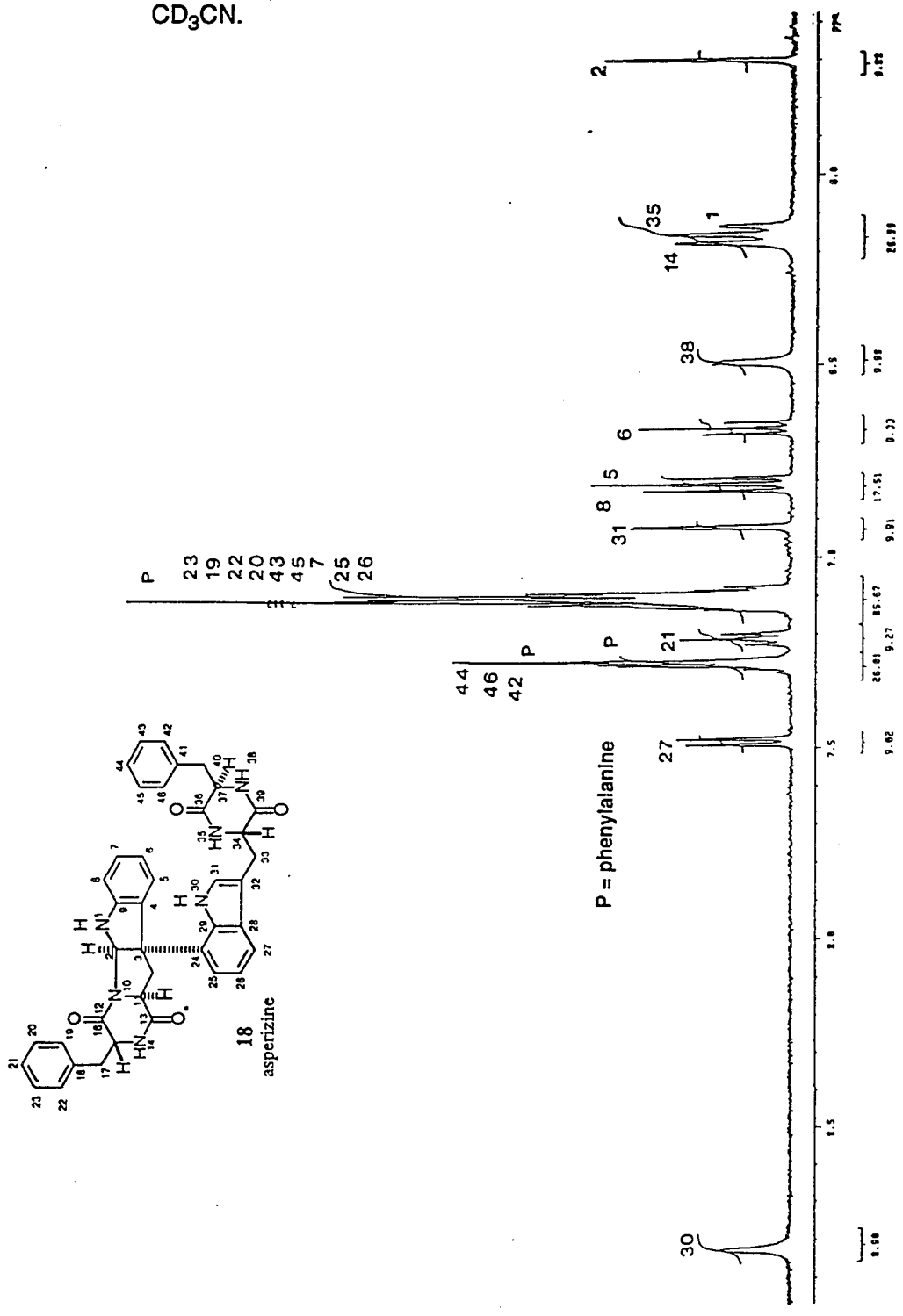


Figure 5.22. HMBC spectrum of asperizine (**18**), correlations to *, 500 MHz, CD_3CN.

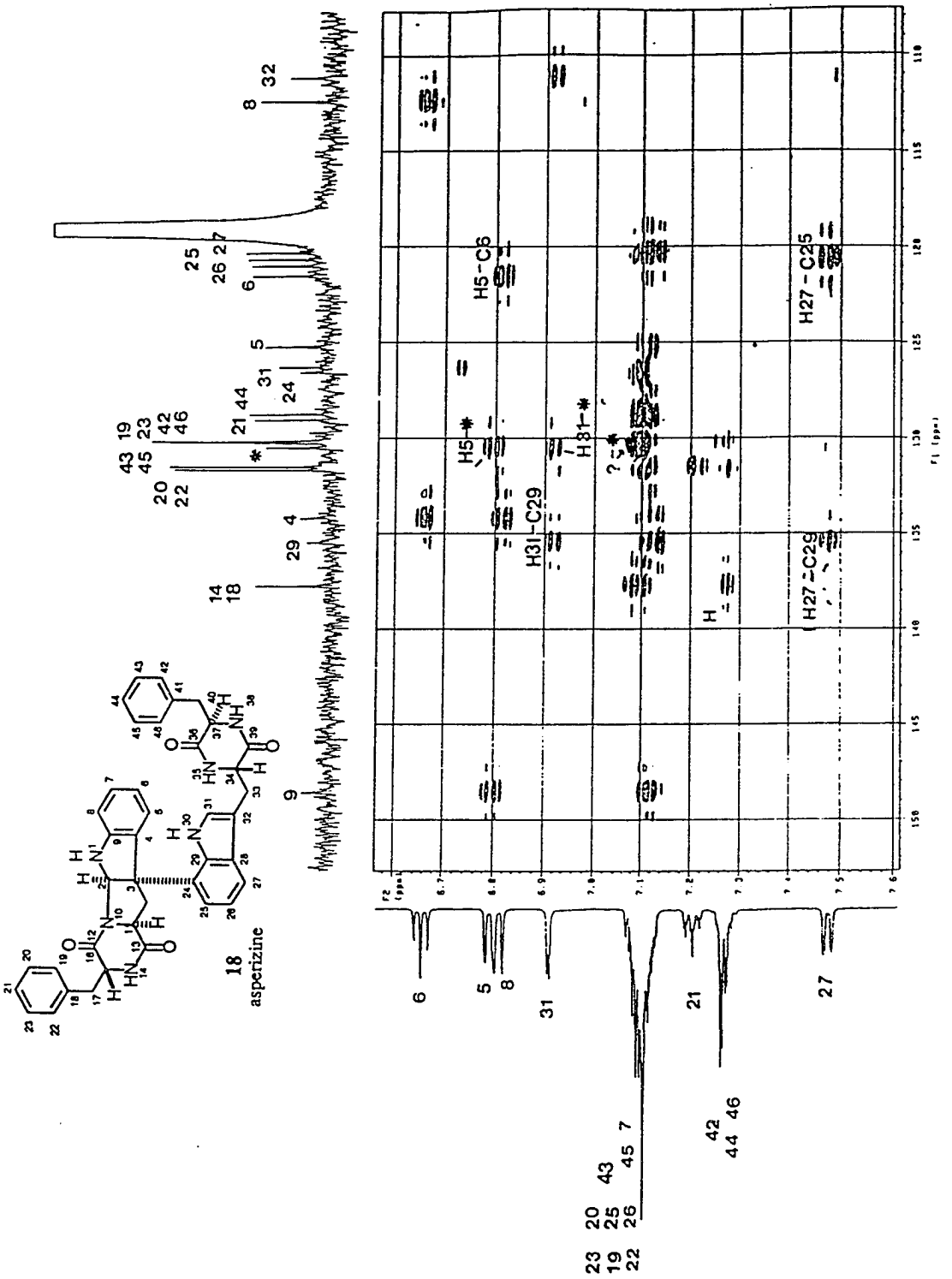


Figure 5.23. LRFABMS and HRFABMS of asperizine (**18**), dithiothreotol/ dithioerythrotol matrix.

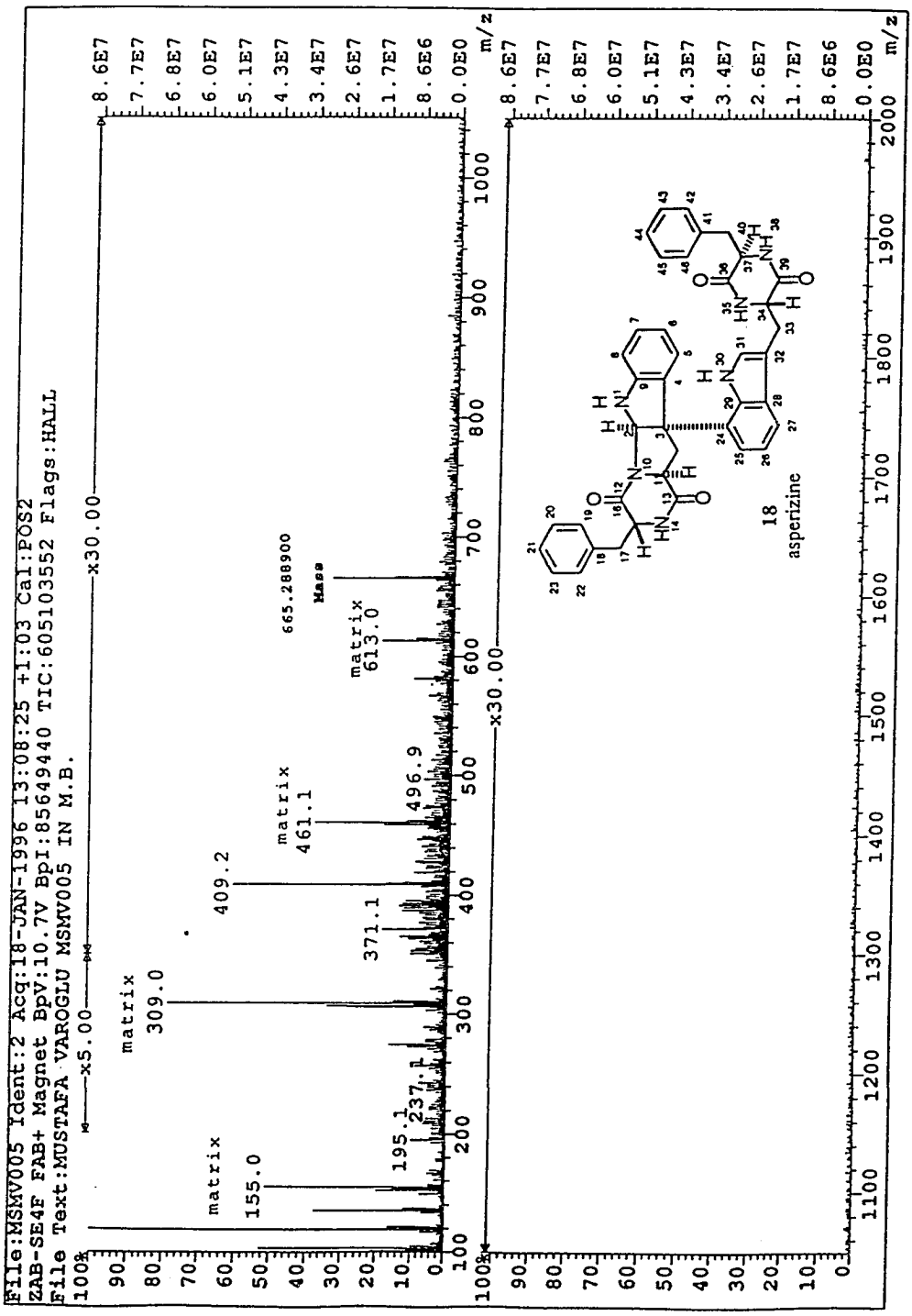


Figure 5.24. ^{1}H-^{1}H COSY spectrum of asperizine (**18**), 6.4-7.8 ppm expansion 500 MHz, CD_3CN.

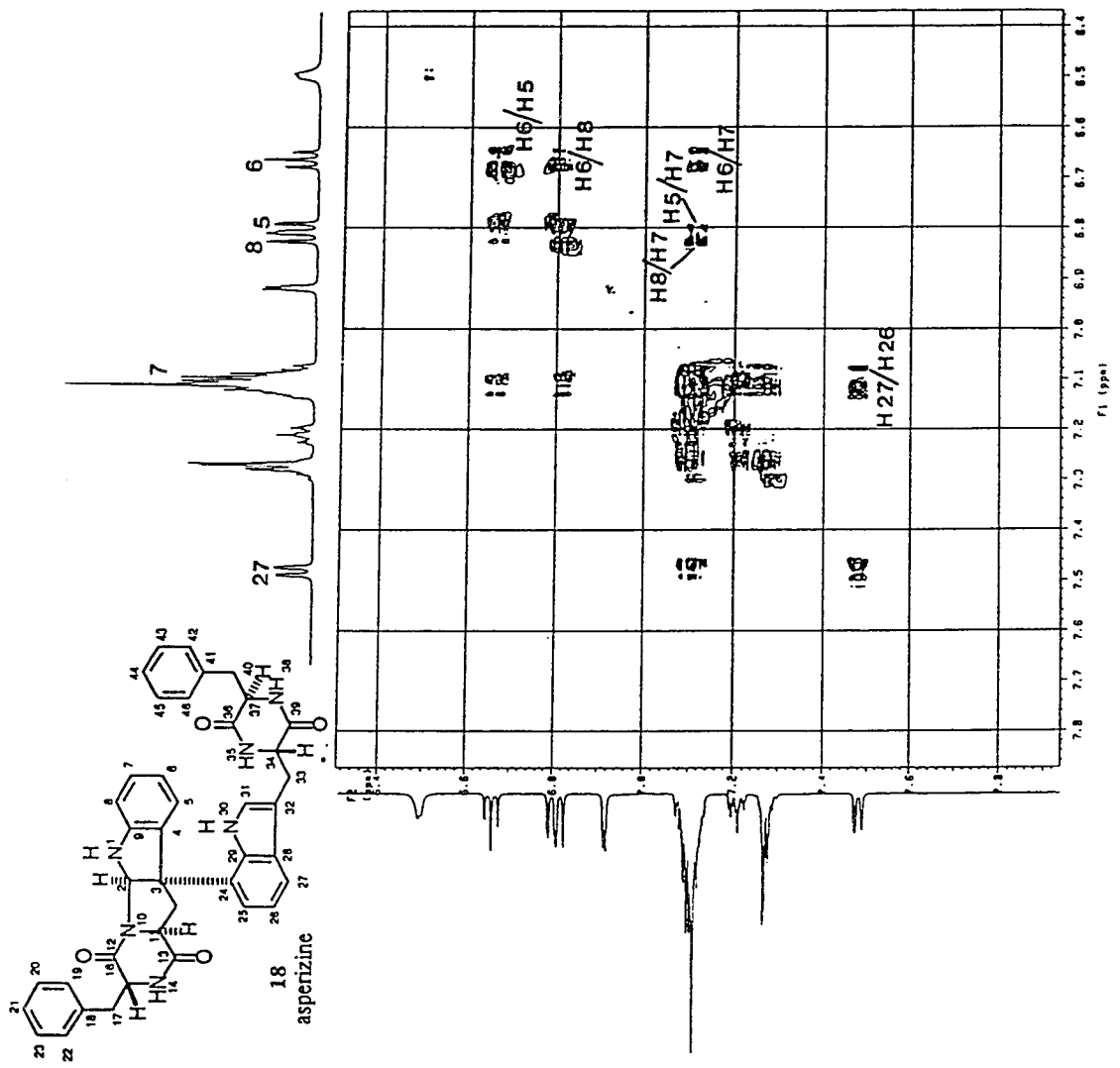
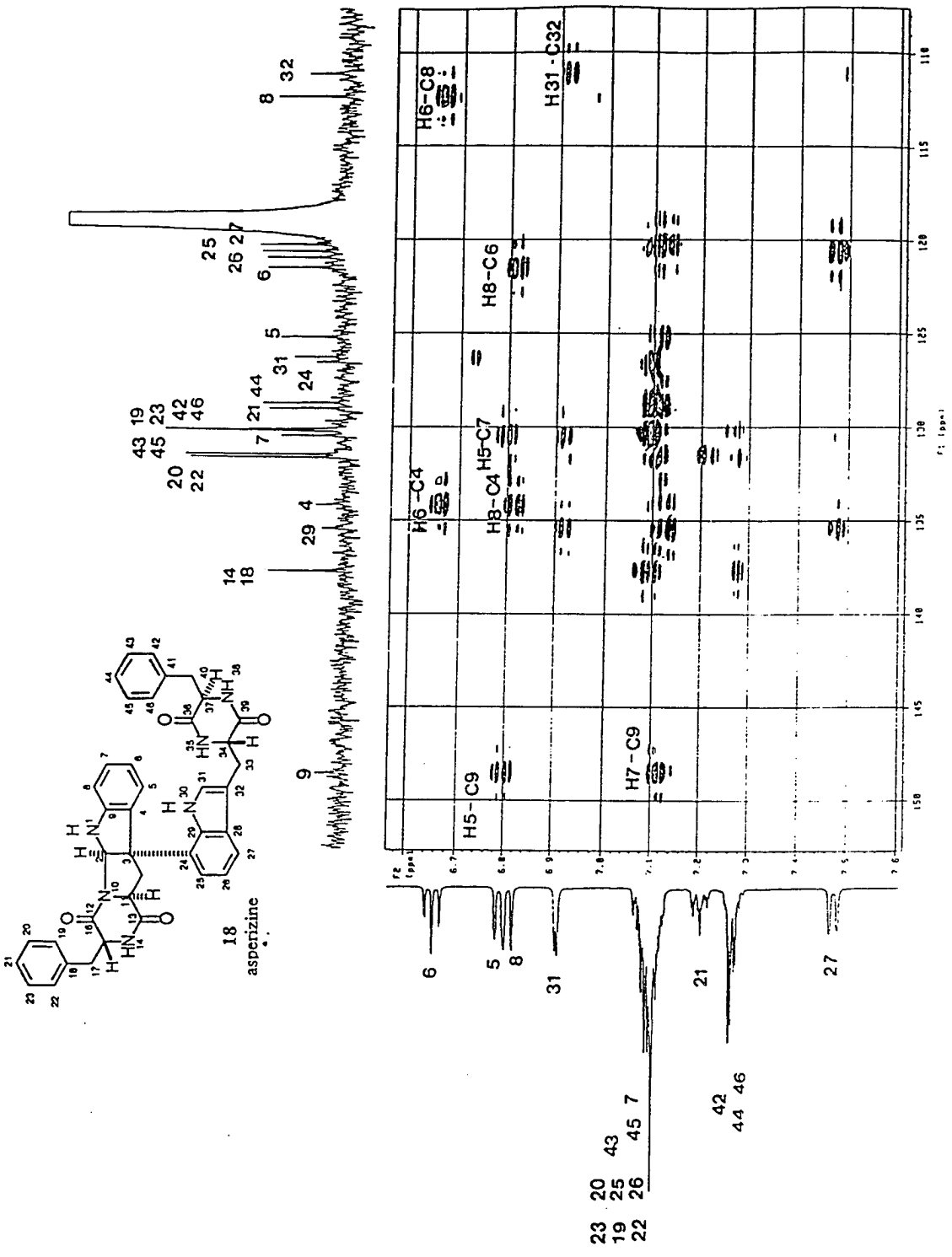
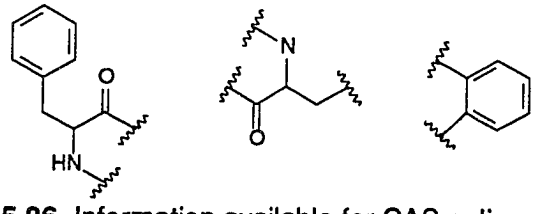


Figure 5.25. HMBC spectrum of asperizine (**18**), 500 MHz, CD_3CN.



Low resolution electrospray ionization (ESI) and low resolution fast atom bombardment (FAB) mass spectrometry both provided an $[M+H]^+ = 665$ *m/z*, Figure 5.23. The high resolution FAB peak at *m/z* = 665.2889 indicated a potential formula of $C_{40}H_{36}N_6O_4$ (Δ 1.3 mmu of calcd), Figure 5.23. The preliminary formula of $C_{39}H_{36}N_4O_4$ was ammended with two nitrogens and one more carbon to match $C_{40}H_{36}N_6O_4$. Four of the six nitrogens could be accounted for in the amide bonds. Therefore, the remaining two nitrogens were part of the sidechains of the other two amino acids.

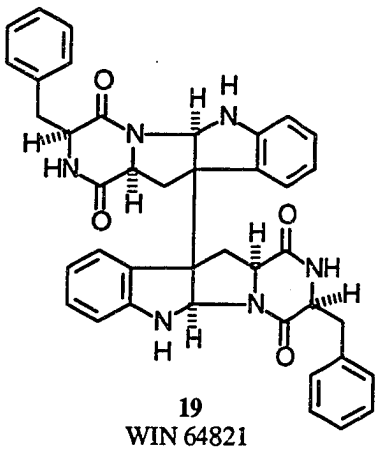
The information gathered to this point was summarized in order to perform a Chemical Abstracts Service online search. The information available for searching with and identifying matches to our compounds consisted of the formula $C_{40}H_{36}N_6O_4$ and the substructures shown in Figure 5.26.



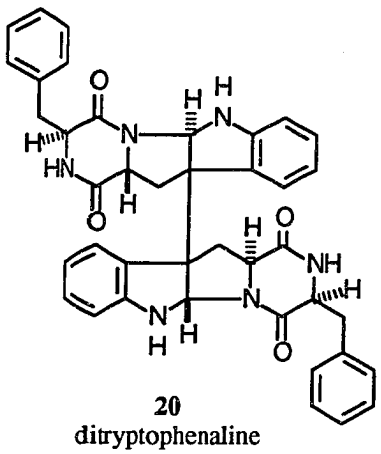
$MF = C_{40}H_{36}N_6O_4$

Figure 5.26. Information available for CAS online search.

A search of the molecular formula $C_{40}H_{36}N_6O_4$ returned the compounds WIN 64821 (**19**)[19] and ditryptophenaline (**20**).[20,21] Both WIN 64821 (**19**) and ditryptophenaline (**20**) are symmetrical dimers composed of tryptophan and phenylalanine with an additional ring formed from the amide nitrogen of tryptophan to the indole rings. WIN 64821 (**19**) possesses C2 symmetry while ditryptophenaline (**20**) is a meso compound with a mirror plane. Ditryptophenaline (**20**) was isolated from a terrestrial *Aspergillus flavus* fungal culture and the structure was solved by X-ray crystallography. WIN 64821 (**19**) was isolated from an unidentified species of terrestrial *Aspergillus* fungus and the structure was solved by NMR and mass spectrometry. The NMR data obtained for WIN 64821 (**19**) was helpful in the further structure elucidation of asperizine (**18**).



19
WIN 64821



20
ditryptophenaline

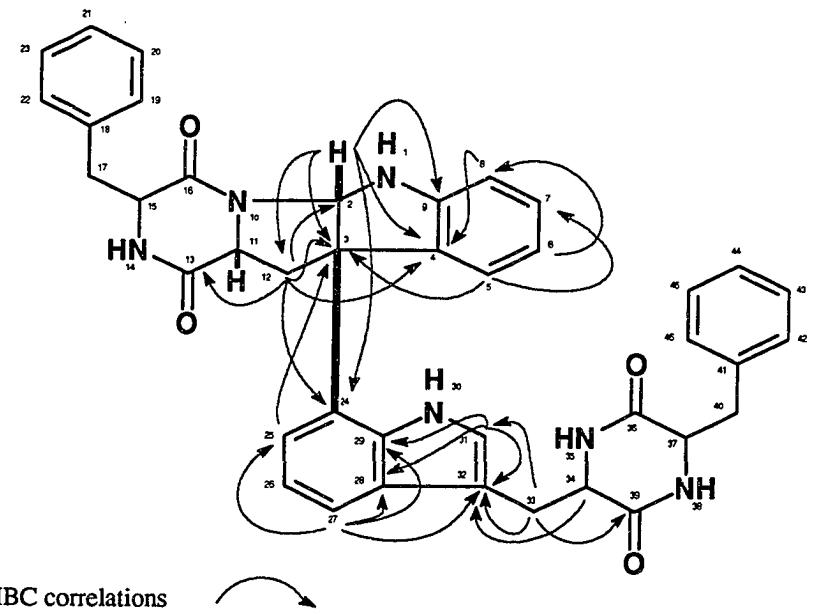
The most striking difference in the NMR data reported for WIN 64821 (**19**) and the data obtained for asperizine (**18**) is the number of signals in both the ^{13}C NMR and ^{1}H NMR, Table 5.4. Due to the symmetrical nature of WIN 64821 (**19**) only 20 signals are present in the ^{13}C NMR spectrum and only 18 protons are found through integration of the ^{1}H NMR spectrum. Asperizine (**18**) possesses 35 distinct ^{13}C NMR resonances that accounted for the 40 carbons present, Figure 5.16. While asperizine (**18**) was clearly related to both WIN 64821 (**19**) and ditryptophenaline (**20**) it did not possess the symmetry of these previously discovered compounds. Both WIN 64821 (**19**) and ditryptophenaline (**20**) have only four protonated nitrogens with two additional rings formed from the indole moieties of the tryptophans cyclized with the amide nitrogens of the tryptophans. Asperizine (**18**) has five protons on nitrogens. The extra nitrogenous proton possessed a very low field ^{1}H NMR shift at 8.81 ppm typical of a proton on the nitrogen of an aromatic indole moiety, Figure 5.18. The other major differences between asperizine (**18**) and both WIN 64821 (**19**) and ditryptophenaline (**20**) were the presence of two extra sp^2 ^{13}C NMR signals at 125.20 (C31) and 111.15 ppm (C32). Thus, asperizine (**18**) was a new compound with an identical formula and the same subunits as WIN 64821 (**19**) and ditryptophenaline (**20**). The extra two sp^2 carbons suggested asperizine (**18**) has an additional double bond and one less ring than the known compounds.

Table 5.4. Comparison of ^{13}C and ^{1}H NMR of asperizine (**18**) and WIN 64821 (**19**), 500 MHz, CD_3CN.

Atom	NMR shift for asperizine (**18**)		Literature values for WIN 64821 (**19**)	
	^{13}C NMR δ, m	^{1}H NMR, δ, m, (*J*)	^{13}C NMR δ, m	^{1}H NMR, δ, m, (*J*)
1 (NH)		6.14, br s		
2	84.20, d	5.71, d, (1)	80.82, d	4.85, d, (1)
3	59.28, s		61.14, s	
4	134.16, s		131.90, s	
5	121.48, d	6.82, d, (7, 1)	126.53, d	7.34, d, (8.0)
6	120.93, d	6.65, dt, (7, 1)	120.86, d	6.73, td, (8.0, 1.2)
7	130.45, d	7.12, m	130.60, d	7.10, m
8	112.41, d	6.82, d, (7.5)	110.82, d	6.67, d, (8.0)
9	148.57, s		150.56, s	
11	57.79, d	3.32, br t	57.93, d	4.05, t, (8.5)
12	39.55, t	3.25, dd, (14, 5.5)	36.88, t	2.98, dd, (14.0, 8)
		2.40, dd, (14, 6.5)		2.50, dd, (14.0, 7.5)
13	169.98, s		170.36, s	
14 (NH)		6.18, br s		
15	57.14, d	3.59, br t	57.17, d	4.15, br t, (5.5)
16	168.89, s		169.55, s	
17	40.40, t	3.01, dd, (13.5, 6)	36.17, t	3.09, dd, (14.5, 5)
		2.95, dd, (13.5, 5)		2.98, dd, (14.5, 6)
18	137.72, s		137.88, s	
19	131.46, d	7.12, m	130.82, d	7.13, m
20	130.09, d	7.12, m	129.84, d	7.13, m
21	128.98, d	7.20, m	128.11, d	7.13, m
22	130.09, d	7.12, m	129.84, d	7.13, m
23	131.46, d	7.12, m	130.82, d	7.13, m
24	126.31, s			
25	120.24, d	7.12, m		
26	120.61, d	7.12, m		
27	119.02, d	7.48, dd, (7, 1)		
28	130.45, s			
29	135.45, s			
30 (NH)		8.61, br s		
31	125.20, d	6.92, d, (2.5)		
32	111.15, s			
33	30.20, t	3.08, m		
34	56.36, d	3.51, br t		
35 (NH)		6.16, br s		
36	169.64, s			
37	60.46, d	4.12, ddd, (5, 6, 4.5)		
38 (NH)		6.49, d, (4.5)		
39	168.84, s			
40	40.72, t	3.06, dd, (14, 5.5)		
		2.93, dd, (13.5, 5)		
41	137.72, s			
42	131.63, d	7.12, m		
43	130.15, d	7.12, m		
44	128.72, d	7.27, m		
45	130.15, d	7.12, m		
46	131.63, d	7.12, m		

The molecular formula required that another carbon atom be identified in the ^{13}C spectrum. Placement of the extra carbon at 130.46 ppm, the position of the * in Figures 5.20 and 5.22, would resolve the perplexing HMBC correlations to this signal. The portions of asperizine (**18**) that were identical to WIN 64821 (**19**) and ditryptophenaline (**20**) could be confirmed by HMBC and ^{1}H-^{1}H COSY correlations. As the difference in the two molecules was present in the tryptophan portions, HMBC correlations were used to unequivocally solve this part of the molecule.

Unlike WIN 64821 (**19**) and ditryptophenaline (**20**), the tryptophan units in asperizine (**18**) are each modified in a different manner. It was determined by HMBC and ^{1}H-^{1}H COSY experiments that one of the tryptophans had the same arrangement as the known compounds. HMBC correlations from H-2 to C12, C24, C4 and C9 placed C24 adjacent to C3, Figures 5.27 and 5.28. Additional two and three bond correlations from H12/12' to C24, C2, C3, C4 and C13 helped confirm the juxtaposition of C24 and C3, Figures 5.27 and 5.29.



HMBC correlations

Figure 5.27. Selected HMBC correlations connecting two subunits of asperizine (**18**).

Figure 5.28. HMBC correlations of C2 of asperizine (**18**), 500 MHz, CD_3CN.

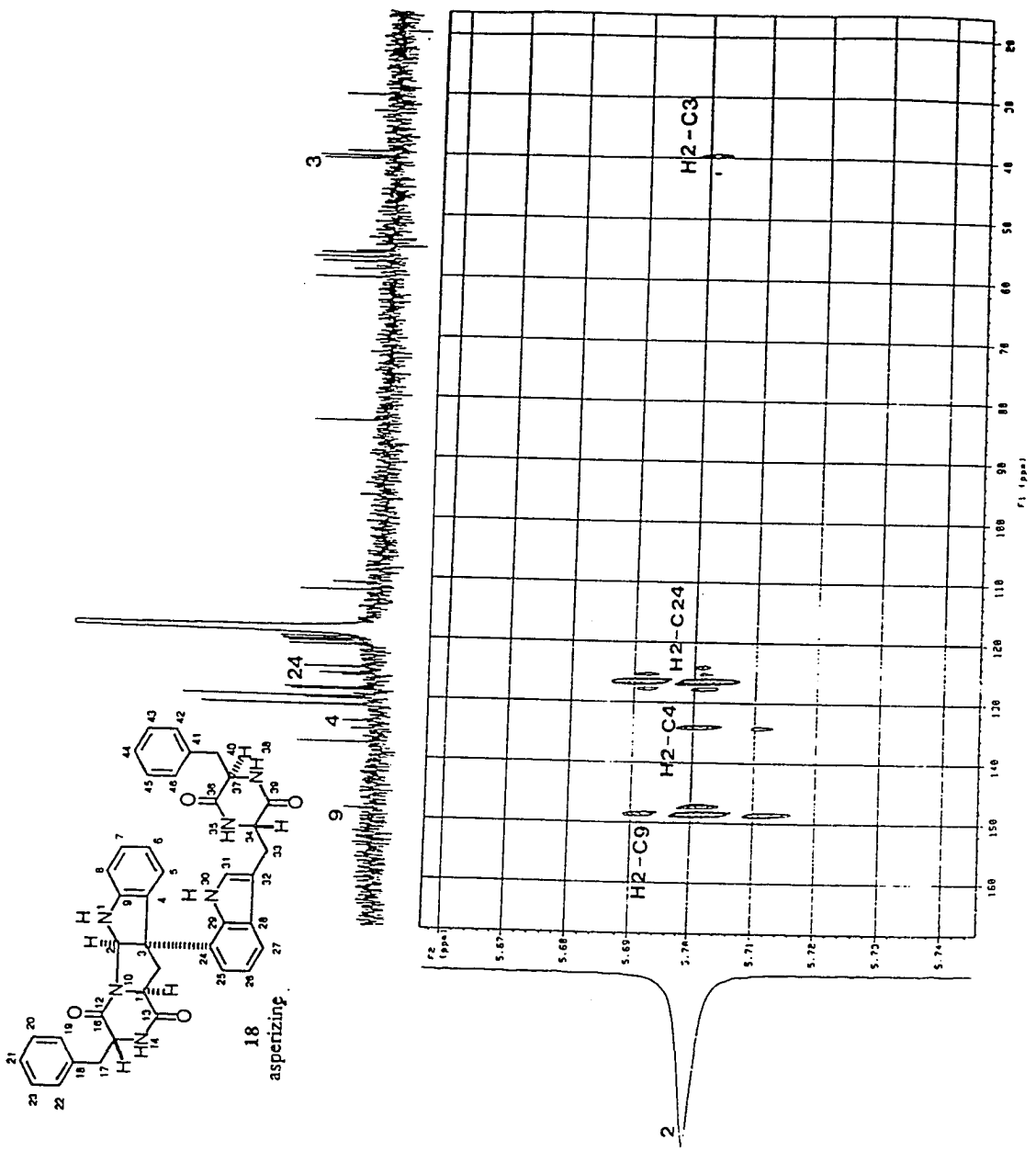
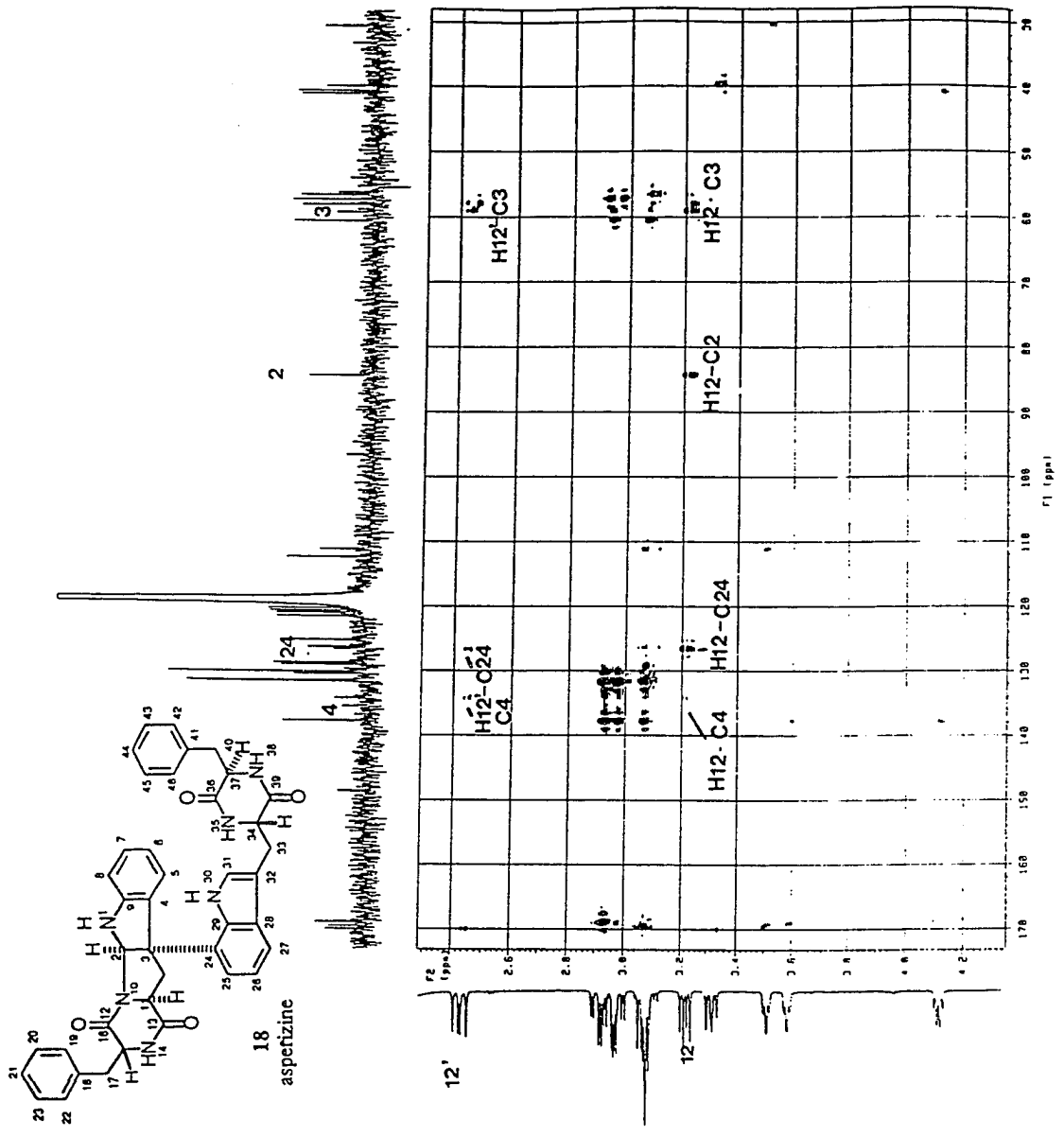


Figure 5.29. HMBC spectrum of asperizine (**18**), 500 MHz, CD_3CN.



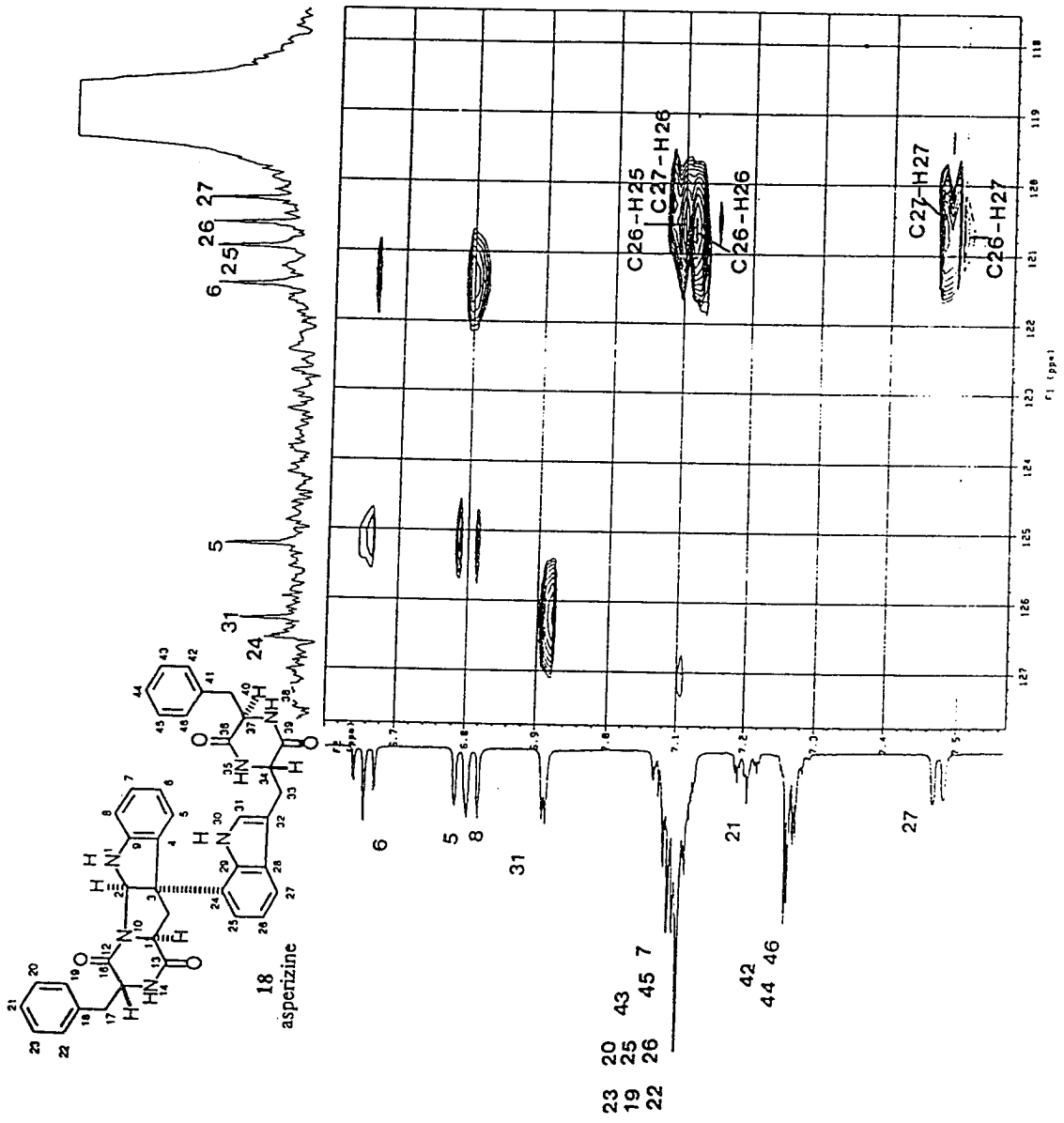
The arrangement of the second tryptophan unit was necessary to elucidate how the two halves of the molecule were linked together. The two extra sp^2 carbons were placed in this portion of the molecule by HMBC correlations from H34 to C32 and from H33 to C32 and C31, Figure 5.27. The aromatic indole proton H31 confirmed that the aryl ring was present by HMBC correlations from H31 to C29 and C28. Correlations from H27 to C32, C28 and C29 confirmed the fusion of the two rings.

The connection between the two tryptophan moieties was found to be through the aryl ring of the second tryptophan. Examination of the aryl portion of the tryptophan indicated only three methine carbons with an extra quaternary aryl carbon present at 126.31 ppm (C24) instead of the usual four methine carbons of tryptophan. The correlation from H2 and H12 to C24 confirmed that the connection was indeed through the aryl ring of the lower tryptophan unit, Figures 5.27-5.29.

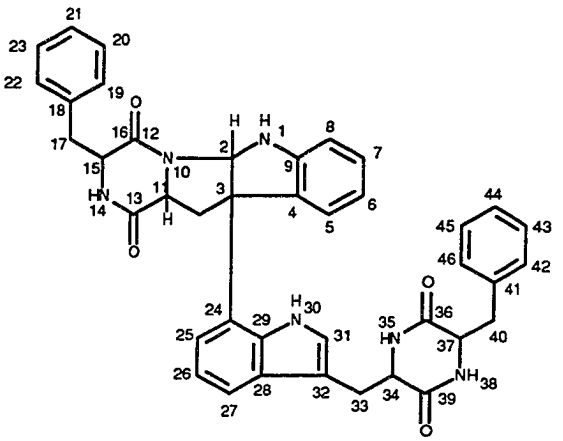
The substitution pattern of the second tryptophan moiety was determined by analysis of the aromatic ^{1}H and ^{13}C NMR signals. The identification of a three proton spin system would corroborate the proposed structure. This was difficult to do by ^{1}H-^{1}H COSY because the resonances of H25 and H26 overlapped in a large multiplet at 7.12 ppm. While the ^{1}H NMR signals overlapped, the ^{13}C NMR signals were well resolved. To solve this problem and take advantage of the spread of the ^{13}C NMR signals, an HMQC-TOCSY spectrum was analyzed, Figure 5.30.

The anchor point for the HMQC-TOCSY was the well resolved proton signal H27 (dd, 1, 1.5 Hz) which was definitively placed adjacent to quaternary carbon C28 by a strong three bond HMBC correlation to C32 and a weak correlation to C28. HMQC-TOCSY allowed assignment of the nearest protonated neighbor of H27 as C26. Determining whether H26 was adjacent to one or two protons would identify the site of attachment to the other half of the molecule. When the HMQC-TOCSY for C26 was examined it was found to have three correlations, Figure 5.30. These were the C26-H26 self correlation plus correlations to protons H27 and H25 from C26. This

Figure 5.30. HMQC-TOCSY correlations of H6 of asperizine (**18**) 500 MHz, CD_3CN.



determined that H26 had two protonated neighborsand confirmed the presence of a three proton spin system. This completed the planar structure of asperizine (**18**).



18 asperizine

The relative stereochemistry of asperizine (**18**) was determined through NOESY and 1D difference nOe experiments. Nuclear Overhauser effects were identified from the aromatic nitrogen proton H30 to H2. This was observed both in a NOESY, Figure 5.31, and 1D nOe difference experiments, Figure 5.32. To bring these two protons near each other in space requires a syn arrangement of the proton on C2 and the aromatic group at C3. A 1D difference nOe was observed from H11 to H2 indicating that both protons were on the same side of the ring, Figure 5.32.

To determine the relative stereochemistry of the diketopiperazine rings, the coupling constants of the α protons were examined. A five bond proton-proton coupling of approximately 1 Hz exists in diketopiperazines when the α protons are in a syn configuration.[22-25] To identify whether this 1 Hz coupling was present, the ^{1}H NMR signal for H11 was examined. The proton H11 is the X of an ABX spin system due to the tertiary nature of the adjacent nitrogen, Figure 5.33. It is clear H11 only couples to H12 and H12' and does not couple to H15. A simulated spectra with a 1 Hz coupling showed a clearly distinguishable splitting at H11, Figure 5.34, which was not observed in the real spectrum. Thus, it was decided that H11 and H15 had an anti

Figure 5.31. NOESY spectrum of asperizine (**18**), 500 MHz, CD_3CN.

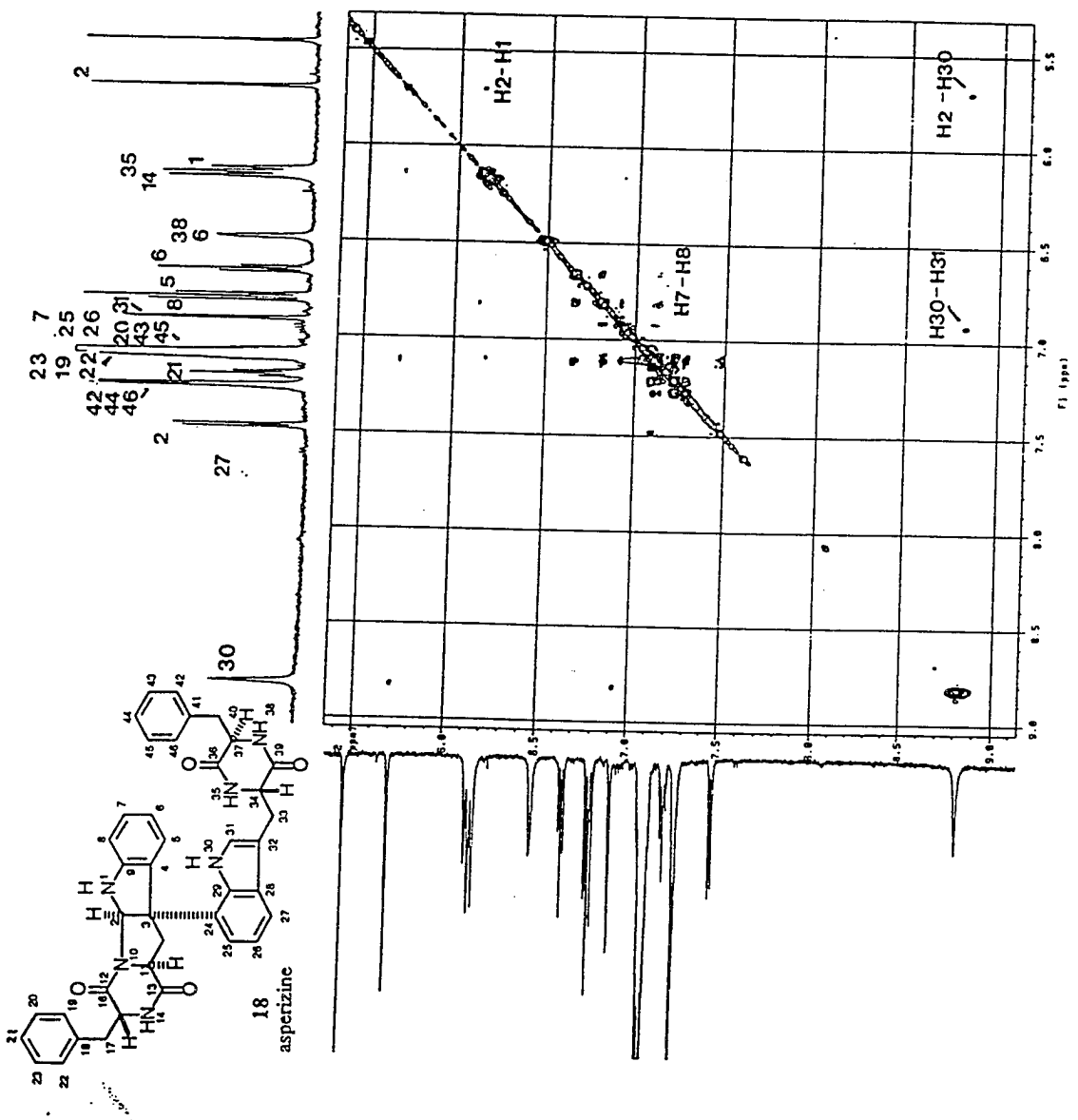


Figure 5.32. Difference nOe, irradiate H11 - observe H2 of asperizine (**18**), 500 MHz, CD_3CN.

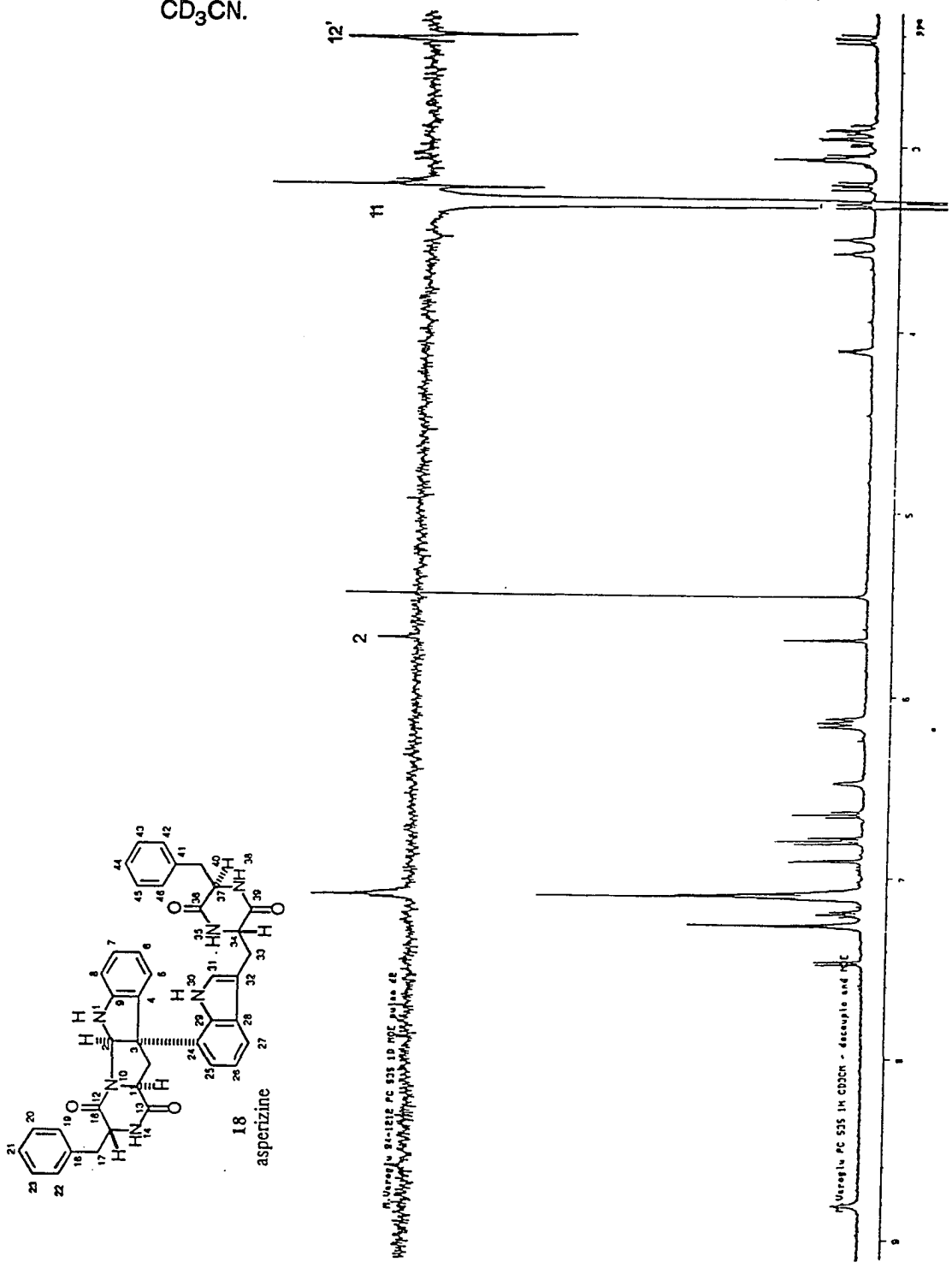


Figure 5.33. ^1H NMR spectrum of asperizine (**18**), expansion of high field and H11 signal, 500 MHz, CD_3CN.

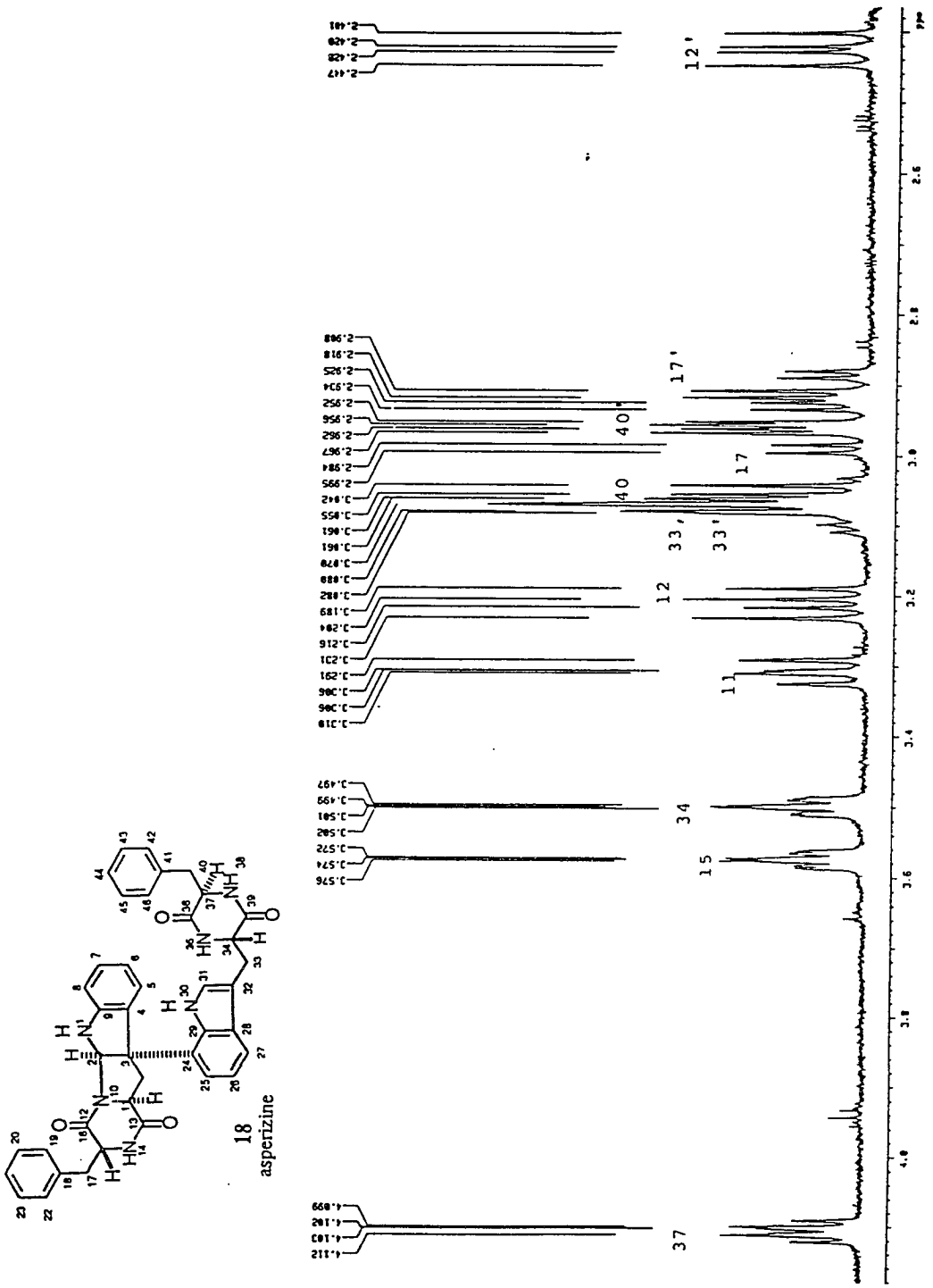
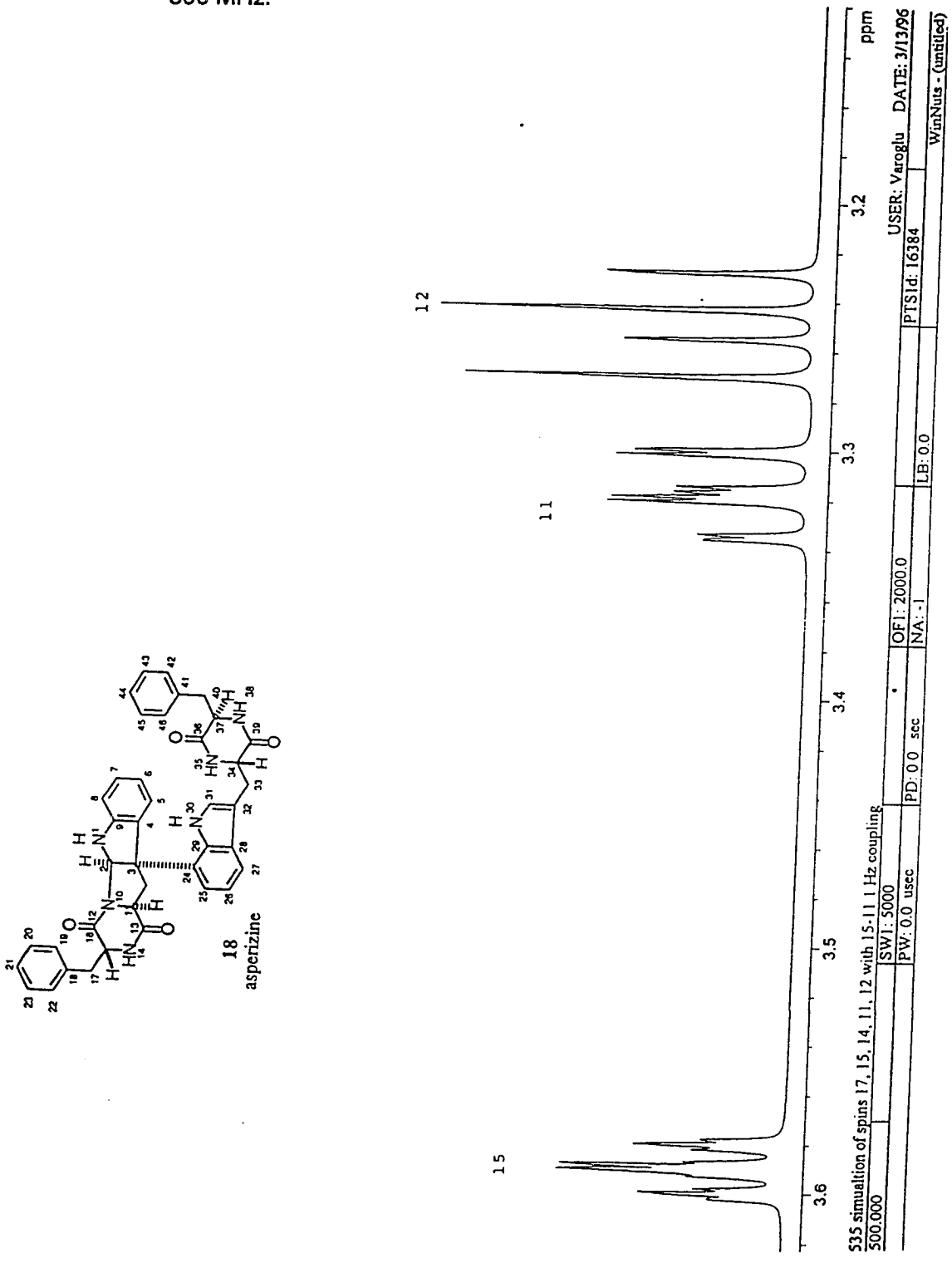


Figure 5.34. ^{1}H NMR spectral simulation of H11-H15 1 Hz coupling of asperizine (**18**), 500 MHz.



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asperizine

relationship to each other. In the previous studies on the identical ring system of WIN 64821 (**19**) and ditryptophenaline (**20**) this coupling was observed.

Determination of the relative stereochemistry of H34 and H37 was more difficult as each proton is part of an ABMX system with an additional coupling from the adjacent nitrogen amide protons. In the case of asperizine (**18**) the NH couplings in CD_3CN were considerable. The H34-H35 coupling is 2.5 Hz while the H37-H38 coupling is 4.5 Hz. Spectral simulation was used to explain the shape of the resonances for H37 and H34. The simulation of spectra without ^5J coupling from H34 to H37 matched the measured spectra, Figure 5.35. To complement the spectral simulation decoupling experiments were performed. Fortunately, H38 is located in an uncrowded region of the spectrum. Thus, very high power decoupling (decoupler power 34 dB) of H38 reduced the spin system to an ABX system with H37 appearing as a double doublet, Figure 5.36. The simplifed signal of H37 had no coupling beyond those from H38/H38'. The line width of the resonance was approximately 1.5 Hz and thus it is expected that a 1 Hz coupling would be readily apparent. To further confirm that H34 and H37 were uncoupled, H37 was irradiated and H34 was examined. The spectra before and after the decoupling contained the same multiplicity, Figure 5.37. For this reason H34 and H37 have been determined to have an anti relationship based on the lack of an observable coupling between H34 and H37.

Knowledge of the relative stereochemistry of the α protons allowed absolute stereochemistry to be determined by hydrolysis of asperizine (**18**) and chiral TLC of the free amino acids. The hydrolysis reaction was performed on 0.1 mg asperizine (**18**) in a 20 μL capillary tube[26] with 6 M HCl solution containing 1% w/w phenol. Phenol has been found to prevent overoxidation of tryptophan.[27] Asperizine (**18**), D and L tryptophan, D and L phenylalanine and a blank were placed in capillary tubes. After heating in a 150 °C oven for 90 minutes, the capillary tubes were flushed out with water and acetonitrile and the resulting solution was dried under nitrogen gas. After removal of HCl and phenol with high vacuum, the samples were spotted on

Figure 5.35. ^{1}H NMR spectral simulation of H34-37 spin systems of asperizine (**18**), 500 MHz.

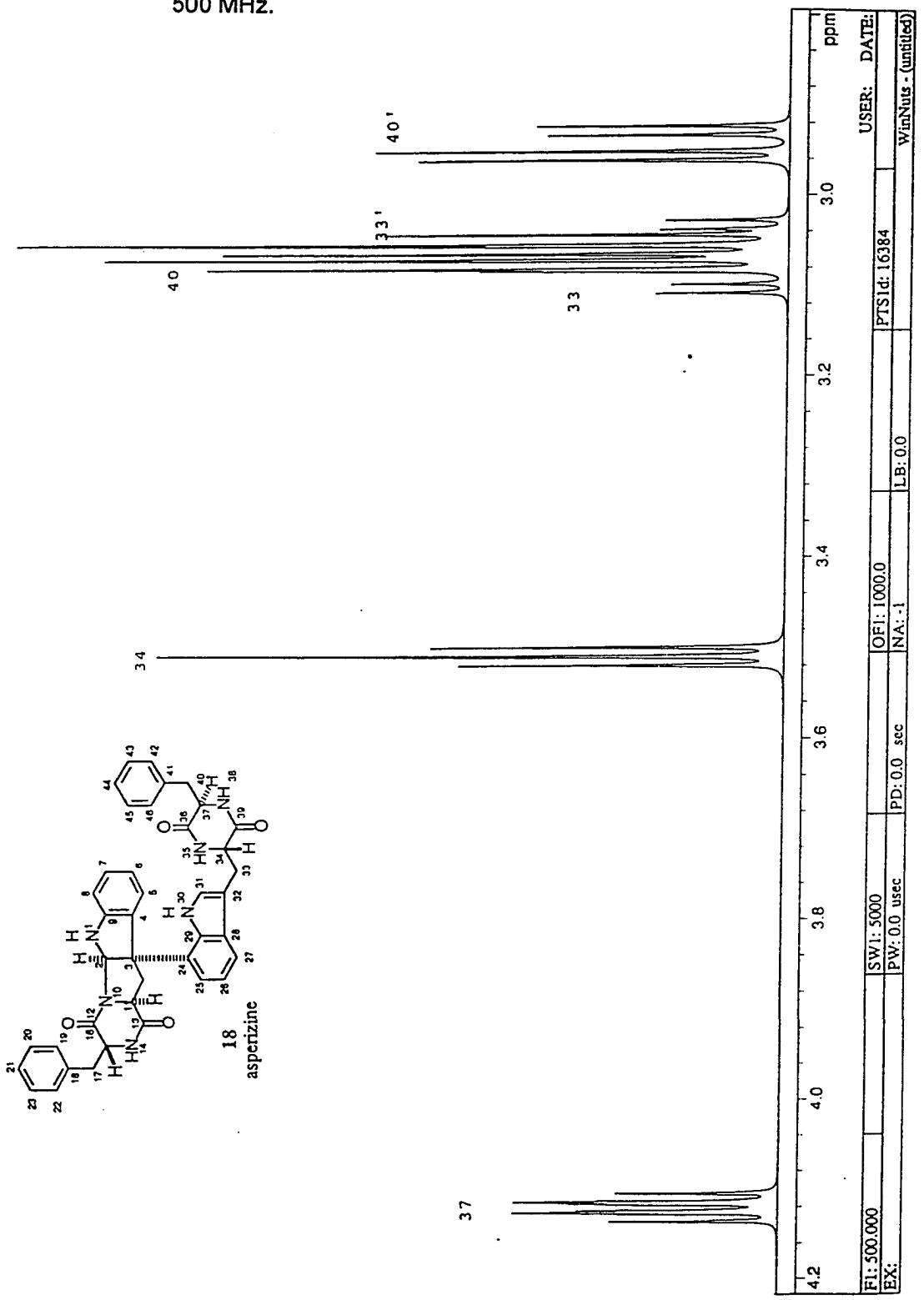


Figure 5.36. ^{1}H NMR selective irradiation of H38 to decouple H37 (top spectra) + normal ^{1}H NMR of asperizine (**18**) (bottom), 500 MHz, CD_3CN.

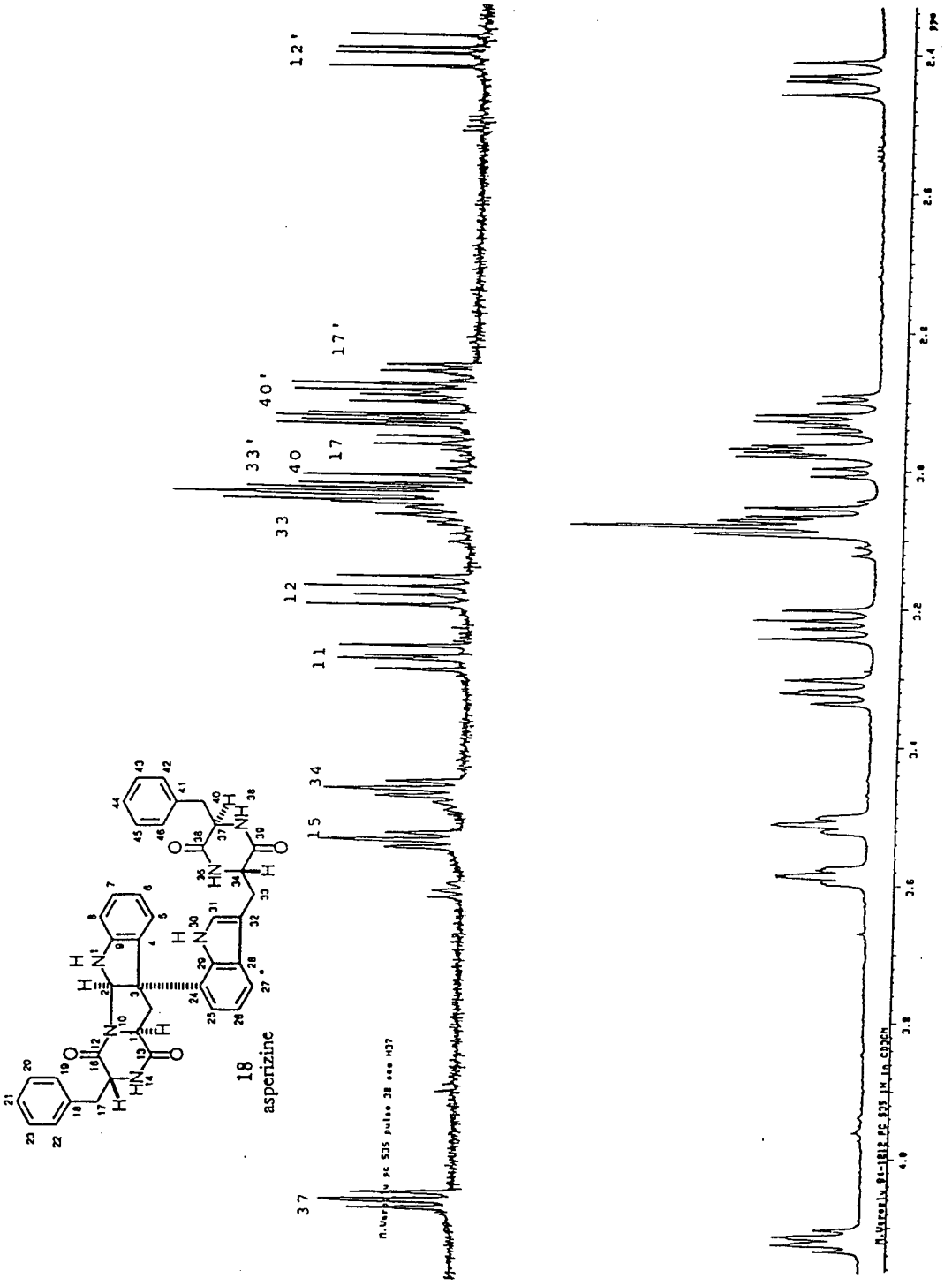
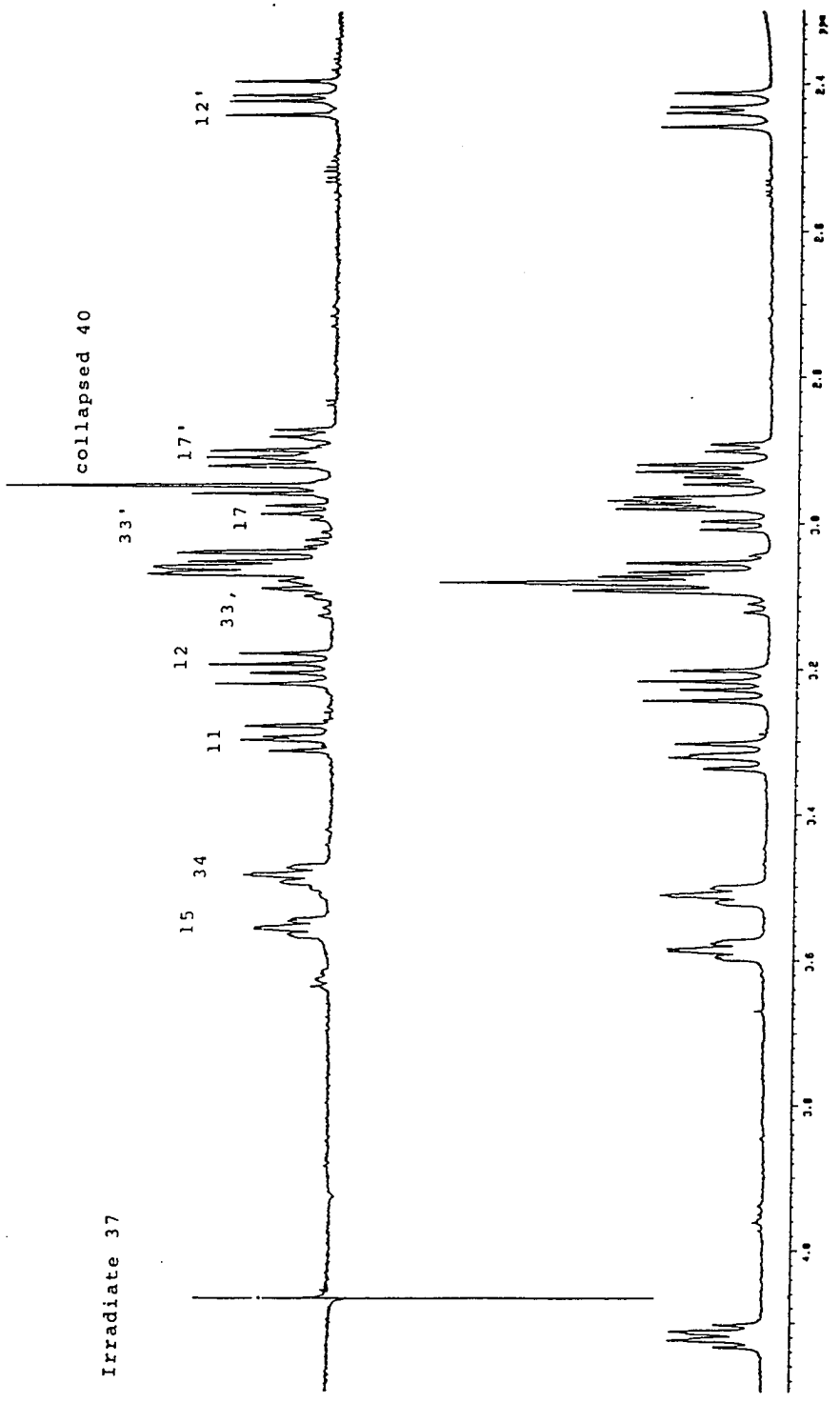
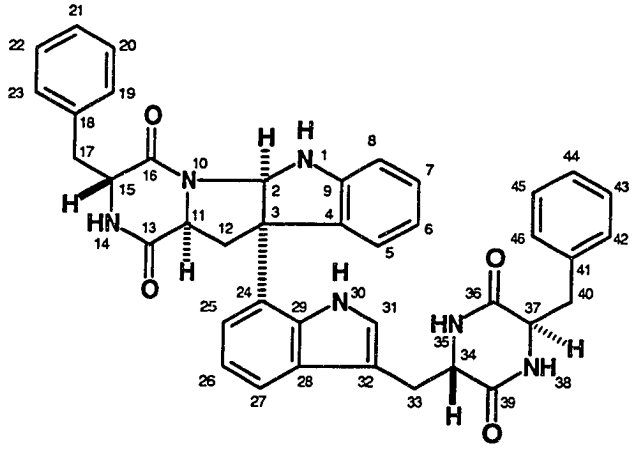


Figure 5.37. Decoupled ^1H NMR of asperizine (**18**). Selective irradiation of H37 to probe H34 (top spectra), normal ^1H NMR (bottom), 500 MHz, CD_3CN.



both normal phase and chiral reverse phase TLC plates.[28] Development of the normal phase plates indicated that the only free amino acid present was phenylalanine. Only D-(*R*)- phenylalanine, $R_f = 0.43$, (standard D-phenylalanine $R_f = 0.44$; L-Phenylalanine $R_f = 0.55$) was observed on the chiral TLC plate.

The absolute stereochemistry of the rest of the molecule was based on the previously determined relative stereochemistry. Thus, the tryptophan units posses *S* stereochemistry at C11 and C34. The absolute stereochemistry at C2 and C3 is assigned as *R* and *S*, respectively. The absolute stereochemistry of all the stereogenic centers in asperizine (**18**) may be summarized as 2*R*, 3*R*, 11*S*, 15*R*, 34*S* and 37*R*.



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asperizine

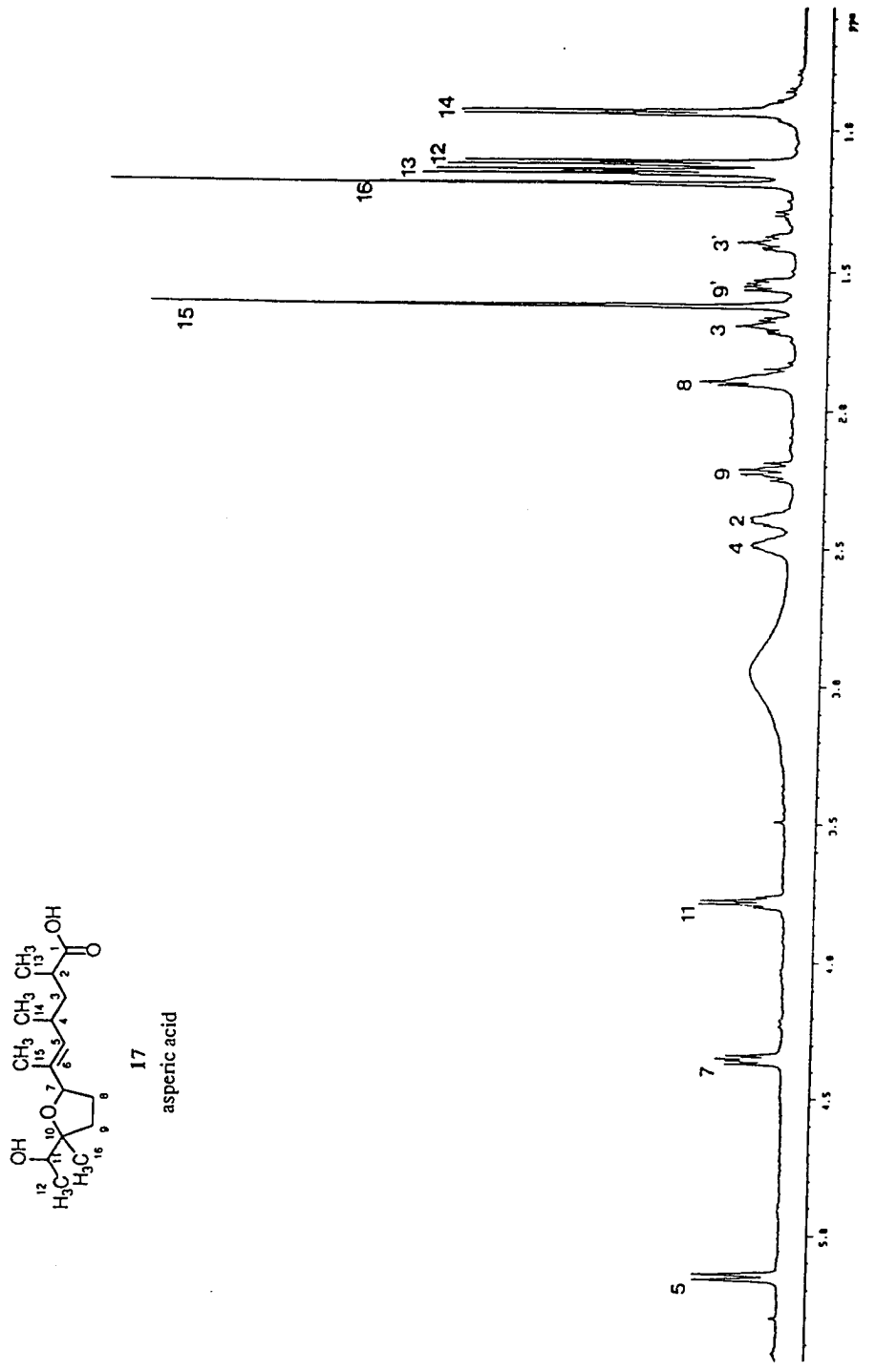
Structure elucidation of asperic acid (17)

A preliminary molecular formula of $C_{16}H_{26}$ was obtained from ^{1}H NMR, Figure 5.38, ^{13}C NMR, Figure 5.39, and a DEPT experiment, Figure 5.40. The ^{13}C and ^{1}H NMR shifts are summarized in Table 5.5. A carbonyl was indicated by a ^{13}C NMR shift at 181 ppm (C1) and three oxygen substituted carbons were implied by ^{13}C resonances at 87.69 (C10), 87.16 (C7) and 72.05 (C11). At this point a low resolution fast atom bombardment mass spectra provided a peak for $[M+H]^+ = 285$ *m/z* indicating a potential molecular weight of 284 g/mol, Figure 5.41. Using the molecular formula found from the NMR experiments and adding the correct number of protons and oxygens a tentative molecular formula of $C_{16}H_{28}O_4$ was determined to fit the available data. This molecular formula was confirmed by high resolution FAB mass spectroscopy ($[M+H]^+ =$ 285.2059 Δ 0.7 mmu of calcd) after the structure of asperic acid (**17**) was solved, Figure 5.41. This working molecular formula provided an unsaturation number of three. The carbonyl and a double bond indicated by vinylic peaks in both the ^{1}H NMR and ^{13}C NMR spectra accounted for two of the unsaturations. The final unsaturation was assigned to a ring in the molecule.

Table 5.5. NMR data for asperic acid (**17**), 500 MHz, in $CDCl_3$.

Atom	^{13}C ppm, δ, m	^{1}H ppm δ, m, (J)
1	180.57, s	
2	36.90, d	2.40, m
3	40.68, t	1.69, m
		1.39, m
4	29.92, d	2.49, m
6	133.77, s	
5	133.24, d	5.16, d, (9.5)
7	87.20, d	4.35, dd, (10, 6)
8	30.66, t	1.89, m
9	30.34, t	2.22, dt, (6.5, 12)
		1.55, m
10	87.67, s	
11	72.05, d	3.79, dd, (6.5, 12.5)
12	17.66, q	1.15, d, (7)
13	16.68, q	1.18, d, (6.5)
14	21.49, q	0.94, d, (6.5)
15	11.23, q	1.62, s
16	24.66, q	1.19, s

Figure 5.38. ^{1}H NMR spectrum of asperic acid (**17**), 500 MHz, $CDCl_3$.



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asperic acid

Figure 5.39. ^{13}C NMR spectrum of asperic acid (**17**), 125 MHz, $CDCl_3$.

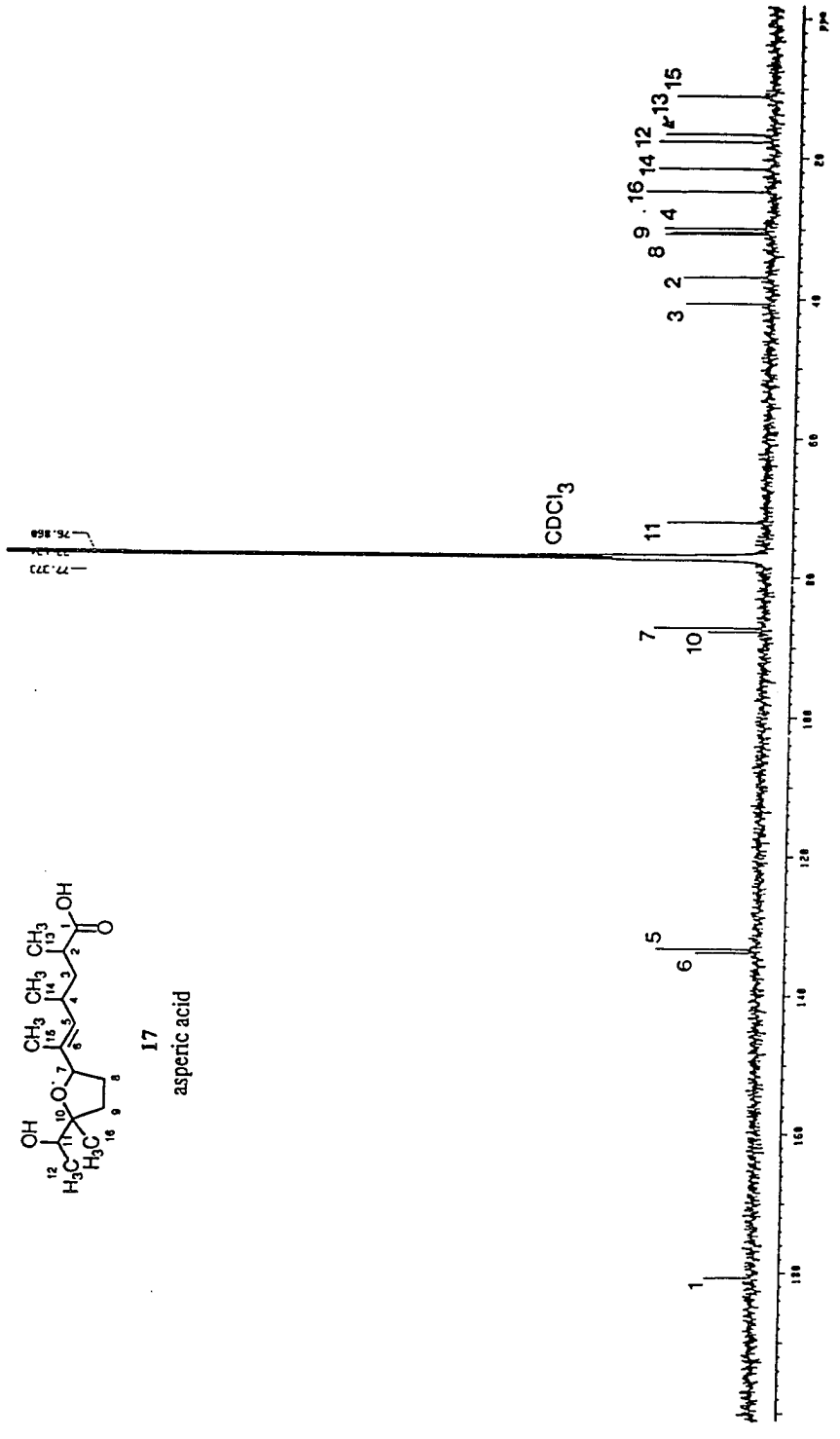


Figure 5.40. DEPT 135 spectrum of asperic acid (**17**), CH_3 and CH up, 500 MHz, $CDCl_3$.

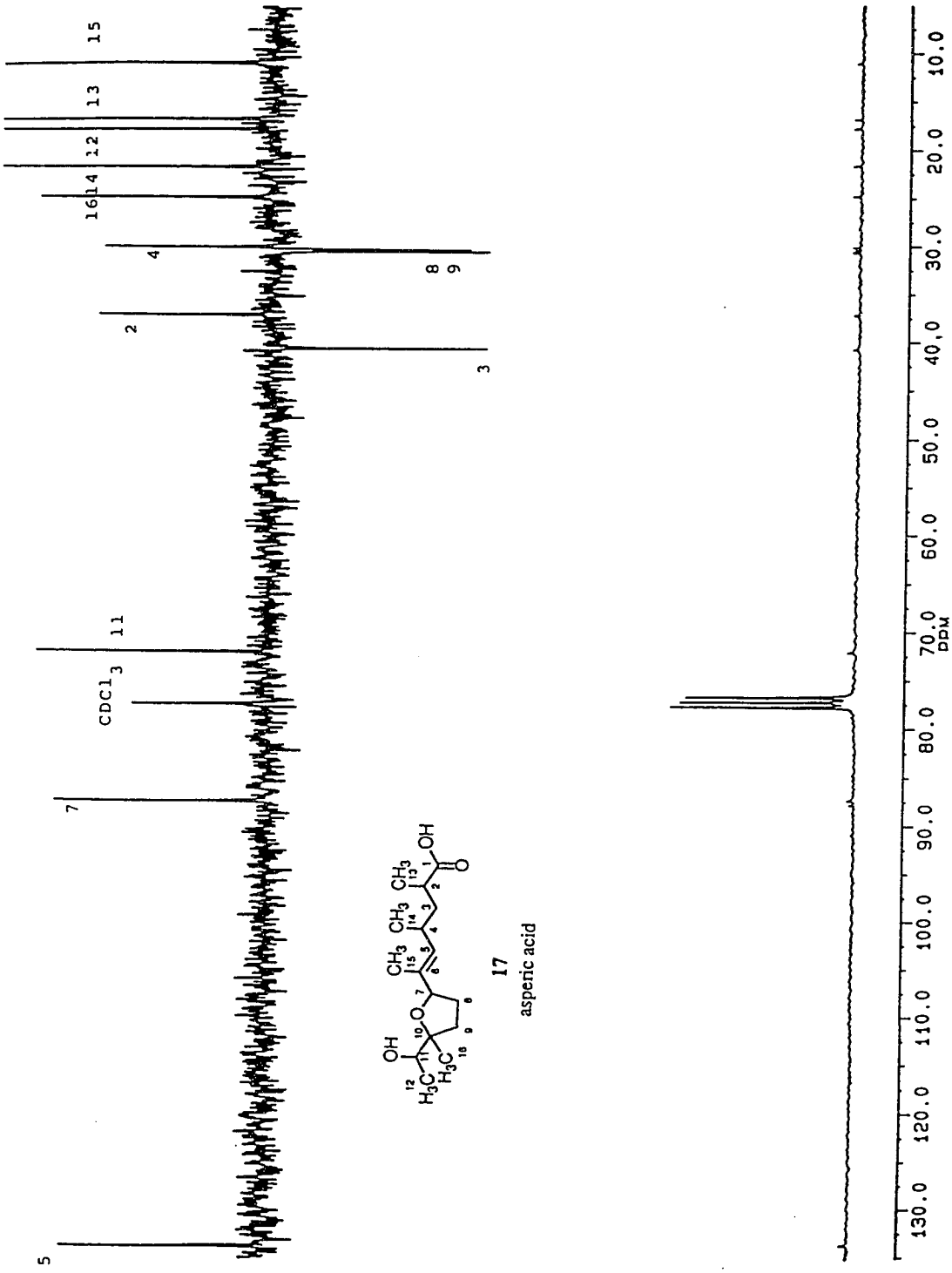
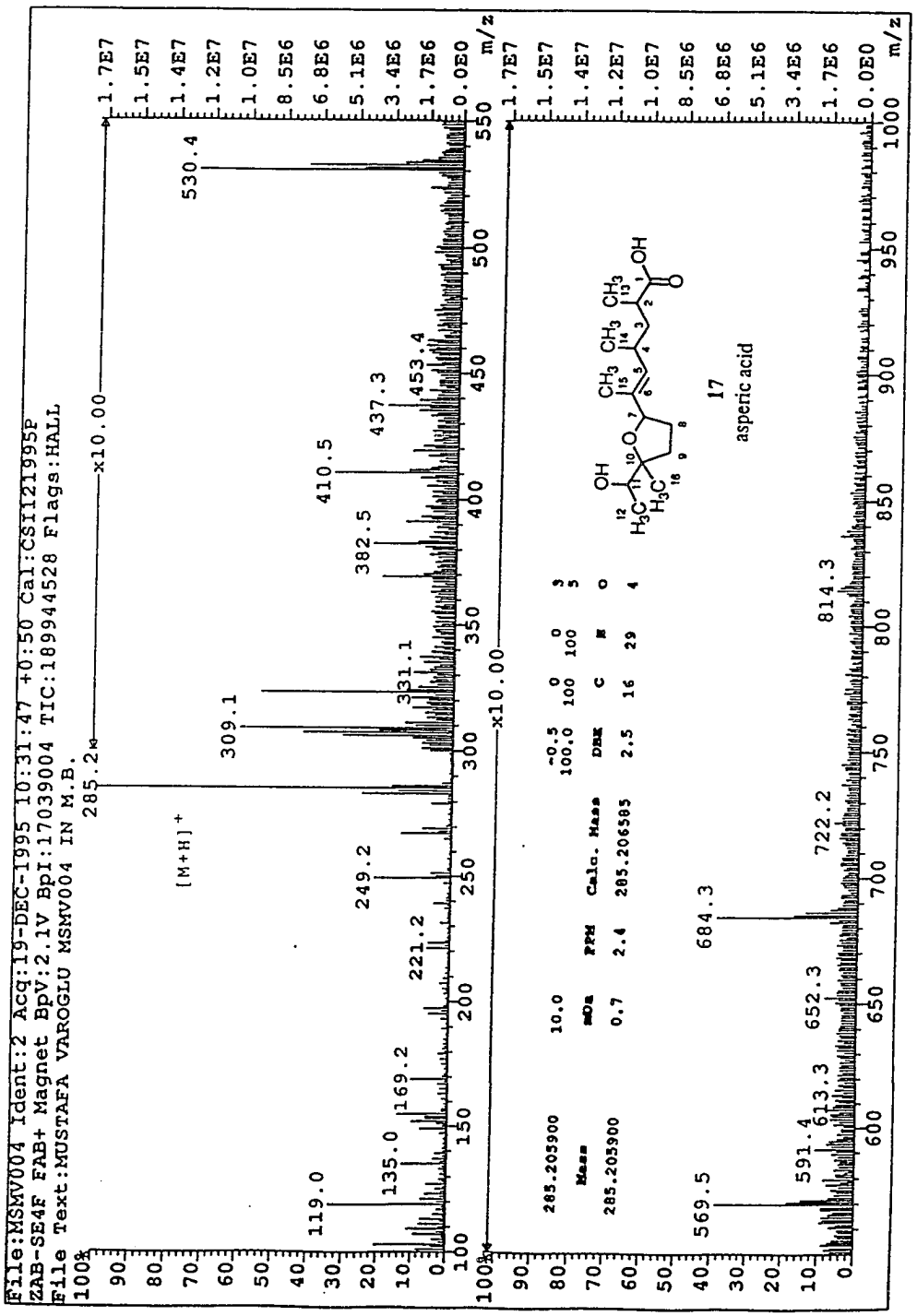


Figure 5.41. LRFABMS and HRFABMS of asperic acid (**17**), dithiothreotol/ dithioerythrotol matrix.



One double bond was indicated by ^{13}C NMR shifts at 133.77 (s) and 133.24 (d) ppm. From the presence of only one vinylic signal in the ^{1}H NMR and the DEPT assigned multiplicities of the sp^2 carbons it was clear that the double bond was tri-substituted. Analysis of the ^{1}H-^{1}H COSY spectrum provided six spin systems, Figure 5.42. Strong ^{1}H-^{1}H COSY correlations from the vinylic hydrogen H5 to H4, from H4 to H3/3' and methyl-14, from H3 and H3' to H12 and from H2 to methyl-13 defined the largest spin system. These ^{1}H-^{1}H COSY correlations are shown in the upper half of the spectrum in Figure 5.42. A long range allylic coupling to the vinylic methyl-15 from H5 was observed. This defined both ends of the double bond.

The next largest spin system derived from ^{1}H-^{1}H COSY correlations was shown by the correlation from H7 to H8/8', and from H8 and H8' to H9/9', Figure 5.42. The H11-H12 spin system was determined by a correlation from H11 to methyl-12 as the final information available from the ^{1}H-^{1}H COSY experiment. These smaller spin systems are labeled on the bottom half of the ^{1}H-^{1}H COSY spectrum, Figure 5.42. Methyl-16, carbonyl-1 and the quaternary carbon-10 were identified as being singlets. This provided the six spin systems summarized in Figure 5.43

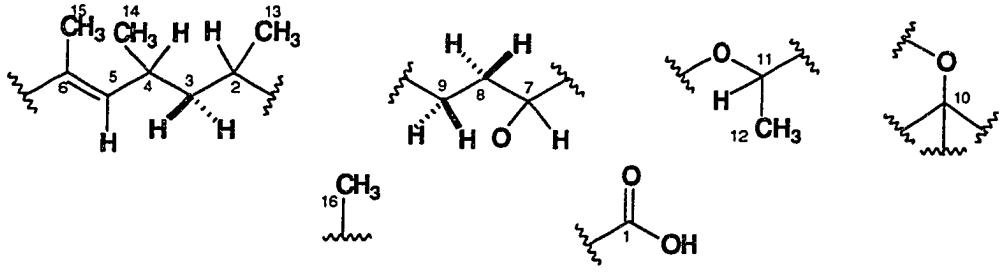


Figure 5.43. ^{1}H-^{1}H COSY derived spin systems for asperic acid (**17**).

These substructures were attached together in a linear sequence by HMBC correlations, Figure 5.44, to yield a working structure for the molecule. The carbonyl was attached to the H2-H6 spin system by a three bond correlation from C1 to H2, H3, H3' and methyl-13, Figure 5.44. The H7-H9 spin system was connected to the quaternary sp^2 carbon of the double bond by an HMBC correlation from C6 to H7 and H8. This connection was also supported by a three bond

Figure 5.42. ^{1}H-^{1}H COSY of asperic acid (**17**), 500 MHz, $CDCl_3$.

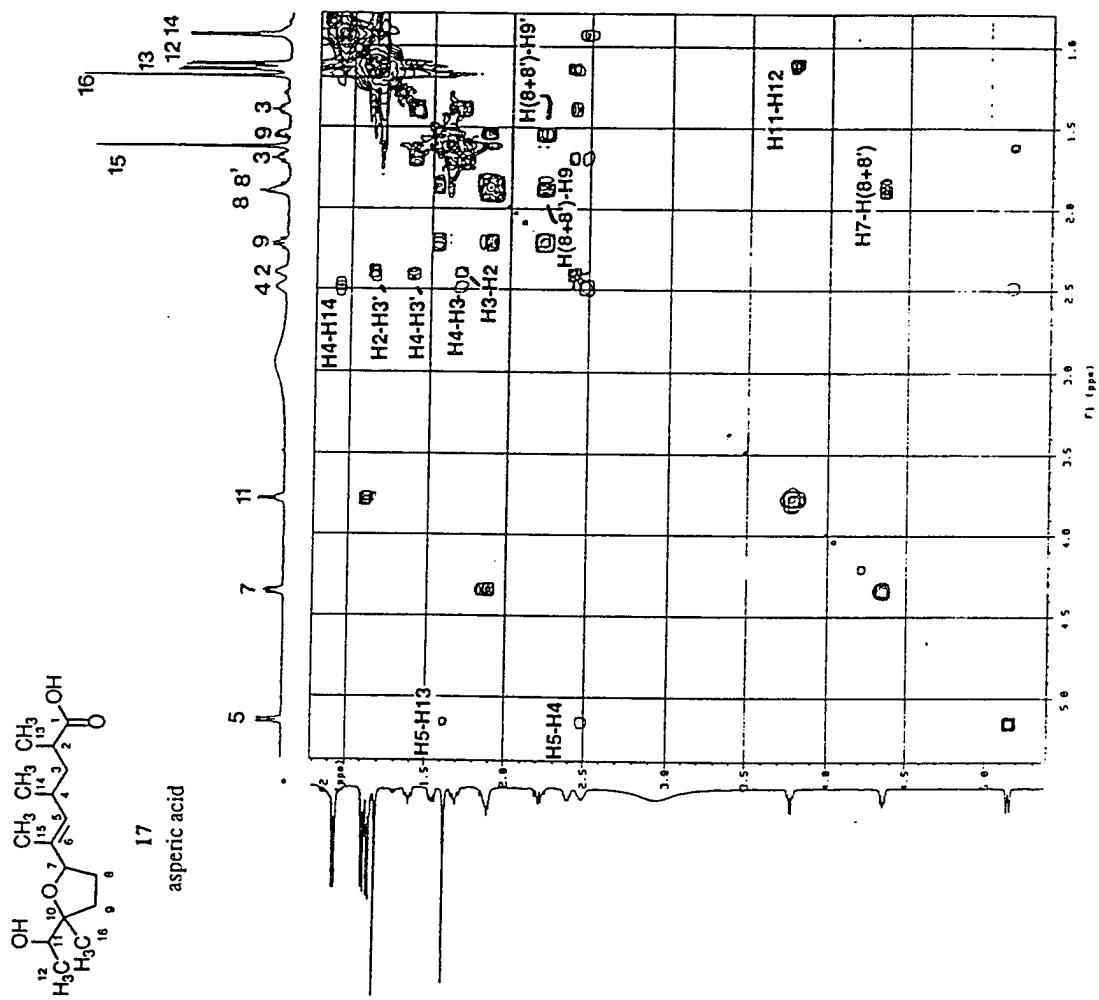
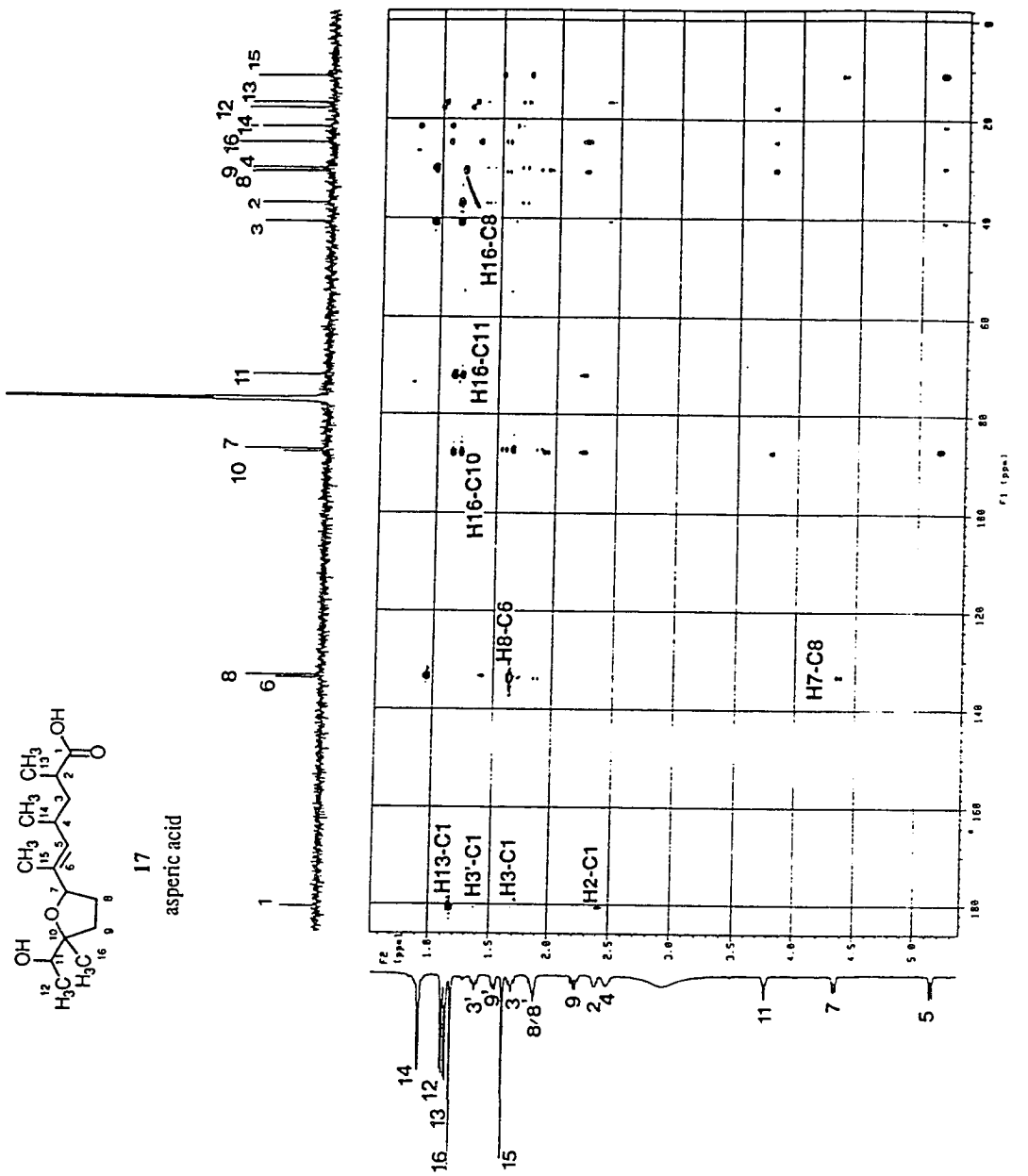


Figure 5.44. HMBC spectrum of asperic acid (**17**), 500 MHz, $CDCl_3$.



correlation from methyl-15 to C7. Methyl-16 was linked to quaternary carbon-10 by an HMBC correlation, and this subunit bridged the H7-H9 and the H11-methyl-12 spin system, Figure 5.44. Carbon hydrogen long range correlations to confirm this were observed from methyl-16 to C9 and C11. Altogether, these HMBC correlations led to the generation of the linear structure of the molecule as seen in Figure 5.45.

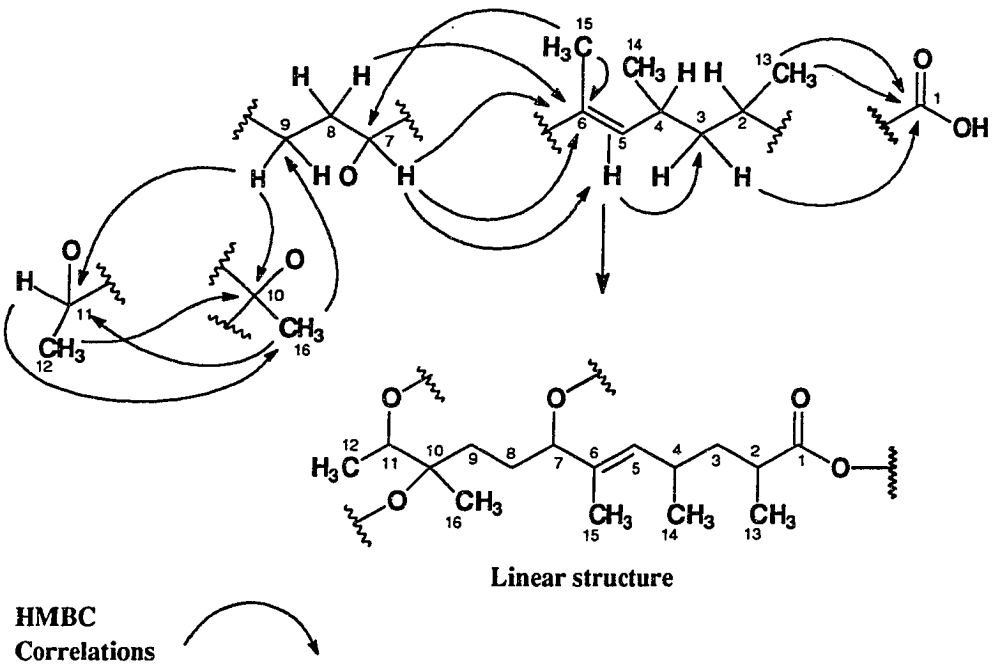


Figure 5.45. Important HMBC correlation linking linear sequence of asperic acid (**17**).

Considering the oxygenated carbons and the carbonyl functionality there were six possible ways to place a ring in the molecule. The presence of a lactone ring was discounted because the carbonyl shift was at 180.57 ppm and there were no ester α protons in the 5 ppm range to support an ester bond. An epoxide, five-, or six-membered ether was possible using oxygen atoms in the linear structure. An epoxide ring was eliminated because the carbons resonance of C10 and C11 were too far downfield. This left two possibilities for the final molecule, Figure 5.46.

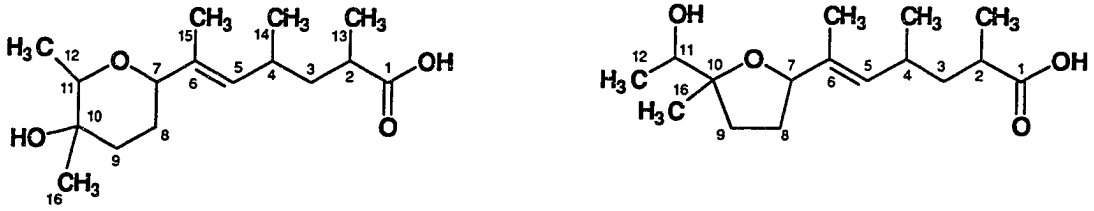


Figure 5.46. Possible structures of asperic acid (**17**).

In order to decide between the remaining two structures it was necessary to obtain further ^{1}H NMR, Figure 5.47, HMBC, and NOESY spectra in dioxane-d_8. In dioxane-d_8 the signals of the hydrogens attached to oxygens are observable in detail. The HMBC correlation from OH11 to C12, shown in Figure 5.48, was only possible if the structure possesses a five-membered ether ring. In the case of a six-membered ether ring the proton would have to be on the alcohol of C10 and the correlation to C12 would be through four bonds. The NOESY experiment, Figure 5.49, provided further confirmation of the five membered ether ring by a correlation from OH11 to H7 across the top of the ring. In the case of the six-membered ether ring, this correlation would have been a 1-4 correlation and would have been too far to observe.

The NOESY experiment in dioxane-d_8 was also used to determine the relative stereochemistry of the asperic acid (**17**) ether ring. The previously described OH11 to H7 correlation indicated the syn relative stereochemistry of the ring at C7 and C10. This stereochemistry was supported by an nOe correlation from H16 to H8' on the other side of the ring.

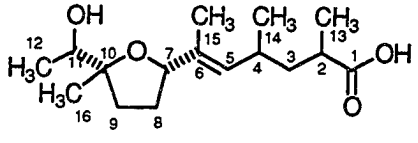


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Figure 5.47. ^{1}H NMR of asperic acid (**17**), 500 MHz, dioxane-d_8.

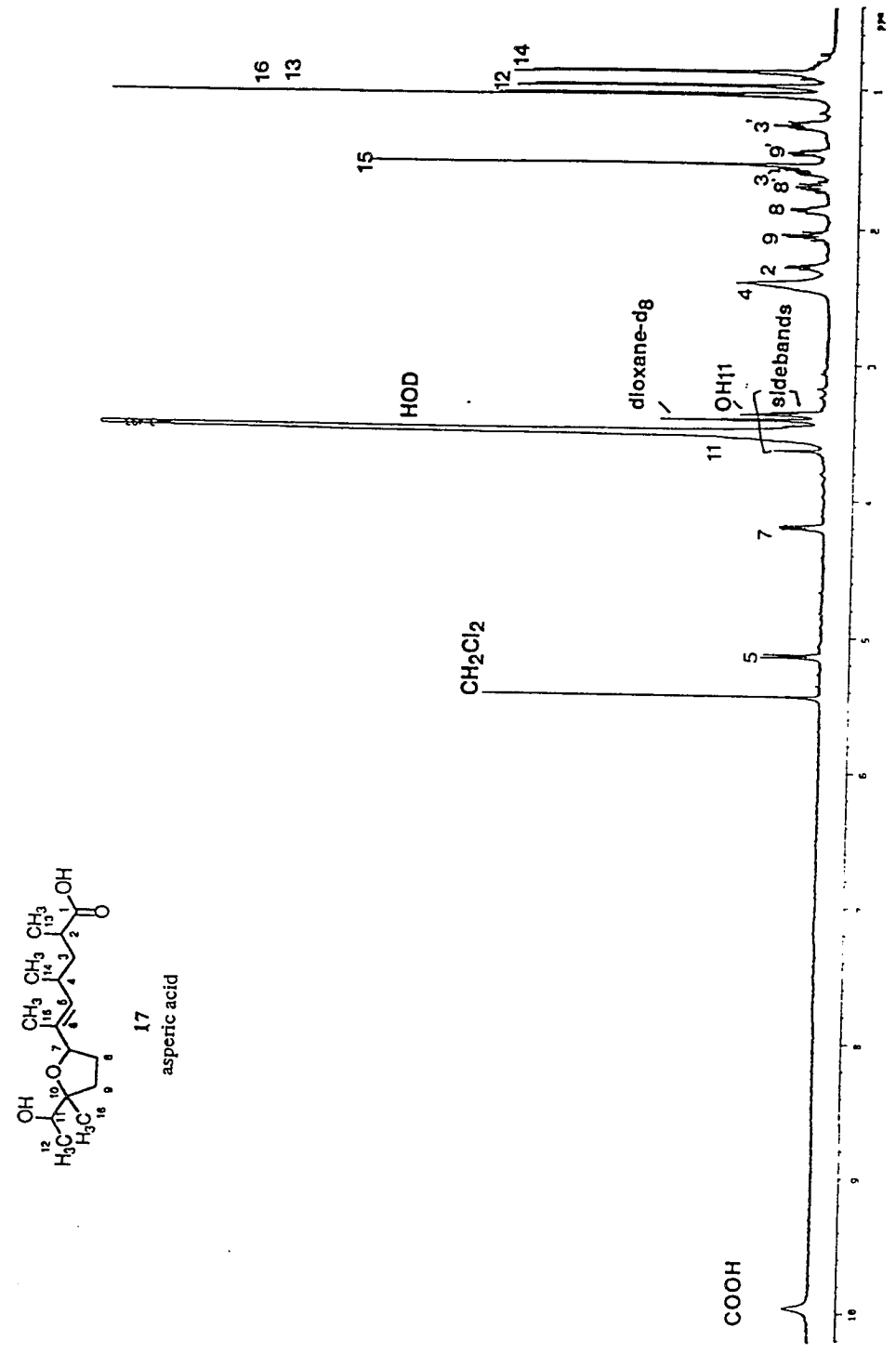


Figure 5.48. HMBC correlation of OH11 to C12 of asperic acid (**17**), 500 MHz, dioxane-d_8.

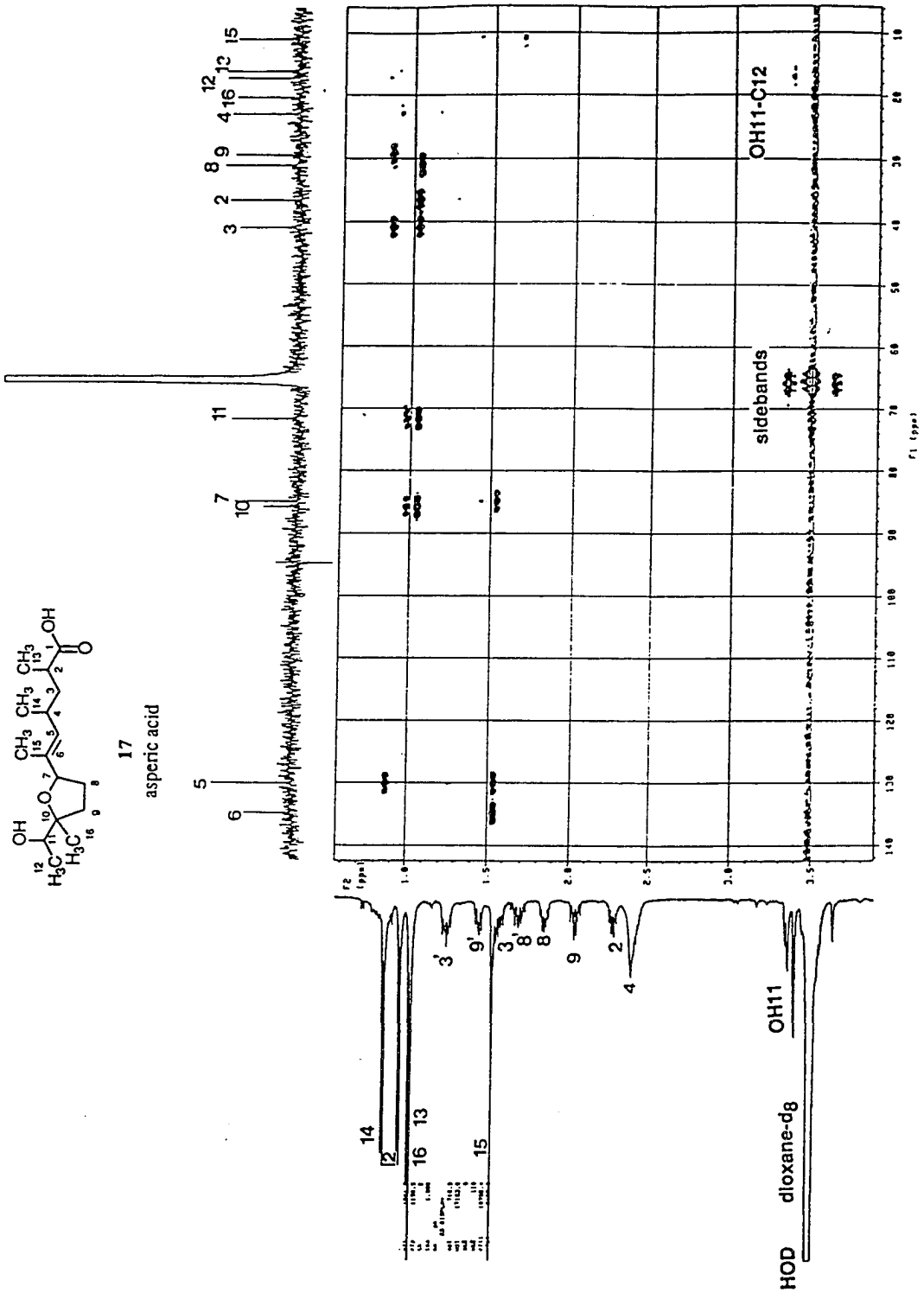
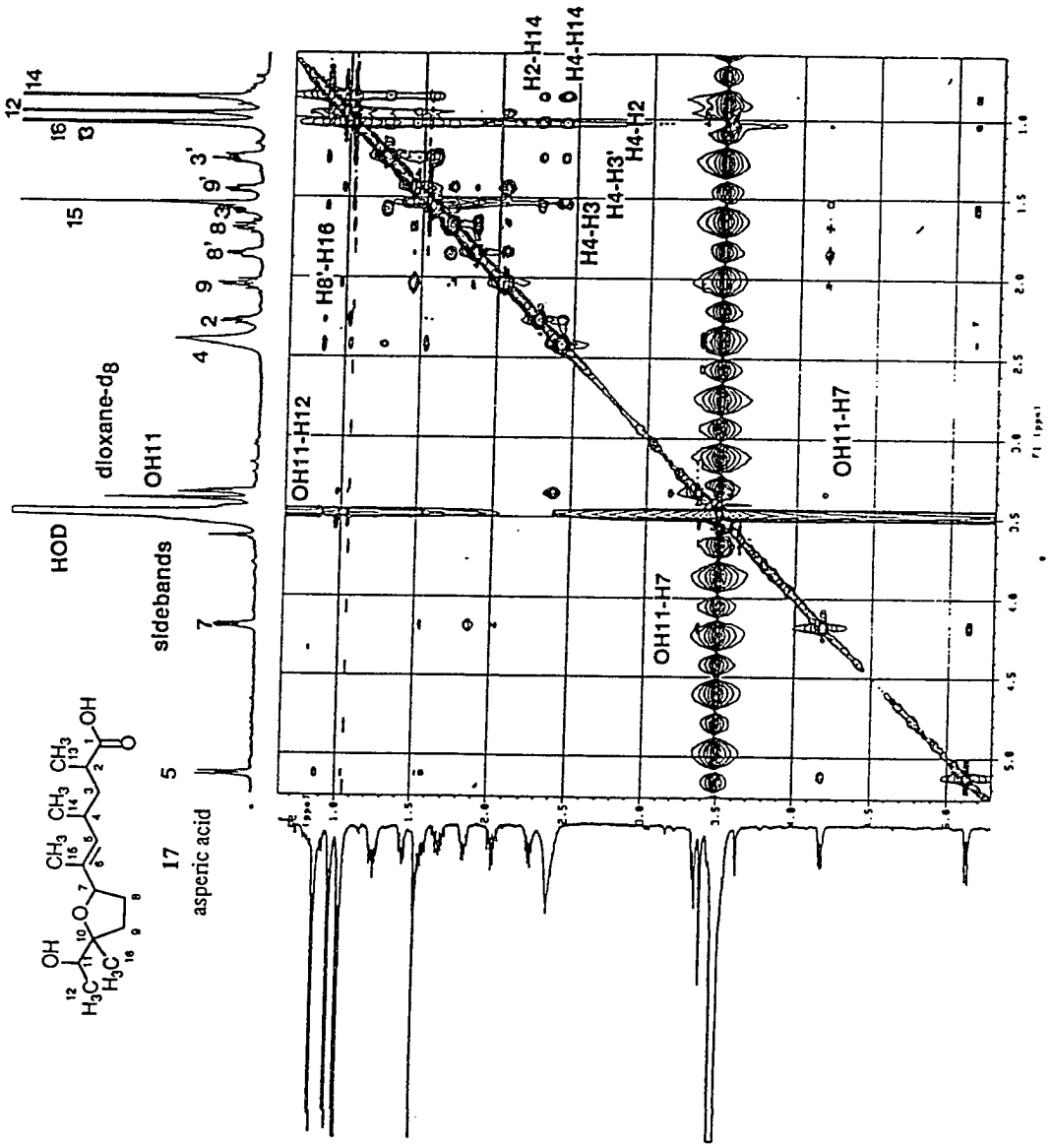


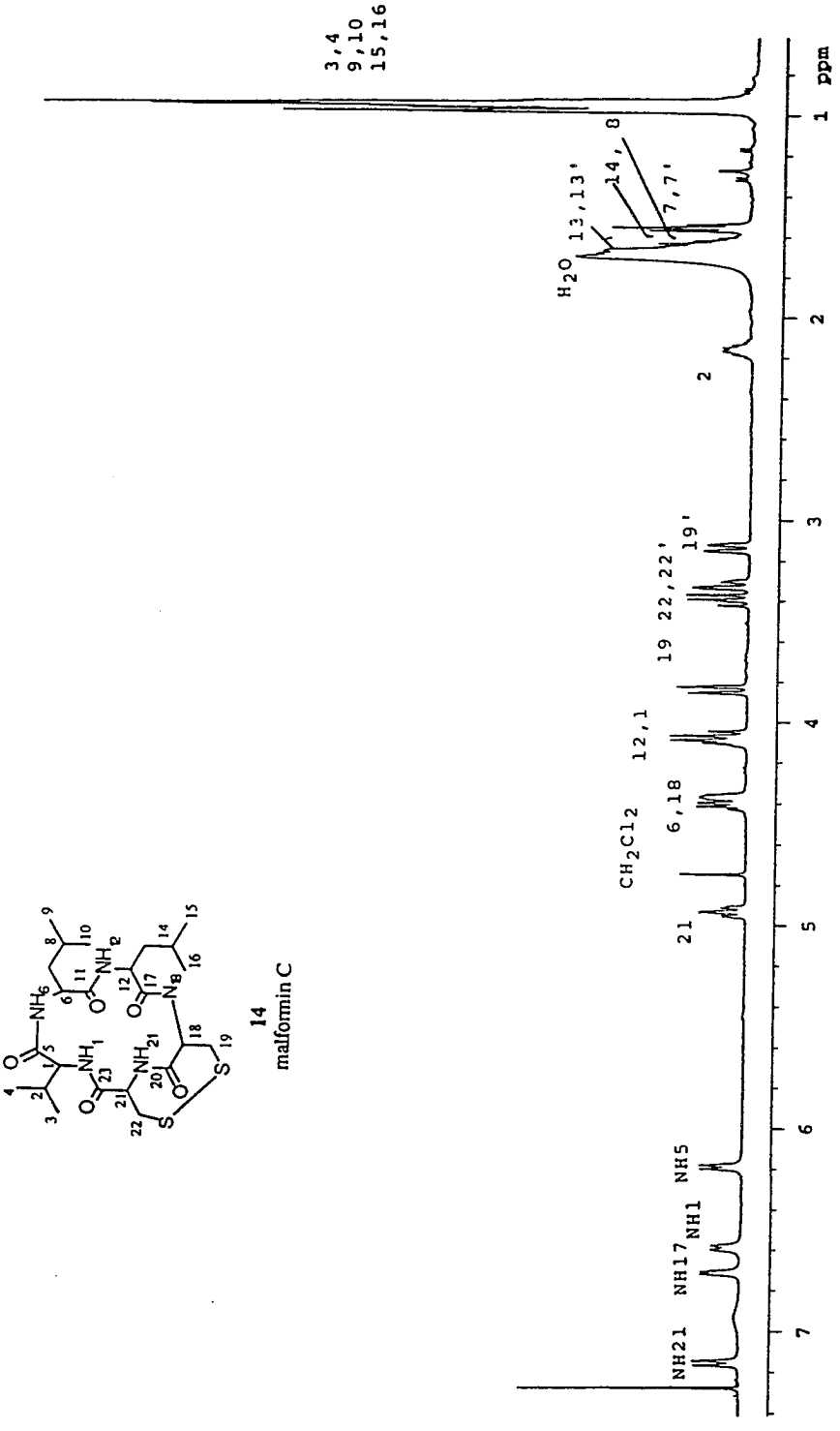
Figure 5.49. NOESY correlation of OH11 to H7 of asperic acid (**17**), 500 MHz, dioxane-d_8.



Dereplication and NMR resonance assignment of malformin C (14)

The ^{13}C and ^{1}H NMR spectra of malformin C (**14**), Figures 5.50 and 5.51, were examined to determine if the compound isolated was the same as the literature structure. If the compound was known, then the NMR data of malformin C (**14**) would be assigned for the first time. A DEPT formula of $C_{23}H_{34}$ was determined, Figure 5.52 and an HMQC experiment, Figure 5.53, was used to assign the 34 protons to their carbons. The five protons that did not correlate to any of the carbons were assigned as the protons on amide nitrogens. Including the carbonyls present in the ^{13}C NMR spectra this led to a formula of $C_{23}H_{39}H_5O_5$. The $[M+H]^+$ peak was observed for this formula by electrospray mass spectrometry at *m/z* =530, Figure 5.54. This preliminary formula of $C_{23}H_{39}H_5O_5$ matched the literature formula of malformin C (**14**). Five of the seven unsaturations calculated from this formula were accounted for by the five carbonyl peaks. The NMR data is summarized in Table 5.6.

Figure 5.50. ^{1}H NMR spectrum of malformin C (**14**), 500 MHz, $CDCl_3$.



14
malformin C

Figure 5.51. ^{13}C NMR spectrum of malformin C (**14**), 125 MHz, $CDCl_3$.

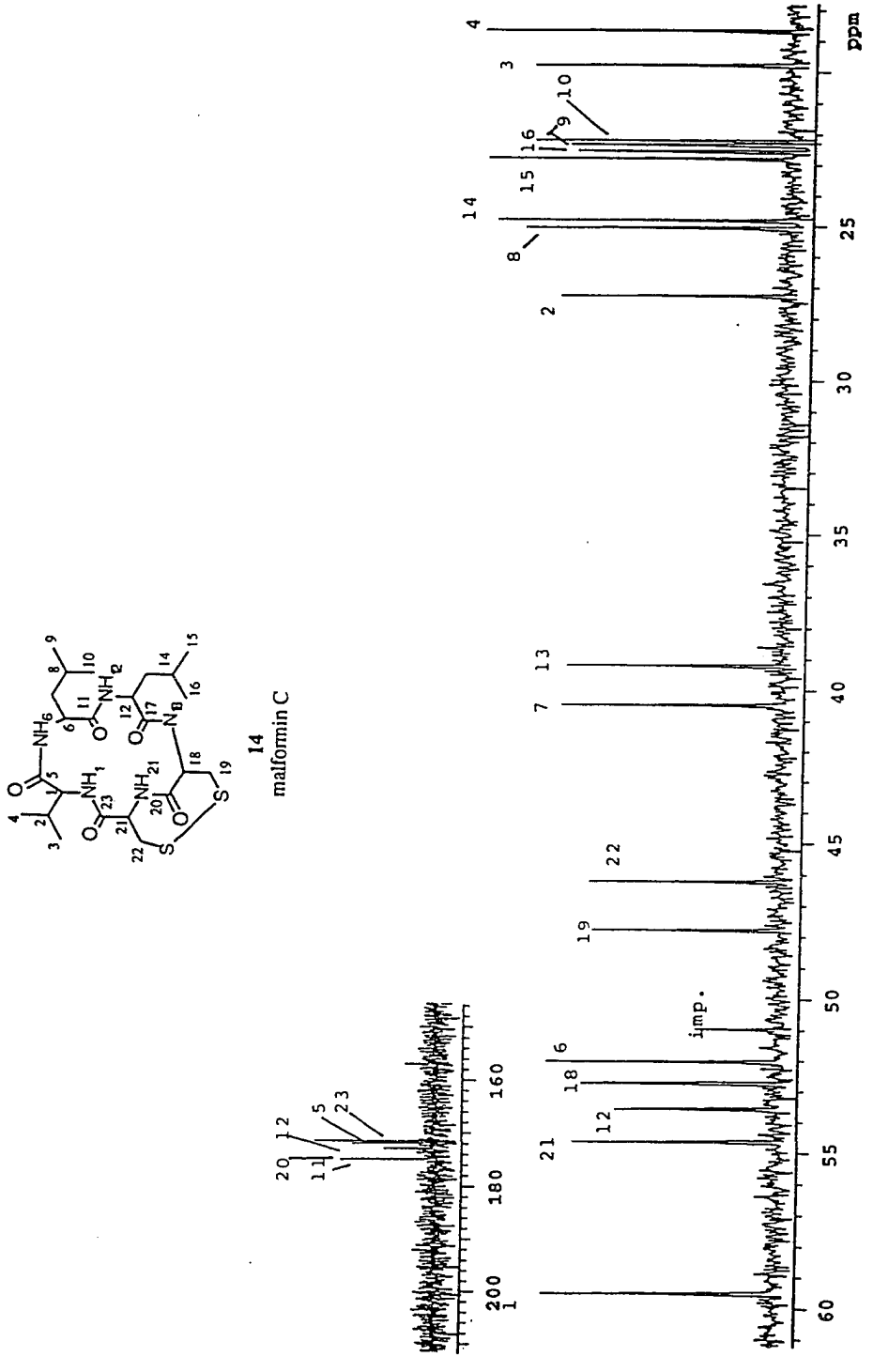


Figure 5.52. DEPT 135 spectrum of malformin C (**14**), CH_3,CH up, 62.9MHz, $CDCl_3$.

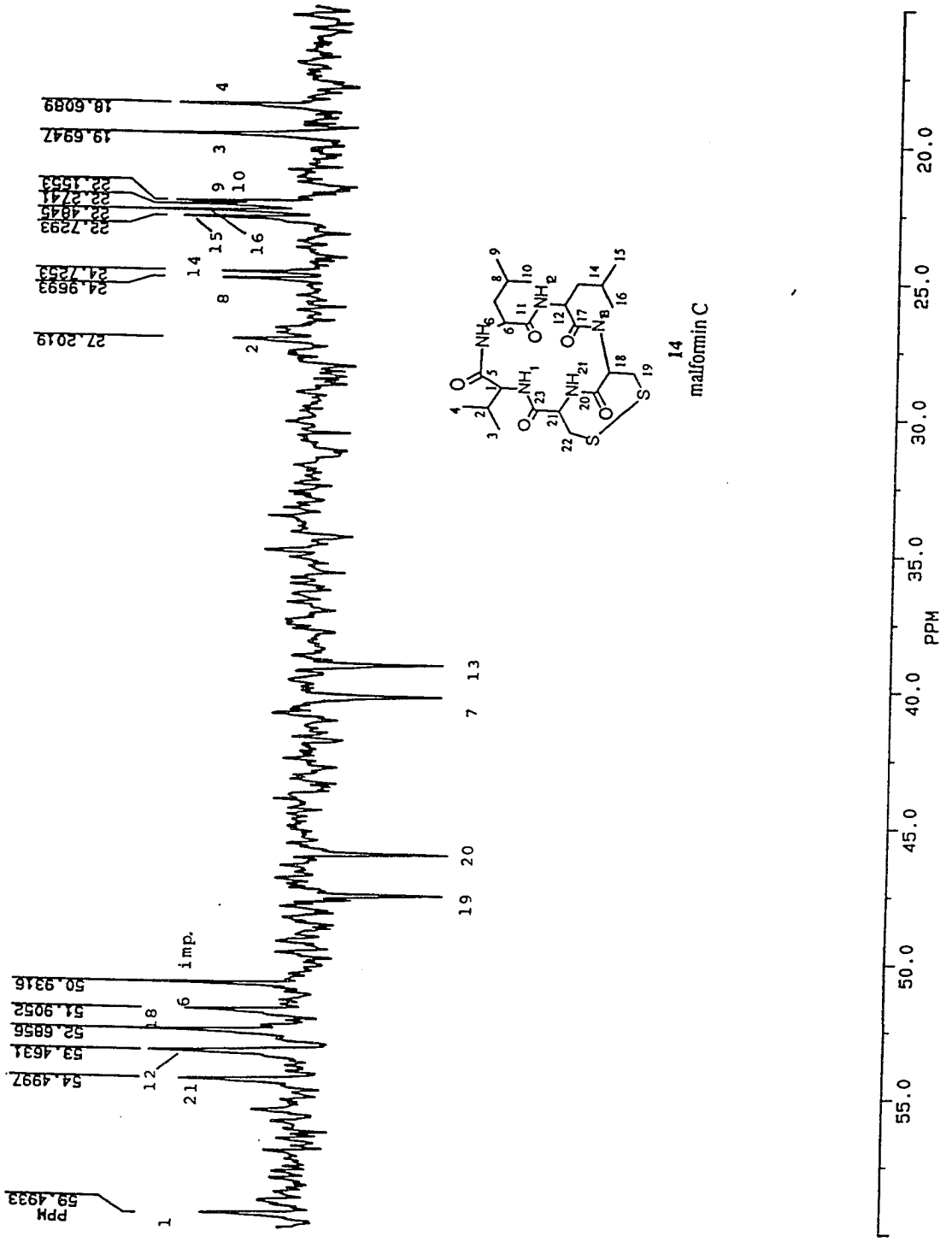


Figure 5.53. HMQC spectrum of malformin C (14), 500 MHz, $CDCl_3$.

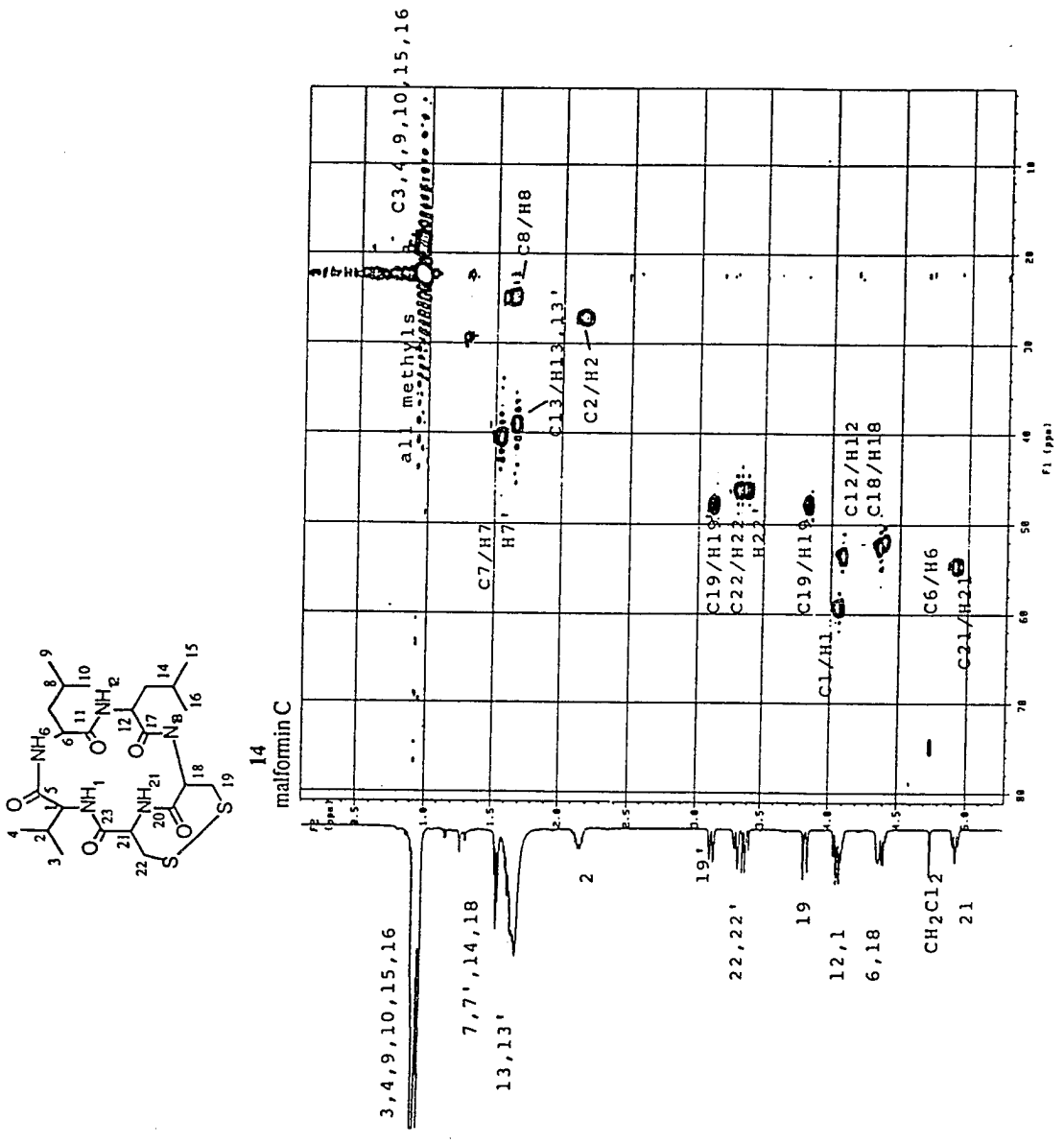


Figure 5.54. LRFAB MS of malformin C (14), dithiothreotol/ dithioerythrotol matrix.

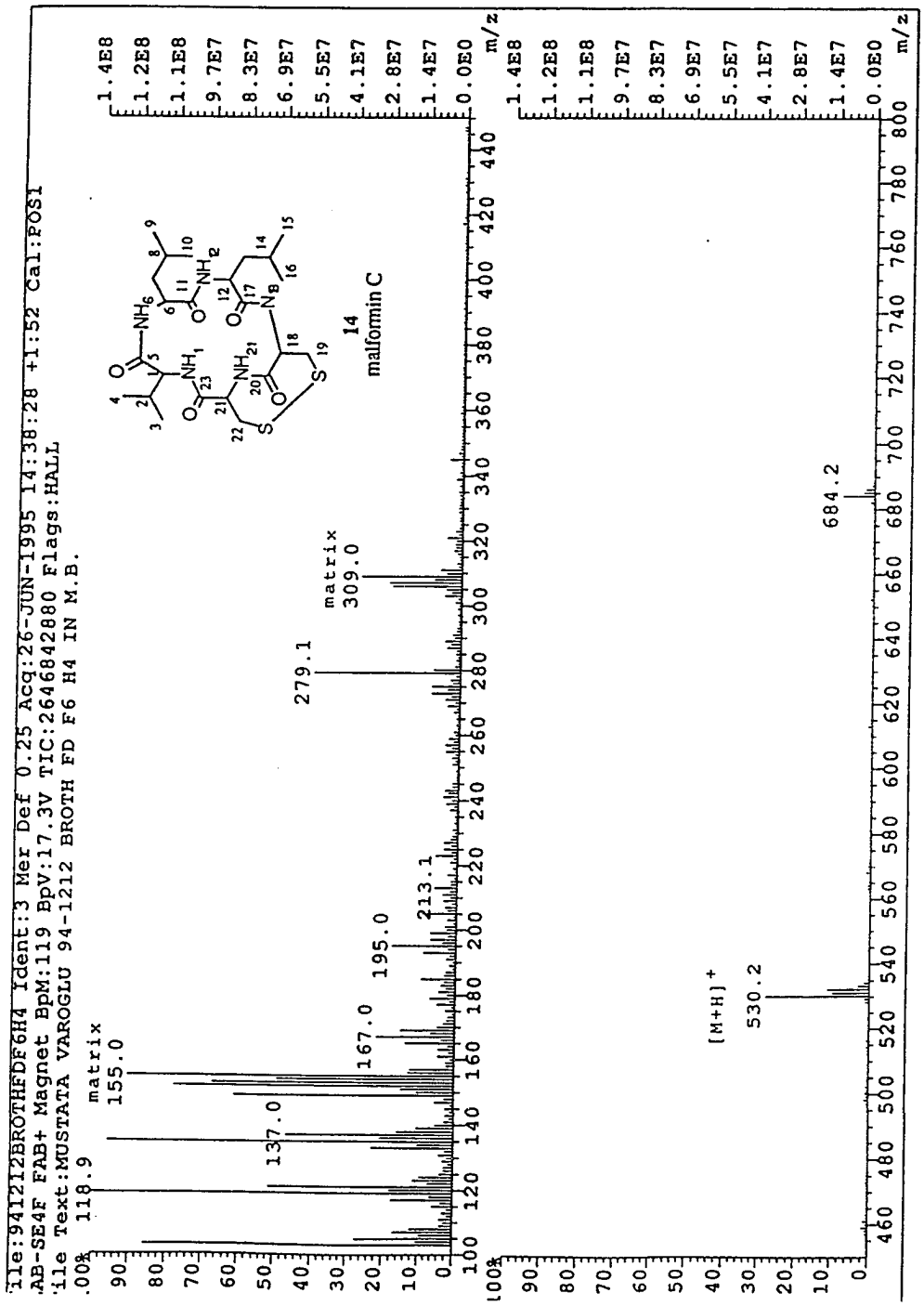


Table 5.6. ^{13}C NMR and ^{1}H NMR of malformin C (**14**).

Atom	^{13}C ppm, δ, m	^{1}H ppm δ, m, (*J*)
1	59.52, d	4.04, dd
2	27.26, d	2.15, m
3	19.76, q	0.93-0.98
4	18.64, q	0.93-0.98
5	171.83, s	
6	52.03, d	4.41, dd
7	40.45, t	1.56, t, (6.5)
8	25.05, d	1.61, m
9	22.32, q	0.93-0.98
10	22.19, q	0.93-0.98
11	174.85, s	
12	172.79, s	
12	53.58, d	4.10, m
13	39.18, t	1.66, m
14	24.80, d	1.61, m
15	22.78, q	0.93-0.98
16	22.53, q	0.93-0.98
18	52.74,d	4.36, m
19	47.46, t	3.83, dd
		3.13, dd
20	174.85, s	
21	54.64, d	4.94, dt
22	46.21, t	3.36, m
23	171.39, s	
NH21		7.15, d, (12)
NH17		6.71, d, (6)
NH1		6.58, d, (10)
NH5		6.18, d, (9)

The identity of the amino acids in the pure compound were initially determined by matching the ^{13}C NMR shifts, Figure 5.51, of the isolated sample with the literature values for amino acids.[17] The ^{13}C NMR methyl resonances for leucine and valine were diagnostic. The two ^{13}C NMR peaks at 19.76 (C3) and 18.64 (C4) ppm were indicative of a valine amino acid, while the four peaks at 22.78 (C10), 22.53 (C11), 22.32 (C15) and 22.19 (C16) ppm demonstrated that

there were two leucine groups present. The possibility of an isoleucine was eliminated because of the lack of a ^{13}C NMR resonance at 11 ppm. The presence of two cysteines was suggested by two methylene carbons at 47.76 (C19) and 46.21 (C22) ppm.

In order to connect the five amino acids together the spin system of each amino acid was identified by a ^{1}H-^{1}H COSY experiment. The α protons were the logical starting place for this exercise as they were among the less crowded signals in the ^{1}H NMR spectra. This provided the substructures with the assignments shown below, Figure 5.55. It was possible to correlate the α proton to the β protons in all of the amino acids. However, for the leucine and valine groups it was not possible to differentiate the methyl groups in the proton spectra due to the severe crowding of six methyl doublets in the region of 0.8-1.0 ppm. It was essential to have the β protons of the amino acids assigned because only from these protons could unequivocal assignment of the carbonyl resonances be made. Thus, the individual amino acids had all of their resonances assigned. HMBC correlations from the β proton of valine (H2), one of the cysteines (H19) and one of the leucines (H7) were used to assign carbonyls C5, C20 and C11 respectively, Figure 5.55.

Once a carbonyl shift was assigned to a specific amino acid, correlations were from the α proton of the N terminal nearest neighbor was used to place the nearest adjacent amino acids. Using the α protons of the nearest neighboring carbonyl carbons provided three amino acid fragments composed of one and two amino acids. The HMBC correlation of H6 to C5 and H21 to C20 led to the substructures shown in Figure 5.55

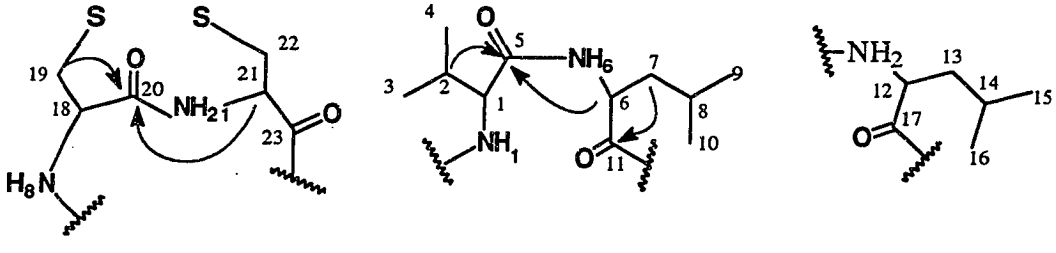


Figure 5.55. Amino acid substructures of malformin C (**14**).

Connection of these substructures to each other was by use of the amino protons to correlate the carbonyl of the N terminal neighbors, H18 to C17 and H1 to C23, which could not be determined by HMBC correlations from the neighboring α protons. This led to the construction of a five amino acid chain, Figure 5.56. The two rings present in the molecule accounted for the last two unsaturations. The cysteine amino acids were determined to possess a disulfide bond due to weak correlations in the RELAY experiment across the sulfurs from H19 to H22.

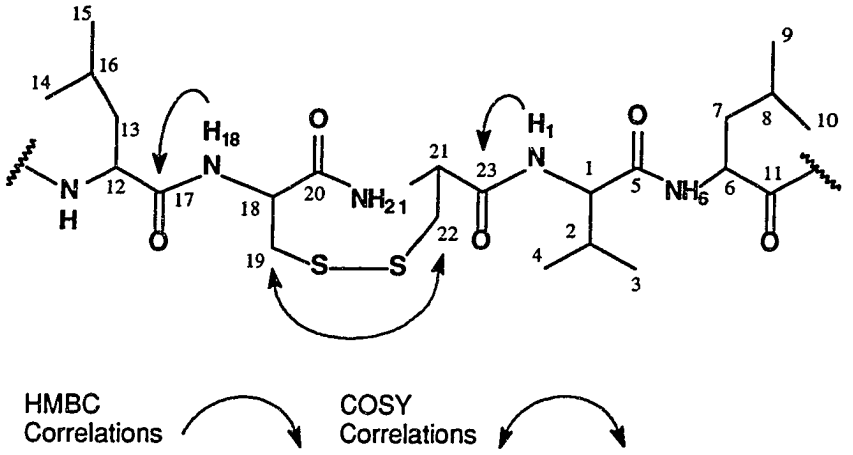
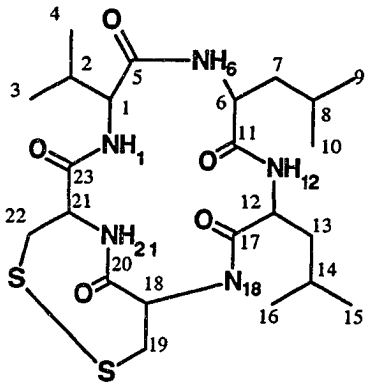


Figure 5.56. Linear chain of malformin C (**14**).

This left one remaining ring : the connection of the amino acid chain back on itself to form a cyclic peptide. Using this reasoning both the ^{1}H and ^{13}C NMR of malformin C (**14**) were assigned.



14 malformin C

Dereplication and NMR resonance assignment of pyrophen (15)

Analysis of the ^{13}C NMR, Figure 5.57, ^{1}H NMR, Figure 5.58, DEPT, Figure 5.59, and HMQC, Figure 5.60, spectra summarized in Table 5.7 of pyrophen (**15**) provided a partial molecular formula of $C_{16}H_{16}$. An additional proton was clearly visible in the proton spectra at 6.06 ppm. This proton was assumed to be attached to a nitrogen to provide a working formula of $C_{16}H_{17}N$. While this formula did not account for the oxygens present in the molecule it was used to derive the correct unsaturation number of nine.

Table 5.7. NMR data of pyrophen (**15**), 500 MHz, $CDCl_3$.

Atom	^{13}C ppm, δ, m	^{1}H ppm δ, m, (J)
1	164.4, s	
2	88.5, d	5.42, d, (2.2)
3	170.5, s	
4	101.2, d	5.75, d, (2.2)
5	161.3, s	
6	52.4, d	5.00, d, (7.5)
7	38.9, t	3.08, dd, (7.7, 8.0)
8	135.8, s	
9	129.1, d	7.15, m
10	128.7, d	7.30, m
11	127.2	7.22, m
12	128.7, d	7.30, m
13	129.1, d	7.15, m
14	169.6, s	
15	23.2, q	1.96, s
16	56.0, q	3.75, s
NH		6.06, d, (8)

A strong hint to the structure of pyrophen (**15**) was obtained by examination of the lowfield ^{13}C NMR shifts. The multiplicity and values of the shifts, especially 88.5 (C2) and 101.2 (C4) suggested a pyrone moiety with an O-methoxy group, 56.0 (C16), attached in the third position, 170.54 (C3).[12] This assumption was supported by the HMBC correlations from H2 to C3 and C4, and from H4 to C3 and C5. An ABMX spin system consisting of NH, H6 and H7/7' was identified from ^{1}H-^{1}H COSY correlations, Figure 5.61. This spin system was linked to the

Figure 5.57. ^{13}C NMR spectrum of pyrophen (**15**), 62.9 MHz, $CDCl_3$.

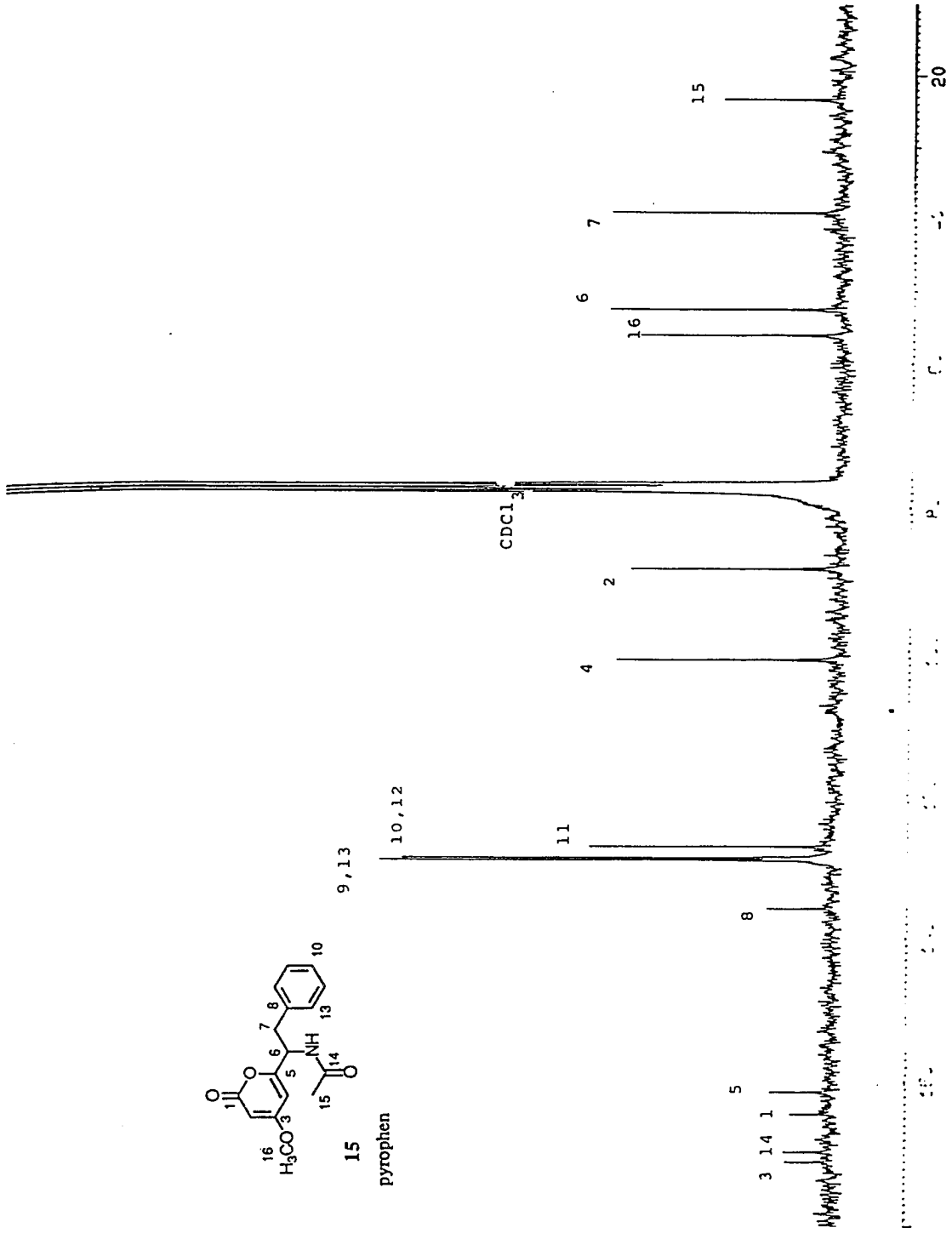


Figure 5.58. ^{1}H NMR spectrum of pyrophen (**15**), 250 MHz, $CDCl_3$.

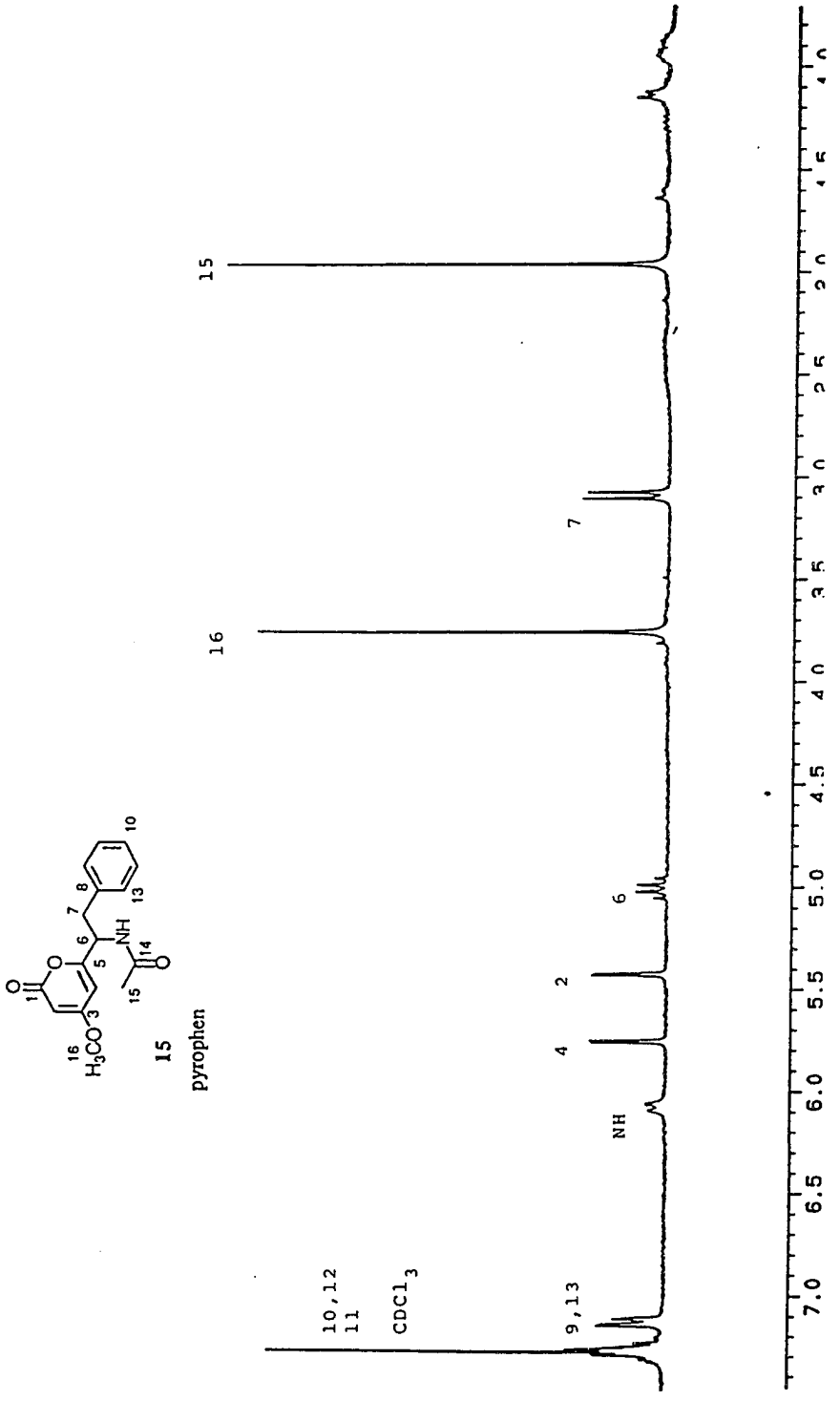


Figure 5.59. DEPT 135 spectrum of pyrophen (**15**), CH_3, CH up, 62.9 MHz, $CDCl_3$.

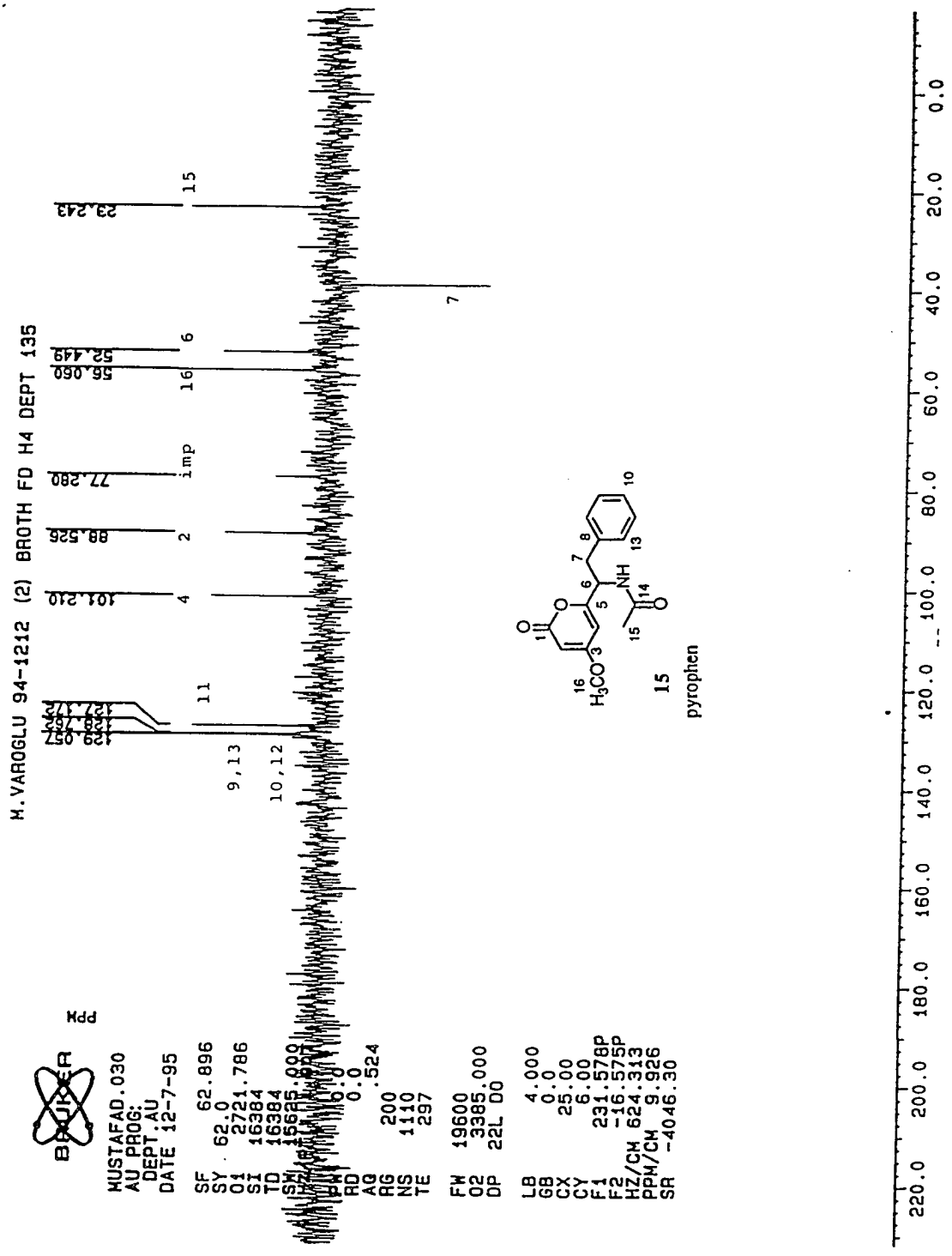


Figure 5.60. HMQC spectrum of pyrophen (**15**), 500 MHz, $CDCl_3$.

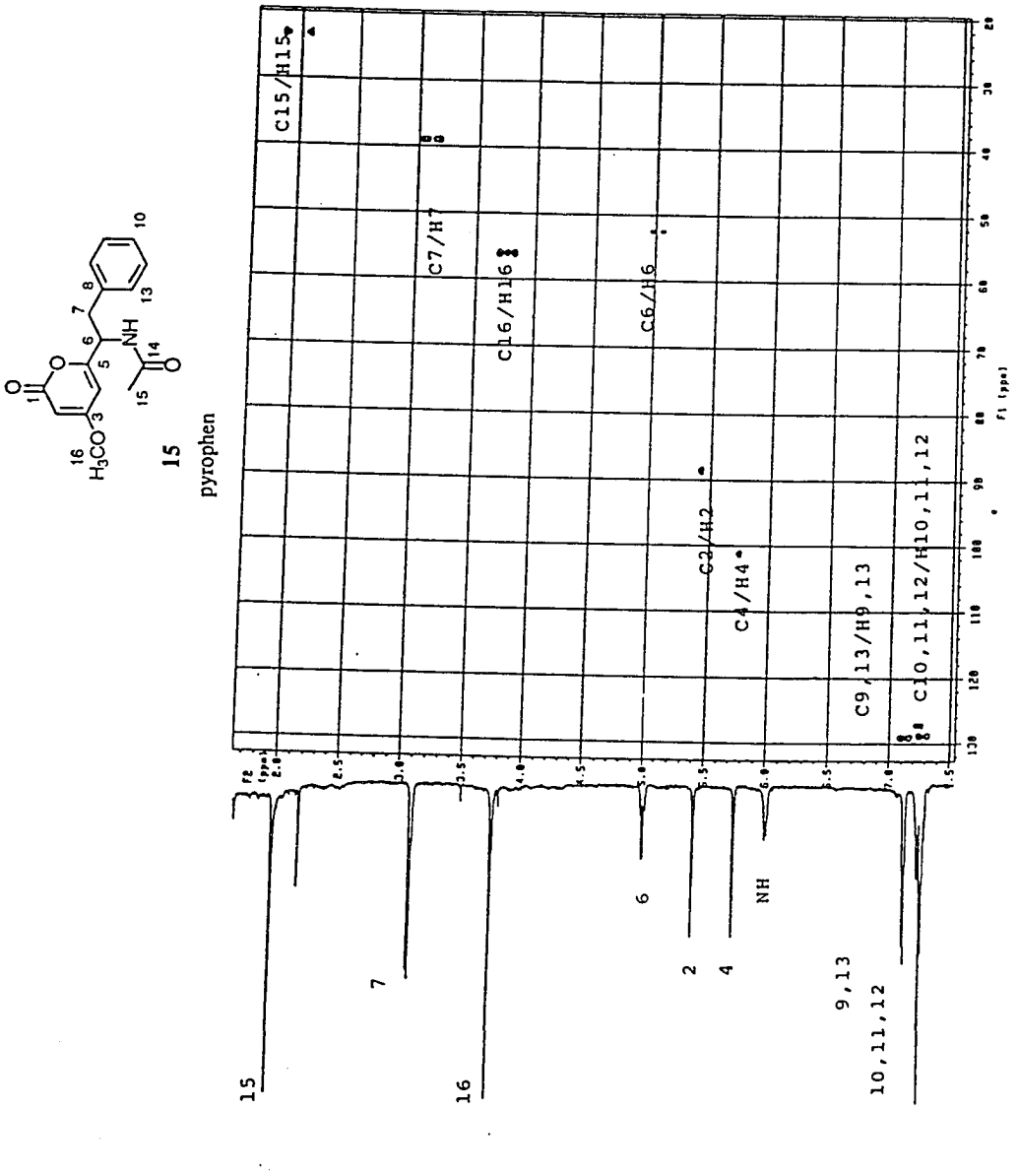
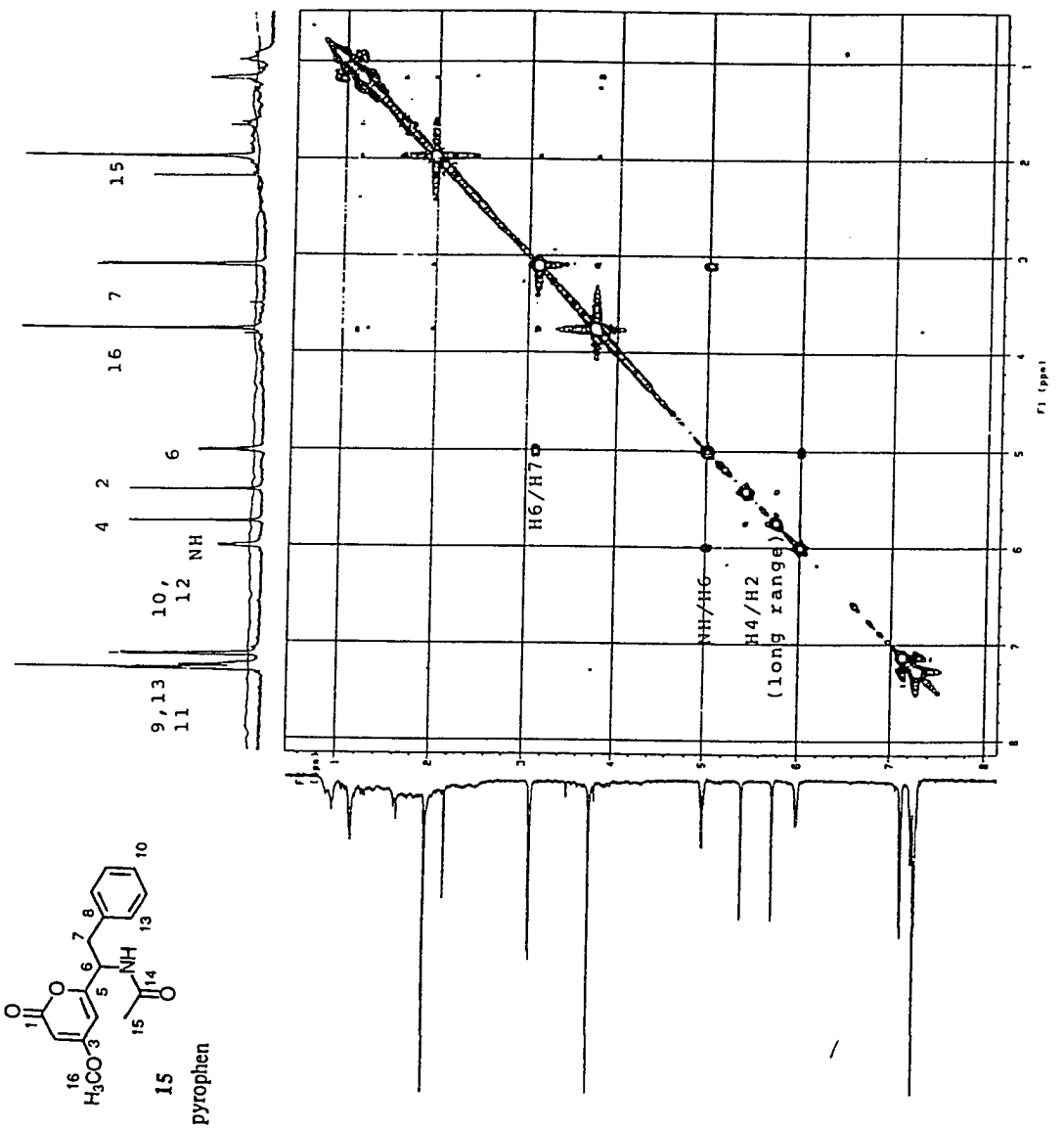


Figure 5.61. ^{1}H-^{1}H COSY spectrum of pyrophen (**15**), 500 MHz, $CDCl_3$.



15 pyrophen

pyrone substructure by an HMBC correlation from C4 to H6, Figure 5.62. The attachment of a phenyl group to C7 was confirmed by the three bond coupling from H6 to C8, and H13 and H9 to C7. The connection of a carbonyl to the nitrogen was determined by the HMBC correlation from H6 to C14. Methyl-15 was placed on carbonyl C14 by a three bond carbon hydrogen correlation from H15 to C14.

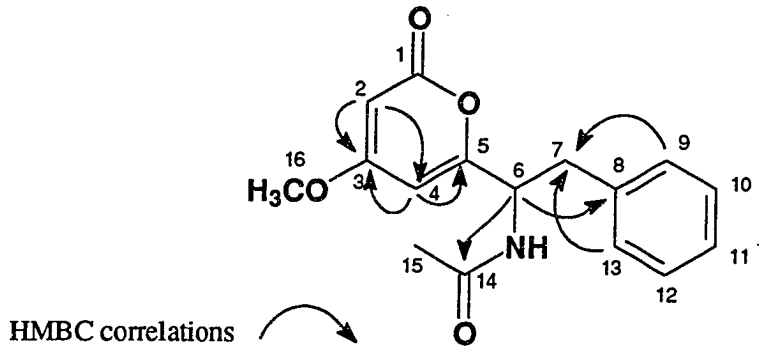
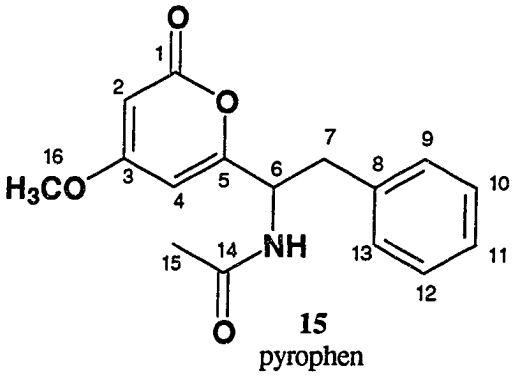


Figure 5.62. Important HMBC correlations of pyrophen (**15**).

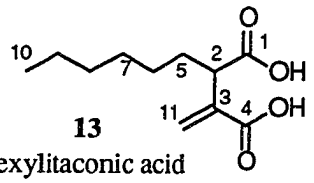
After solving the structure, a CAS online substructure search was performed and it was found that pyrophen (**15**) had been previously isolated. The previous isolation was from a terrestrial culture of *Aspergillus niger* and was solved by X-ray crystallography. The structure elucidation of pyrophen (**15**) by NMR spectroscopy provides the first NMR assignment of the structure.



15
pyrophen

Dereplication of hexylitaconic acid (13)

Examination of the ^{1}H NMR, Figure 5.63, the ^{13}C NMR, Figure 5.64 and DEPT spectrum, Figure 5.65, led to a partial molecular formula of $C_{11}H_{16}$ for hexylitaconic acid (**13**). A seven carbon spin system was determined by ^{1}H-^{1}H COSY correlations, Figure 5.66. This spin system was accompanied by two carbonyls and a quaternary vinylic carbon. The presence of an exomethylene was determined by the methylene signal at 129.2 ppm whose protons were at 6.54 and 5.84 ppm. HMBC correlations from these exomethylene protons were used to determine the juxtaposition of the carbonyl and quaternary double bond carbon, Figure 5.67. A substructure search was made on Chemical Abstracts Services online service and the structure of hexylitaconic acid (**13**) was found.



13
hexylitaconic acid

Hexylitaconic acid (**13**) was previously identified in 1984 as a plant growth regulator and the structure was solved by NMR spectroscopy. Comparison of the reported spectra for hexylitaconic acid (**13**) with the spectra of the isolated compound indicated hexylitaconic acid (**13**) matched the isolated compound in hand, Table 5.8

Table 5.8. Measured NMR data for hexylitaconic acid (**13**),250 MHz, $CDCl_3$.

	isolated compound		hexylitaconic acid (**13**) literature values	
Atom	^{13}C ppm, δ, m	^{1}H ppm δ, m, (*J*)	^{13}C ppm, δ, m	^{1}H ppm δ, m, (*J*)
1	178.5,s		179.6, s	
2	46.8, d	3.42, t, (7)	47.0, t	3.35, t, (7)
3	137.6, s		137.5, s	
3	29.0, t	1.29, m	29.0, t	1.29, m
4	170.5,s		171.7, s	
5	30.3, t	1.90, m, 1.74, m	29.9, t	1.80, m
6	27.4, t	1.29, m	27.4, t	1.29, m
8	31.6, t	1.29, m	31.7, t	1.29, m
9	22.6, t	1.29, m	22.6, t	1.29, m
10	14.1, q	0.87, t, (6)	14.1, q	0.83, t, (6)
11	129.2, t	6.54, br s, 5.84, br s	129.6, t	6.40, s5.80, s

Figure 5.63. ^{1}H NMR spectrum of hexylitaconic acid (**13**), 250 MHz, dioxane-d_8.

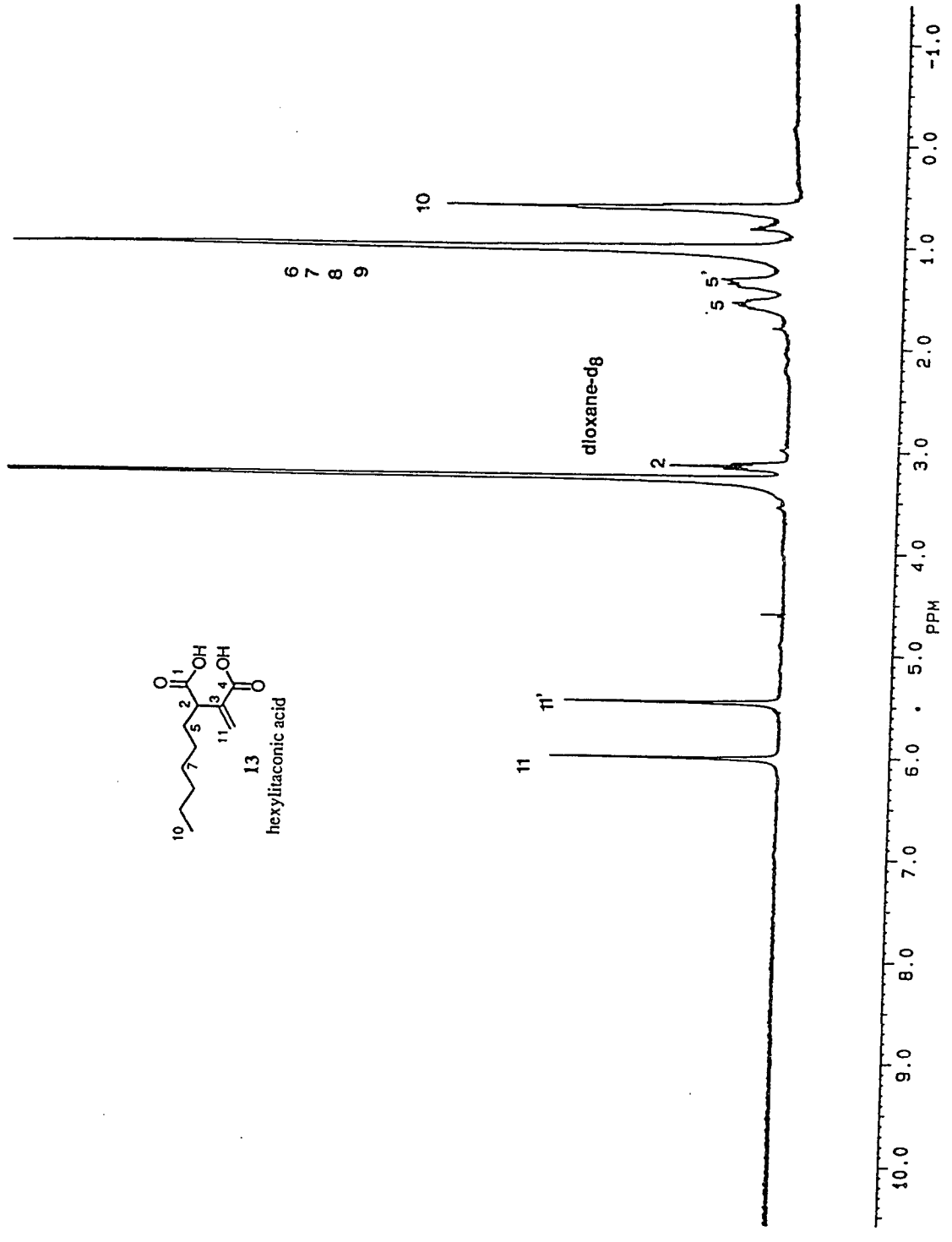


Figure 5.64. ^{13}C NMR spectrum of hexylitaconic acid (**13**), 125 MHz, $CDCl_3$.

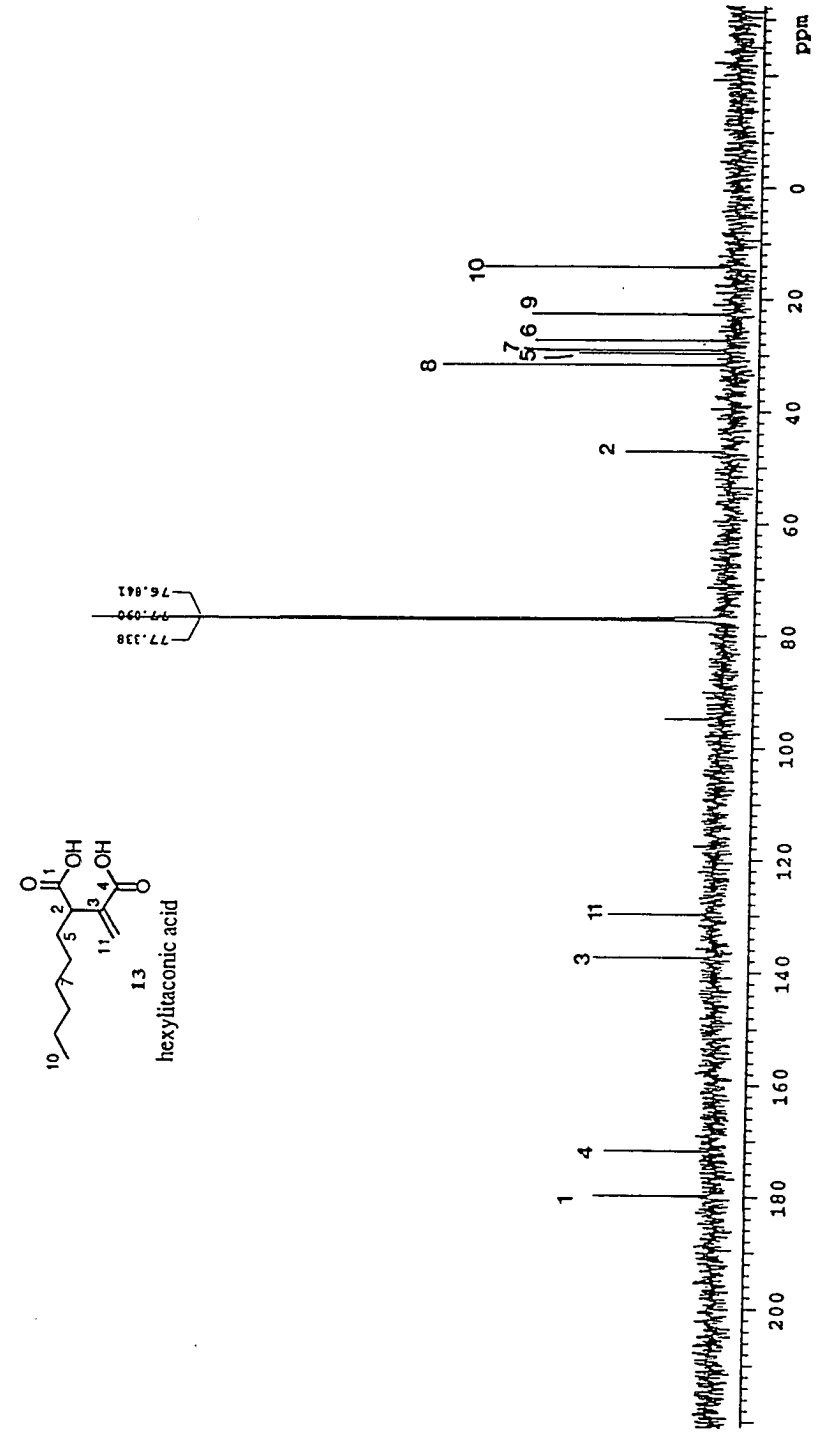


Figure 5.65. DEPT 135 spectrum of hexylitaconic acid (**13**), 62.9 MHz, $CDCl_3$.

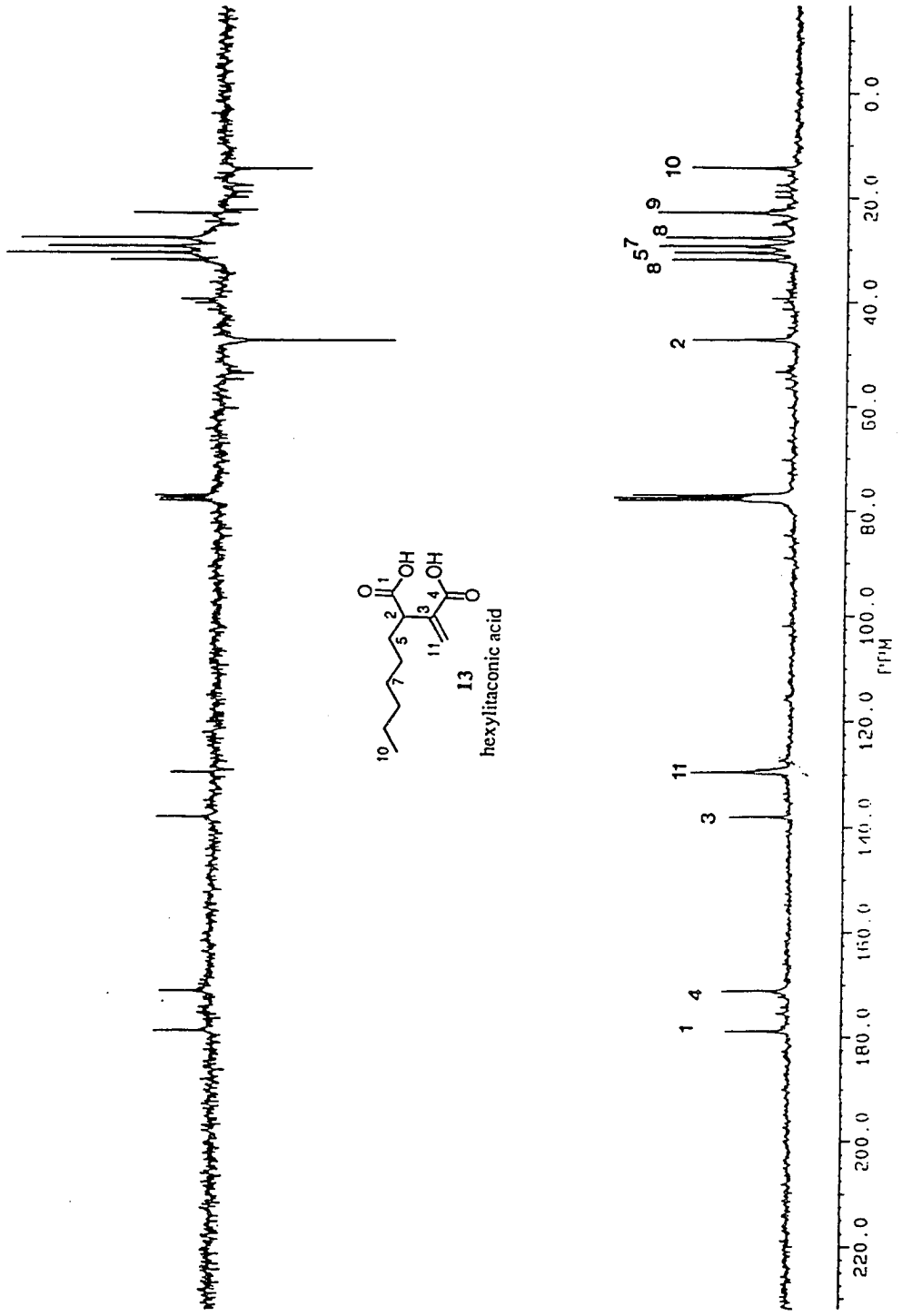


Figure 5.66. ^{1}H-^{1}H COSY spectrum of hexylitaconic acid (**13**), 250 MHz, $CDCl_3$.

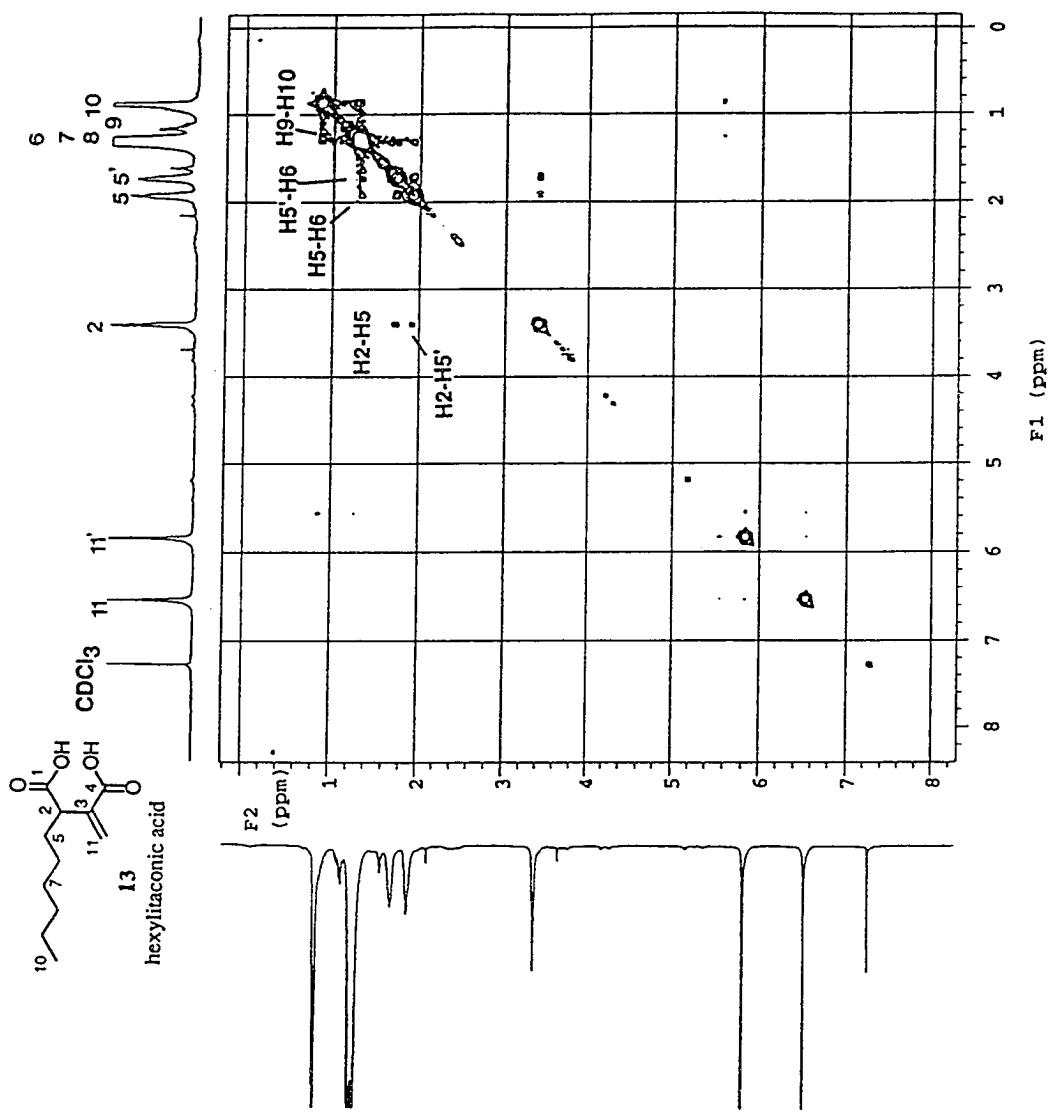
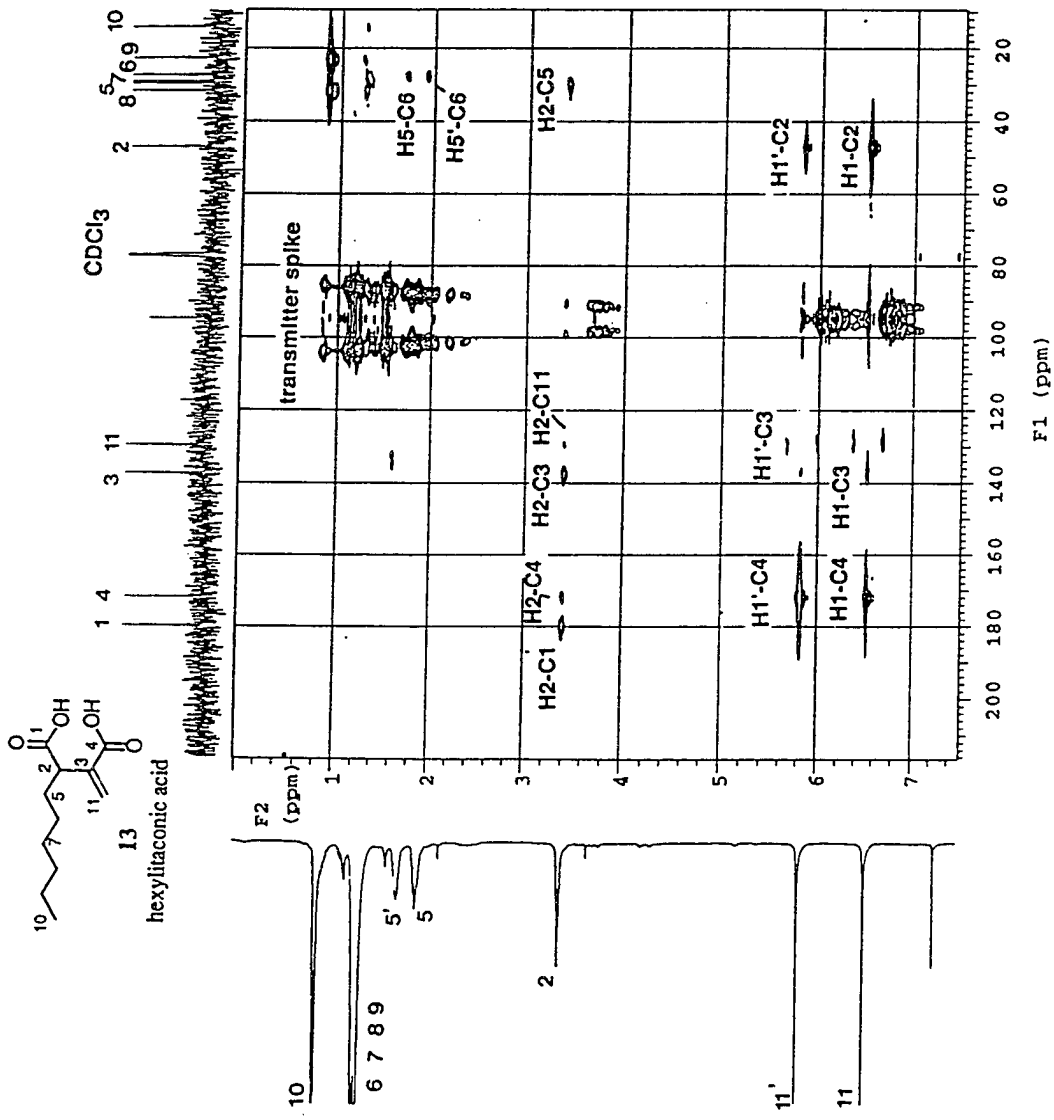


Figure 5.67. HMBC spectrum of hexylitaconic acid (**13**), 500 MHz, $CDCl_3$.



DISCUSSION

The issues raised at the onset of this project were :

- Can new natural products be obtained from a sponge derived culture of *Aspergillus niger*?
- Were the secondary metabolites isolated from the fungus similar to *Hyrtios proteus* sponge compounds?
- What accounts for the changing distribution of the compounds isolated?
- Was electrospray ionization mass spectrometry effective in this project?

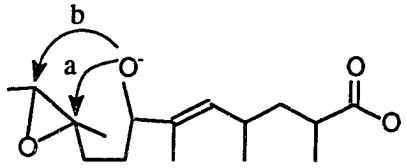
The isolation of hexylitaconic acid (**13**), malformin C (**14**), pyrophen (**15**), asperic acid (**17**), and asperizine (**18**) provides some answers to these questions.

Both known and novel secondary metabolites were isolated from the culture of *Aspergillus niger* examined. While the known compounds hexylitaconic acid (**13**), pyrophen (**15**) and malformin C (**14**), purified in this culture have been found in previous terrestrial cultures of *Aspergillus niger*, there are no reports identifying all of these known compounds simultaneously from a single culture. Even more unusual is that the compounds produced by this culture are members of three different biosynthetic groups. This culture was able to produce TCA cycle derivatives, polyketides, amino acid and amino acid polyketide conjugate natural products. This range of biosynthetic capability has not been reported from a single culture of *A. niger*. If the culture was composed of several different strains of *A. niger* this would account for the biosynthetic diversity in the compounds isolated. While culture was taxonomically identified as *Aspergillus niger*, the specific strain was not conclusively determined. If more than one strain is present, there has been at least a common strain present in each sample analyzed as some malformin C (**14**) has been isolated from every culture grown to date.

The new compounds isolated demonstrate important and unique variations not observed before in terrestrial fungal natural products. Asperizine (**18**) represents an unprecedented

arrangement of the known cyclized di-tryptophan moiety. All previous compounds of this type (see Chapter Four for the melinicidins and leptosins) are linked through the 3-position of the tryptophan portion in a symmetrical manner as represented by WIN 64821 (**19**). Asperizine (**18**) is the first diketopiperazine dimer with a linkage through the phenyl moiety. This rearrangement of a known skeleton parallels the chemistry obtained for chloriolin A (Chapter Four - **105**) and observed in the dendryphiellins (Chapter Four - **63-67**). The marine fungi studied to date have repeatedly demonstrated the ability to rearrange a known terrestrial skeleton. Both ditryptophenaline (**20**) and WIN 64821 (**19**) contain L-tryptophan and L-phenylalanine. The presence of D-phenylalanine in asperizine (**18**) is unprecedented in previously reported related structures.

The structure of asperic acid (**17**) also provides unexpected variations compared to other polyketides isolated from fungi. Asperic acid (**17**) is postulated to be a polyketide derived from malonate and methylmalonate units. While the structure could have been cyclized by the alcohol on C12 to the carboxylic acid to form a macrocyclic polyketide, this has not occurred. The structure of asperic acid (**17**) allows for the possibility of a five- or six-membered ether ring, Figure 5.68. In a previously studied fungal polyketide asperlactone (**21**) a similar possibility of either five- or six-membered lactones has been identified.[29,30] In the case of asperlactone (**21**), the six-membered lactone rings are formed exclusively after the tenth day of culture while only the five-membered lactones (**22**) are produced before day ten, Figure 5.69. In the case of the ether compound, asperic acid (**17**), only the five-membered ring is produced in the 21 day culture period. It would be interesting to examine the culture at an earlier stage to attempt to locate the six-membered ring product. The observed variations in secondary metabolitic pathways confirms the ability of *A. niger* to produce novel compounds as suggested by the creativity index described earlier.



Putative asperic acid precursor

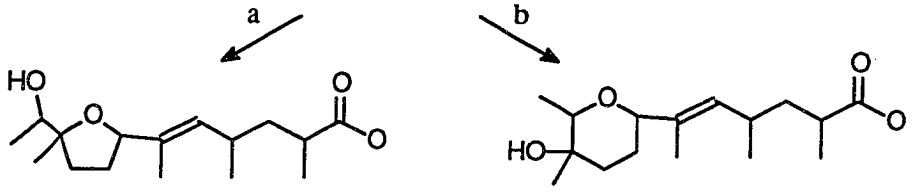
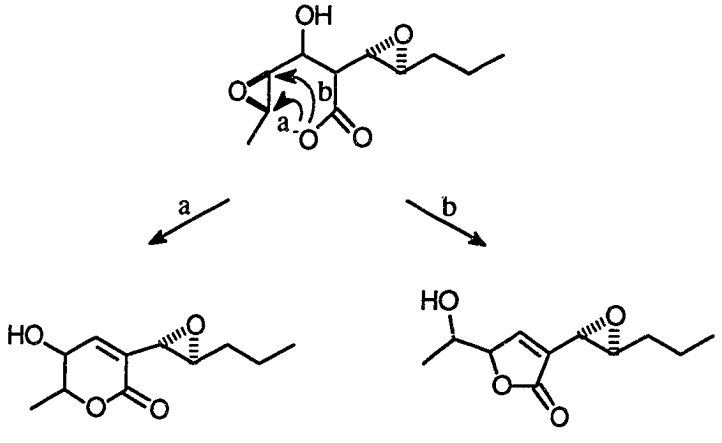


Figure 5.68. Possible cyclizations for the formation of asperic acid (**17**).



21
asperlactone

Produced before day 10
of culture growth

22
aspyrone

Produced after day 10
of culture growth

Figure 5.69. Parallel cyclization possibilities from terrestrial *A. melleus*.

While a wide variety of compounds were isolated from the large scale liquid broths of the *Aspergillus niger*, they did not resemble the terpene (**1-2**) or indole natural products (**3-4**) isolated from *Hyrtios proteus*. Compounds of polyketide, amino acid and TCA cycle derivatives were

isolated, however, no terpenes were isolated. The only possible correlation to *Hyrtios* compounds was the presence of the indole ring system present in asperizine (**18**). However, the asperizine (**18**) strongly resembles the known *Aspergillus* secondary metabolites WIN 64821 (**19**) and ditryptophenaline (**20**) much more than it does the indole products produced by *Hyrtios sponges.*

The varying distribution of the compounds isolated is accounted for by the starting inoculum of the large scale growths. The first large scale growth was the only time hexylitaconic acid (**13**) acid was isolated. Correspondingly, this was the only time the plate MEA 2.1 was used as a starting point for the large scale culture of 94-1212. Malformin C (**14**) was also isolated in the first large growth, albeit in very small amounts. Plate MEA 2.2 was used as the inoculum for the first culture and every large scale culture onward in which malformin C (**14**) was isolated. Pyrophen (**15**) was also discovered in the second large scale growth of 94-1212. It is possible trace amounts of pyrophen (**15**) were produced in the first culture in undetectable quantities. The isolation of asperic acid (**17**) and asperizine (**18**) marked the final change in the compound distribution. The large growths 94-1212 (3) and 94-1212 (4) were both derived from a cryopreserved sample of MEA 2.2. The cryopreservation apparently brought forward new biosynthetic pathways for the production of the novel compounds asperizine (**18**) and asperic acid (**17**) while also retaining the old biosynthetic pathways for malformin C (**14**) and pyrophen (**15**). These results are summarized in Figure 5.70.

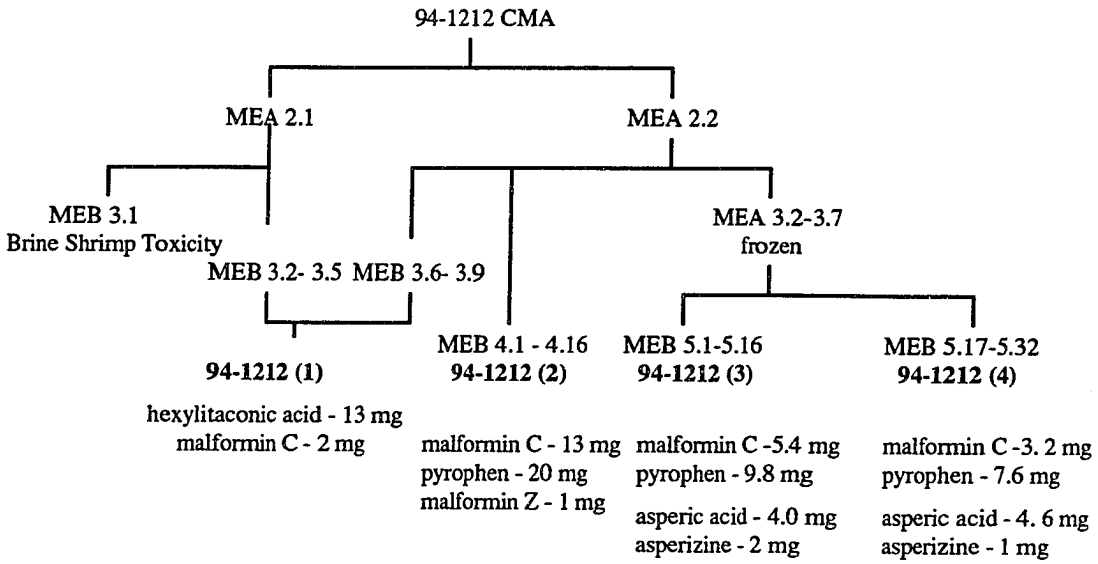
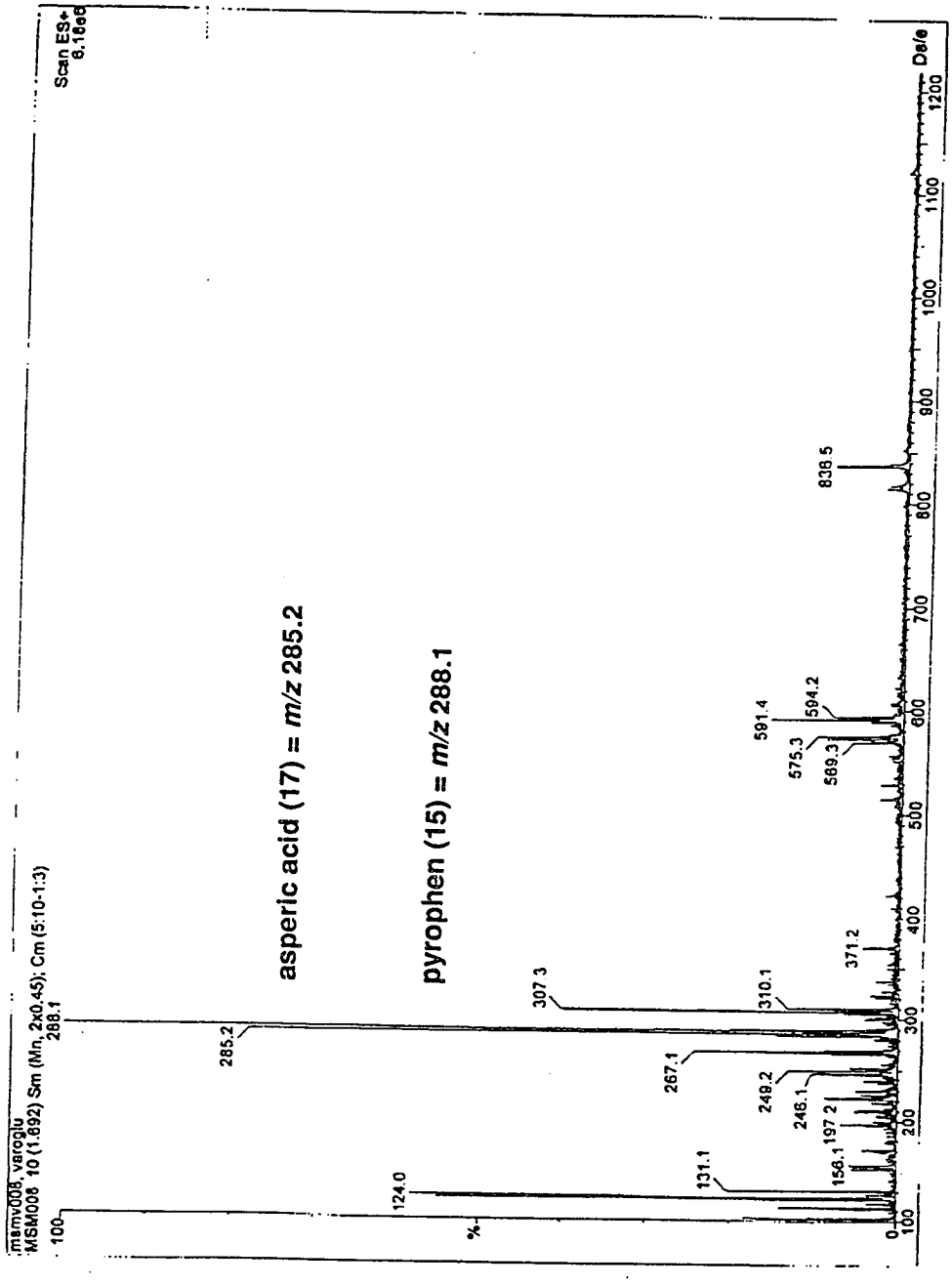


Figure 5.70. Summary of sources and amounts of compounds isolated from large scale growths of 94-1212.

The application of electrospray ionization mass spectrometry (ESIMS) was first used in this project to determine if the flash fraction following hexylitaconic acid (**13**) in the first large growth contained any larger compounds. The observation of the peak at *m/z* =530, Figure 5.7, indicated larger (and more interesting) compounds than hexylitaconic acid (**13**) were present. There was hope that this compound was a novel malformin as several malformins with the same molecular weight are known. Thus, ESIMS very efficiently provided both information on the presence of a sophisticated compound and its possible identification.

The limitations of this method are demonstrated by the peak at *m/z* = 836.5, Figure 5.7. This compound has not been located even though the peak height is almost as great as the peak of malformin C (**14**). In a similar case the ESIMS of 94-1212(4) FD which contained pyrophen (**15**), malformin C (**14**), asperic acid (**17**) and asperizine (**18**) did not indicate the presence of asperizine (**18**), Figure 5.71. This highlights the difficulty of using ESIMS to quantify the proportions of dissimilar compounds in a mixture. While the requirements of protonation of the sample for detection in the positive mode implies that basic or nitrogen containing compounds will ionize

Figure 5.71. ESI mass spectrum of FD partition of 94-1212 (4) broth extract.



readily, the signal for asperizine (**18**), a tetrapeptide with six nitrogens, is still not observed. There exists at this time no empirical collection of ESIMS data on the sensitivity of detection for a wide range of structural classes. These examples of a large ESIMS peak that could not be located chromatographically, and the failure to detect the signal of a compound present in the mixture is analogous to both false positives and false negatives in an assay system.

Once more empirical data can be collected so that the behavior of a large variety of compounds in ESIMS is understood, the technique promises great potential for the early detection of individual compounds in a mixture. When this technique is coupled with a database with a molecular weight index, its full potential of identifying individual compounds in a mixture will be realized.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES—The NMR spectra were recorded at 250 or 500 MHz for ^{1}H and 62.9 and 125.7 MHz for ^{13}C. UV/Vis measurements were performed with a HP 8452 diode array detector. Optical rotations were measured with a Jasco model DIP 370 digital polarimeter. IR spectra were measured with a Perkin-Elmer FT IR 1600 series spectrometer. The assignments of the ^{13}C and ^{1}H NMR data reported for all compounds were made by using HMQC[31,32] data to determine one bond H–C connectivities, HMBC [31] data to determine two and three bond H–C connectivities and NOESY[33] data to interrelate protons with close spatial proximity. High performance liquid chromatography (HPLC) was done using columns of 10 μm ODS. All solvents were distilled and dried for HPLC use and were spectral grade for NMR spectroscopy.

CULTURE OF FUNGI AND EXTRACTION— In the Dry Tortugas, Florida a piece of sponge collected by skin diving was placed in a sterile bag. In the presence of an updraft from an alcohol lamp the sponge sample was blotted on sterile filter paper to remove excess water. Under sterile conditions the sample was cut and placed on four different solid media consisting of malt extract agar (MEA), corn meal agar (CMA), cellulose powder agar (CPA) and potato dextrose agar (PDA) all with 100 mg/L of streptomycin and penicillin G to prevent bacterial growth. Fungal growth originating from the sponge was observed on the corn meal agar plates after 10 days and a sterile transfer was made to two malt extract agar plates, MEA 2.1 and 2.2, without antibacterials, Figure 5.2. An inoculum from MEA 2.2 was used to start a small 75 mL malt extract broth for brine shrimp toxicity assays. The broth was filtered from the mycelium and extracted with ethyl acetate and methanol respectively. The broth extract exhibited brine shrimp toxicity at a level of 2 μg/mL while the mycelium did not exhibit any toxicity.

Using both MEA 2.1 and MEA 2.2 plates eight replicates of the 500 mL malt extract broth (MEB) liquid cultures (MEB 3.2 -3.9) were inoculated and allowed to grow for 21 days, Figure

5.2. This first large scale growth was named 94-1212 (1). During this time the fungus grew as off-white mycelial spheres approximately 4-5 cm across.

ISOLATION OF HEXYLITACONIC ACID (**13**) AND MALFORMIN C (**14**) FROM 94-1212 (1) — The broth was extracted three times with ethyl acetate. This extract was dried and partitioned according to Figure 5.3 to yield 0.151 g of the FD fraction. This crude FD fraction, was chromatographed on a flash columns containing reverse phase C2 silica starting with pure water and decreasing in polarity to pure methanol. The major compound of the fraction hexylitaconic acid (**13**), 15 mg, was eluted at 75% aqueous methanol. Malformin C, 1.5 mg, from the fractions immediately following the hexylitaconic acid (**13**) was purified by analytical reverse phase C8 chromatography eluting with 35 % aqueous methanol.

ISOLATION OF PYROPHEN (**15**), MALFORMIN Z (**16**) AND MALFORMIN C (**14**) FROM 94-1212 (2)—The broth of 94-1212 (2), 8 L, was extracted with ethyl acetate with a continuous liquid liquid extractor for 24 hours. Ethyl acetate was evaporated under reduced pressure and the resulting extract, 60 mg, was applied to a reverse phase C18 HPLC column and eluted with a gradient solvent system that started at 30% aqueous methanol and ended at pure methanol over the period of one hour. Pyrophen (**15**) (5.4 mg), malformin Z (**16**) (1 mg) and malformin C (**14**) (13 mg) were eluted as pure compounds.

ISOLATION OF PYROPHEN (**15**), ASPERIZINE (**18**), ASPERIC ACID (**17**) AND MALFORMIN C (**14**) FROM 94-1212 (3)—The same extraction conditions used for the extraction of 94-1212 (2) were applied to the broth of 94-1212 (3). The concentrated broth extract was placed on a gradient HPLC and the same solvent conditions were used to elute the C18 reverse phase column. Pyrophen(**15**) (9.8), impure asperizine (**18**) (5.8 mg), asperic acid (**17**) (3.7 mg) and malformin C (**14**) (5.4 mg) eluted as the polarity of the solvent decreased. The impure asperizine (**18**) was applied to a C18 reverse phase column eluted isocratically with 24 % aqueous acetonitrile to provide 2.1 mg of pure asperizine (**18**).

ISOLATION OF PYROPHEN (**15**), ASPERIZINE (**18**), ASPERIC ACID (**17**) AND MALFORMIN C (**14**) FROM 94-1212 (4)—The broth of 94-1212 (4) was extracted and chromatographed under the same conditions as 94-1212 (3) to yield pyrophen (**15**) (7.6 mg), asperizine (**18**) (1.0), asperic acid (**17**) (4.6 mg) and malformin C (**14**) (3.2 mg).

TAXONOMIC IDENTIFICATION—Taxonomic analysis was performed by Analytical Services Inc. using both growth characteristics and fatty acid analysis. The sample was plated out on malt extract agar, potato dextrose agar and two varieties of cellulose yeast extract agar SDA. The organism was keyed to the description of *Aspergillus niger* var. *awamori*. However the other similar varieties of *A. niger* are *A. niger* var *niger* and *Aspergillus foetidus*. By fatty acid analysis the sample was identified as *Aspergillus niger*.

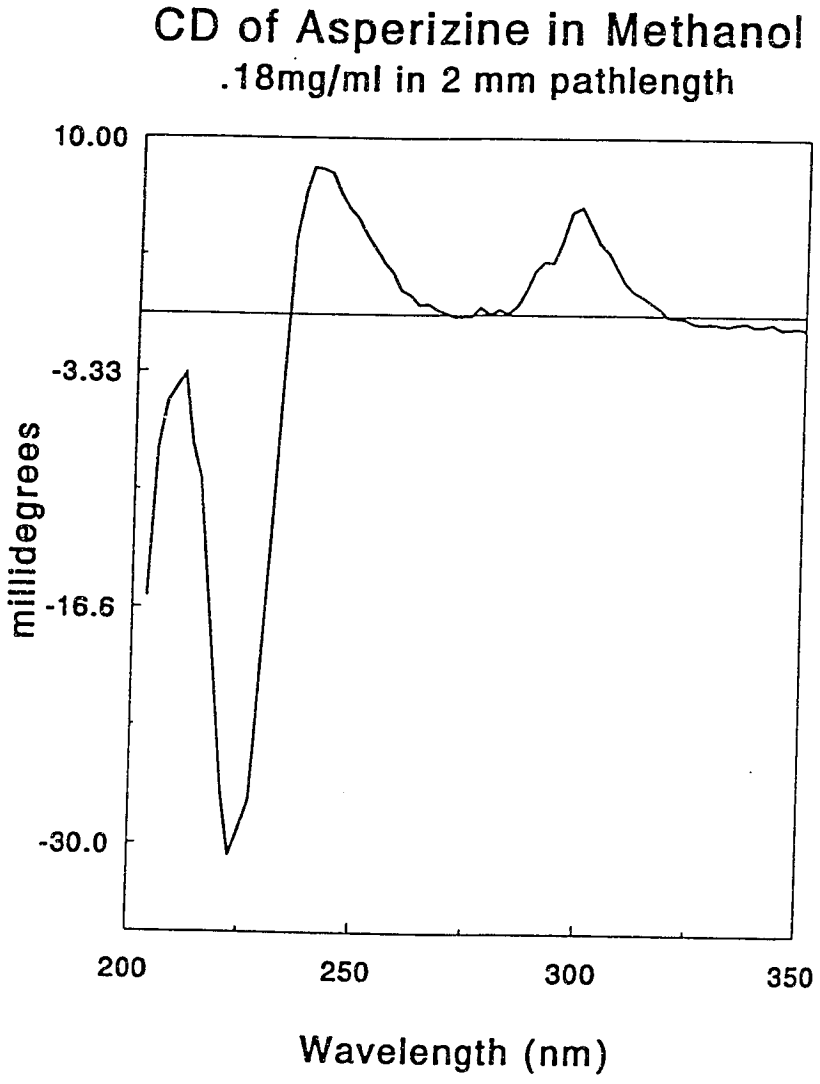
ASPERIZINE (**18**)— White powdery flakes, $[\alpha] = +53°$ (c, 0.2, CH_3OH); IR (film) 3448, 3365, 2931, 1681, 1673, 1667, 1651, 1444, 1314, 1093, 743, 700 cm^{-1}; λ_{max} = 300, 285, 225 nm; CD spectra is in Figure 5.72. LRFABMS, positive ion, *m/z* (relative intensity) 665.3 ($[M+H]^+$, 60), 409 (100); HRFABMS 665.2889 $[M+H]^+ = C_{40}H_{37}N_6O_4$ (Δ 1.3 mmu of calcd); NMR data are presented in Table 5.4.

ASPERIC ACID (**17**)— Yellow wax, $[\alpha] = +35°$ (c, 0.5, CH_2Cl_2); IR (film) 3301, 2967, 2874, 1709, 1672, 1533, 1454, 1370, 1064, 1018 cm^{-1}; λ_{max} = 280, 225 nm LRFABMS, positive ion, *m/z* (relative intensity) 285.2 ($[M+H]^+$, 100); HRFABMS 285.2059 $[M+H]^+ = C_{16}H_{29}O_4$ (Δ 0.7 mmu of calcd); NMR data are presented in Table 5.5.

HYDROLYSIS OF ASPERIZINE (**18**)— Asperizine (**18**), D and L-tryptophan, D and L-phenylalanine (0.100 mg each) in 20 µL of 6M HCl and 1% w/w phenol solution were heated in a 20 µL capillary tube at 150 °C for 90 minutes. After heating, the capillary tubes were flushed with water and acetonitrile and the resulting solution was dried under nitrogen gas. After removal of HCl and phenol with high vacuum the asperizine (**18**) and the standards were spotted on both normal phase and chiral reverse phase TLC plates (Chiralplate from Macherey-Nagle, distributed

by Alltech Co.).[28] Development of the normal phase plates (80/20/20 v/v/v n-butanol/water/glacial acetic acid) indicated that the only free amino acid present was phenylalanine. Only D-(*R*)-phenylalanine, $R_f = 0.43$, (standard D-phenylalanine $R_f = 0.44$; L-Phenylalanine $R_f = 0.55$) was observed on the chiral TLC plate (developed in 80/20/20 v/v/v acetonitrile/water/methanol).

Figure 5.72. CD spectra of asperizine (**18**), 2 mm (c = 0.18 mg/ml)) CH_3OH.



REFERENCES

(1) Amade, P.; Chevelot, L.; Perganowski, H. P.; Scheuer, P. J. *Helv. Chim. Acta* **1983**, *66*, 1672.

(2) Iguchi, K.; Shimada, Y.; Yamada, Y. *J. Org. Chem.* **1992**, *57*, 522.

(3) Kobayashi, J.; Murayama, T.; Ishibashi, M.; Kosuge, S.; Takamatsu, M.; Ohizumi, Y.; Kobayashi, H.; Ohta, T.; Nozoe, S.; Sasaki, T. *Tetrahedron* **1990**, *46*, 7699.

(4) Turner, W. B.; Aldridge, D. C. *Fungal Metabolites II*; Academic Press: San Francisco, 1983.

(5) Bodansky, M.; Stahl, G. L. *Bio-org. Chem.* **1975**, *4*, 93.

(6) Tanaka, H.; Wang, P.-L.; Yamada, O.; Tamura, T. *Agric. Biol. Chem.* **1966**, *30*, 107.

(7) Barton, D. H. R.; Bruun, T. *J. Chem. Soc.* **1951**, 2728.

(8) Baba, S.; Sakaguchi, K. *Agric. Chem. Soc. Japan* **1942**, *18*, 93.

(9) Dreyfuss, M. M.; Chapela, I. H. In *The Discovery of Natural Products with Therapeutic Potential*; Gullo, V. P., Ed.; Bitterworth Heinemann: New York, 1994, pp. 49-80.

(10) Wildman, H. G. *Can. J. Bot.* **1995**, *73 (Suppl. 1)*, S907-S916.

(11) Abrell, L. M.; Borgeson, B.; Crews, P. *Tetrahedron Lett.* **1996**, *37*, 2331-2334.

(12) Abrell, L. M.; Cheng, X.-C.; Crews, P. *Tetrahedron Lett.* **1994**, *35*, 9159-9160.

(13) Isogai, A.; Washizu, M.; Kondo, K.; Murakoshi, S.; Suzuki, A. *Agric. Biol. Chem.* **1984**, *48*, 2607-2609.

(14) Constant, H. L.; Beecher, C. W. W. *Natural Product Letters* **1995**, *6*, 193-196.

(15) Anderegg, R. J.; Biemann, K.; Buchi, G.; Cushman, M. *J. Am. Chem. Soc.* **1986**, *98*, 3365.

(16) Barnes, C. L.; Steiner, J. R.; Torres, E.; Pacheco, R.; Marquez, H. *Int. J. Pept. Protein. Res.* **1990**, *26*, 292-296.

(17) Pretsch, E.; Simon, W.; Seibl, J.; Clerc, T. *Tables of Spectral Data for Structure Determination of Organic Compounds.*; 2nd ed.; Springer-Verlag: New York, 1989.

(18) Kock, M.; Reif, B.; Fenical, W.; Griesinger, C. *Tetrahedron Lett.* **1996**, *37*, 363-366.

(19) Barrow, C. J.; Ping, C.; Snyder, J. K.; Sedlock, D. M.; Sun, H. H.; Cooper, R. *J. Org. Chem.* **1993**, *58*, 6016-6021.

(20) Springer, J. P.; Buchi, G.; Kobbe, B.; Demain, A. L.; Clardy, J. *Tetrahedron Lett.* **1977**, *28*, 2403-2406.

(21) Maes, C. M.; Potgieter, M.; Steyn, P. S. *J. Chem. Soc. Perkin Trans. I* **1986**, 861-866.

(22) Vicar, J.; Budesinsky, M.; Blaha, K. *Collection Czechoslov. Chem. Commun.* **1973**, *38*, 1940-1956.

(23) Davies, D. B.; Khaled, M. A. *J. Chem. Soc. Perkin Trans. II* **1976**, 187-196.

(24) Davies, D. B.; Khaled, M. A. *J. Chem. Soc. Perkin Trans. II* **1976**, 1238-1244.

(25) Hodge, R. P.; Harris, C. M.; Harris, T. M. *J. Nat. Prod.* **1988**, *51*, 66-73.

(26) Liu, T.-Y.; Boykins, R. A. *Analytical Biochemistry* **1989**, *182*, 383-387.

(27) Muramoto, K.; Kamiya, H. *Analytical Biochemistry* **1990**, *189*, 223-230.

(28) Gunther, K.; Martens, J.; Schickendoz, M. *Angew. Chem. Int. Ed. Engl.* **1984**, *23*, 506.

(29) Garson, M. J.; Staunton, J.; Jones, P. G. *J. Chem. Soc. Perkin Trans. I* **1984**, 1021-1026.

(30) Bereton, G. R.; Garson, M. J.; Staunton, J. *Chem. Soc. Perkin Trans. I* **1984**, 1027-1033.

(31) Bax, A.; Sparks, S. W.; Torchin, D. A. *J.Am. Chem. Soc.* **1988**, *110*, 7926.

(32) Bax, A.; Subramanian, S. *J. Magn. Res.* **1986**, *67*, 565.

(33) Hull, W. E. In *Two Dimensional NMR Spectroscopy for Chemists and Biochemists.*; Croasmun, W. R., Carlson, R. M., Eds.; VCH Publishers, Inc.: New York, 1987, p. 155.