Bioactive metabolites of endophytic fungi of the Mexican yew (Taxus globosa) and the isolation and chemical modification of sphaeric acid
by Royce Allen Wilkinson

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Chemistry
Montana State University

Abstract:
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Epicorazine A, a previously known isolate of the fungus, Epicoccum nigrum, with wide spectrum antibiotic activity, was isolated and characterized. Additionally, a novel succinic acid derivative, sphaeric acid, was isolated from a fungus identified as a Sphaeriopsis sp.

Several synthetic modifications of the acid functionalities of sphaeric acid were carried out to explore any structure/function relationships. These modifications included esterifications, amidifications, a reduction, and a subsequent oxidation. A variety of activities were observed among the derivatives in each of the bioassays. However, few consistent trends Were observed throughout the entire range of assays.

Finally, the effect of various culture media on bioactive metabolite production was studied. Several possible bioactive fractions were observed. Succinic acid was isolated as one of the active fractions, and a mixture of triacylglycerols were isolated due to their physical and spectroscopic similarities to sphaeric acid. Several active fractions remain that have the potential of yielding novel bioactive metabolites.

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APPROVAL

of a thesis submitted by

Royce Allen Wilkinson

This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

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ABSTRACT

Over the past decade there has been an increase in the need for novel pharmaceuticals. The development of microbial resistance to current clinical antibiotics, and the increasing number of immunocompromised hosts (AIDS patients, patients on chemotherapy, etc.) are two major reasons new antibiotics are required. New antitumor compounds with greater efficacy and fewer side effects are also in demand.

Endophytic microbes, fungi and bacteria living within the intercellular spaces of higher plants, are one possible source of novel bioactive compounds which are mostly unexplored. The endophytic microbes of yew trees were studied as a novel source of the antitumor drug, taxol, and the collection of microbes isolated in the search for taxol are also being screened for novel bioactive metabolites.

Epicorazine A, a previously known isolate of the fungus, *Epicoccum nigrum*, with wide spectrum antibiotic activity, was isolated and characterized. Additionally, a novel succinic acid derivative, sphaeric acid, was isolated from a fungus identified as a *Sphaeriopsis* sp.

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Chapter 1

INTRODUCTION

The Antibiotic Paradox

The "antibiotic paradox" is a phrase coined by Stuart Levy, M.D. describing the fact that antibiotics have sown the seeds of their own downfall by selecting for strains of bacteria that can resist their activity. These "miracle drugs" achieved their great acclaim by rapidly curing previously fatal infections. However, bacteria responded to the widespread use of antibiotics by finding ways of becoming insensitive to the killing effects of these drugs. Beyond this, many of these resistant traits have been found to be transferable from one resistant bacteria to another; even bacteria of different types.[1]

This has become such a concern that even the popular media has covered this phenomenon. *Time* magazine ran a story in 1994 entitled "The Killers All Around" which listed recent outbreaks by new or mutated bacteria and viruses. These included the "flesh eating" streptococcus-A infections in England, a flare-up of tuberculosis in southern California, an epidemic of pertussis (whooping cough) in Cincinnati, and several unknown viral and bacterial illnesses including the Hantavirus outbreak in the southwestern US. As little as a generation ago, medical researchers had begun to believe the end of infectious diseases was

only a matter of time. As *Time* put it, "the question ceased to be, When will diseases be gone? and became, Where will the next deadly virus appear?"[2]

The leading causes of the "antibiotic paradox" have been the widespread use and misuse of these drugs. The use of antibiotics for ailments for which they have no value[3], the sustained use of a single antibiotic[4], and incomplete treatment, that is, stopping the antibiotic treatment before destroying all infectious bacteria[5], have all been factors in decreasing the susceptibility of these pathogens to the current drugs.

The answer to this problem lies not in abolishing the use of antibiotics, but in the wise use of them, as well as the discovery and development of new antibiotics. A new metabolite with a specific mode of action could have a big impact in antibiotic treatments. Even if the activity is insufficient for use as a therapeutic agent, the natural product could be useful as a biochemical tool as either a starting material or as a template for chemical synthesis. A large number of antibacterial compounds have been discovered, but there is still a need for new types of therapeutic agents. The natural mutation of both pathogenic and antibiotic producing organisms will continue to supply both the need and a source for these agents.[6]

Taxol is a classic example of a compound whose novel mode of action inspired tremendous interest. Unlike other antimitotic agents which inhibit microtubule assembly, taxol actually promotes the assembly of tubulin and stabilizes the microtubules so formed.[7] This unique mode of action, coupled

with taxol's particular efficacy against refractory breast and ovarian cancers, has made this one of the most important antitumor agents of the past two decades.[8]

In addition to the need for new antibacterial compounds, antifungal agents are becoming more important as the occurrence of fungal infections increases among immunocompromised hosts. Aging, acquired immunodeficiency syndrome (AIDS), and treatment with immunosuppressive drugs such as anticancer agents are current factors increasing the frequency of opportunistic fungal infections. In many of these cases, the actual cause of death is fungal growth in the organs and blood, rather than tumors or AIDS itself.[9]

Clinically useful antifungal compounds are less common than antibacterial agents for several reasons. First and foremost, fungi are eucaryotes similar to human and animal cells. Therefore, selective toxicity is less likely to occur with antifungal agents. In addition, potent fungicidal effects are desired, but almost all the antifungal drugs now available for use are fungistatic. Those compounds which show strong fungicidal activity are unfortunately also highly cytotoxic. Finally, the host defense system is still a major factor in successful antimicrobial chemotherapy. However, fungal infections are mostly associated with a depression in host immune action making antifungal therapy less effective.[10]

Why Fungi?

The fungi form one of the largest kingdoms of living organisms with a conservatively estimated total of about 1.5 million species. Based on this

estimated figure, only 4.6% of the world's fungi have been recognized, and secondary metabolites have only been documented in about 5000 (or 7%) of the known species.[11] Therefore, a large pool of unknown fungi exists which will likely be found to exhibit novel chemicals among their secondary metabolites.

Two major areas of bioactivity of fungal produced compounds which have precedence in the literature are antibiotic (antifungal and antibacterial) substances and antitumor agents. Amphotericin B is currently the only antifungal compound of microbial origin which is now available for systemic use.[12] It was isolated from a soil actinomycete, *Streptococcus nodosus*, by W. Gold in 1955. Despite its adverse side effects, it remains the standard treatment for most serious mycoses.[13]

Antibacterial agents from microorganisms are a little more common. Penicillin is probably the most celebrated antibiotic. Vancomycin, a fungal derived glycopeptide, is another commonly used antibiotic isolated from a strain of *Streptomyces* (now *Amycolatopsis*) *orientalis* in 1956.[14] There are also several synthetic analogs of microbial compounds used clinically as antibiotics. Included in these are analogs of the monocyclic β-lactams isolated from various soil bacteria and analogs of cephalosporin produced by the fungus *Cephalosporium acremonium*.[15,16] There have been several new antibacterial compounds isolated from microbes over the last few years. Omura's book, *The Search for Bioactive Compounds from Microorganisms*, contains a table of recently isolated compounds which inhibit bacterial cell wall formation. The

table lists 47 compounds (some previously identified) which were published since 1980.[17]

Actinomycin D, mitomycin C, doxorubicin, and bleomycin are among the microbe-derived compounds that are currently being used clinically as antitumor agents.[18] A major discovery in this area was the isolation of the enediyne class of antitumor compounds including calicheamicin and esperamicin by May Lee at the Lederle Laboratories of the American Cyanamid Company. These are some of the most potent agents ever discovered, with calicheamicin being over 1000 times more potent than clinically useful antitumor antibiotics. The calicheamicins are produced by the fermentation of *Micromonospora echinospora* ssp *calichensis*, a bacterium isolated from a soil sample collected in Texas.[19] Omura's book also contains a list of 58 novel antitumor antibiotics isolated from microorganisms since 1984.[20]

This study was concerned with the isolation of novel bioactive compounds from endophytic fungi of the mexican yew tree (*Taxus globosa*). Endophytes are microbes which commonly live in the intercellular spaces of stems, petioles, roots, and leaves of plants causing no outward manifestation of their presence. The associations of these microbes to their hosts range from mutualistic, to symbiotic, to commensal.[21] The microbes may mimic the plant and make the same secondary metabolites as its host, or it may contribute by producing one or more compounds that may protect the plant (and thus itself) from attack by insects, fungi, bacteria, or other predators. It is probable that some of these

bioactive compounds may also have pharmaceutical applications.[22] Plant endophytes are a particularly unexplored niche of fungi, and of the work done, there is limited knowledge of the metabolites of these particular fungi. Together this makes endophytic fungi possibly one of the largest potential areas for discovery in the plant/microbe world.[23]

The choice of yew trees as a source of endophytic fungi was driven by the high cost and limited supply of taxol. The work done in the Stierle and Strobel laboratories to isolate a fungal source of taxol[24] resulted in a large collection of fungi which could also be examined for other bioactive metabolites.

Preliminary Results

Six fungi isolated from the mexican yew (labled as MA17, MC1, ME1, ME7, ME19, MF1) were chosen for this study. The fungi were purified and grown in 300 ml of sterilized medium containing 10.0 g sucrose, 1.0 g Bacto-Soytone, and 1 ml of a 1.0 M phosphate buffer (pH = 6.8) per liter. The cultures were incubated at room temperature without shaking for twenty-one days.

The mycelia were removed by filtration through eight layers of cheesecloth. The mycelia were then ground and extracted with 1:1 chloroform:methanol for the mycelial/organic (M/O) fraction and then with water for the mycelial/aqueous (M/H_2O) fraction. The filtrate medium was also extracted with three successive portions of chloroform and two portions of ethyl acetate. The organic extracts

were combined for the filtrate/organic (F/O) fraction and the remaining aqueous solution was lyophilized and labeled as the filtrate/aqueous (F/H_2O) fraction.

The crude extracts were subjected to a brine shrimp toxicity assay and to agar-diffusion assays to test for bioactivities (See Chapter 2 for details). The agar-diffusion assays were carried out on 0.5 mg of each extract dissolved in 20 µL of methanol. Positive results are recorded on a scale (+ to +++++) according to the completeness and size of the zone of inhibition. Negative results are recorded as a negative (-) and those with multiple negatives (-- and ---) were zones of apparent growth promotion. The brine shrimp toxicity assay was performed on 1.5 mg of extract in 60 µL of methanol per 3 mL of brine water for the organic extracts. The aqueous extracts were 1.5 to 5 mg of sample in 30 µL of water per 3 mL of brine water. Results are given as the number of dead brine shrimp at each time interval. The results of these bioassays are given in Tables 1.1 and 1.2.

There was very little activity in any of the aqueous fractions. However, several of the organic fractions were active in at least one of the bioassays. The severe brine shrimp activity of fraction MA17 F/O and the antifungal activity of the ME19 F/O fractions led to the isolation of sphaeric acid (Chapter 3) and epicorazine A (Chapter 6) respectively.

Extract	G. cand.	C. alb.	B. sub.	P. aerug.	E. coli
MA17 F/O	-	+	+++	++++	+++
MA17 M/O	-	-	+	-	-
MA17 F/H_2O	-	-	-	-	-
MA17 M/H_2O	-	-	-	-	-
MC1 F/O	-	+	+	-	-
MC1 M/O	+	++	+	-	-
MC1 F/H_2O	-	-	-	-	-
MC1 M/H_2O	+	---	-	-	-
ME1 F/O	-	-	++	-	-
ME1 M/O	-	-	+	-	-
ME1 F/H_2O	-	---	-	-	-
ME1 M/H_2O	+	-	+	-	-
ME7 F/O	+	+	++++	-	-
ME7 M/O	+	+	+	-	-
ME7 F/H_2O	-	--	-	-	-
ME7 M/H_2O	+	-	+	-	-
ME19 F/O	+++++	+++++	+++++	-	-
ME19 M/O	++	++	++	-	-
ME19 F/H_2O	-	+	-	-	-
ME19 M/H_2O	-	+	+	-	-
MF1 F/O	-	+	++++	-	-
MF1 M/O	+	+	+	-	-
MF1 F/H_2O	-	-	-	-	-
MF1 M/H_2O	-	-	+	-	-

Table 1.1 Antibiotic bioassays of crude extracts

Extract	t=0h	t=1h	t=3h	t=6h	t=12h	t=24h	total
MA17 F/O	13	26	45	-	-	-	45
MA17 M/O	0	0	0	0	0	0	44
MA17 F/H_2O	0	0	0	0	1	2	15
MA17 M/H_2O	0	0	0	0	0	0	23
MC1 F/O	0	0	1	11	29	32	32
MC1 M/O	0	0	0	0	1	2	22
MC1 F/H_2O	0	0	0	0	2	2	25
MC1 M/H_2O	0	0	0	0	0	1	16
ME1 F/O	0	0	1	1	1	4	42
ME1 M/O	0	0	0	0	0	13	42
ME1 F/H_2O	0	0	0	0	0	1	23
ME1 M/H_2O	0	0	0	0	5	5	11
ME7 F/O	0	0	2	2	3	14	30
ME7 M/O	0	0	0	0	0	1	37
ME7 F/H_2O	0	0	0	0	0	0	25
ME7 M/H_2O	0	0	0	0	0	1	20
ME19 F/O	0	0	0	0	2	14	24
ME19 M/O	0	0	0	0	0	0	27
ME19 F/H_2O	0	0	0	0	2	2	22
ME19 M/H_2O	0	0	0	0	0	3	22
MF1 F/O	0	0	0	0	4	18	22
MF1 M/O	2	0	0	1	2	2	48
MF1 F/H_2O	0	0	0	0	5	4	34
MF1 M/H_2O	0	0	0	0	0	10	25

Table 1.2 Brine shrimp bioassays of crude extracts

Fungal Identification

The identification of the fungi involved in this study was performed by Dr. Gene Ford. ME19 was identified as an *Epicoccum* sp. based on its spore characteristics. However, the identification of MA17 was not so straightforward. By the time Dr. Ford examined the fungus, it had lost its ability to produce spores in culture under the same conditions that it had when originally isolated. His identification was then based on the initial observations of spores produced in early cultures and their documentation by photomicrographs.

Several authorities on mycological taxonomy examined cultures of this isolate. None of them were able to make a positive identification because of the inability to obtain adequate sporulation of the fungus. However, the fungus produces sterile fruiting structures (dark colored pycnidia with curled setae adorning the area around the ostiole) on many media. All the authorities agreed that the fungus belongs to the *Sphaeropsidales*. Brian C. Sutton believed the fungus is possibly a *Phyllosticta*, but without spores to examine, admitted this is an educated guess based only on hyphal and cultural appearance. R. A. Samson (Centraalbureau voor Schimmelcultures) considered it to possibly be a *Diplodia*, even though he did not observe any two-celled spores in culture. Likewise, Jeffery Stone at Oregon State University cultured the fungus and got a microconidial stage (possibly *Leptodothiorella*) and a pycnidial stage which produced dark single-celled spores which he speculated might be an immature

Diplodia stage. However, he did not observe any two-celled spores. A fourth mycologist from the University of Toronto, David Mallock, speculated that the fungus is *Chaetodiplodia* based on the setae found on the pycnidia. However, this ID also requires the spores to be two-celled.

In light of the inability of these experts to agree on an identification and using our own observations on fruiting body and spore formation, Dr. Ford believes this fungus to be a *Sphaeriopsis* sp. The reasons for this are as follows:

1) Only dark colored, single-celled pycnidiospores were observed, which rules out *Diplodia* which has two-celled spores.

2) Spore measurements showed their size to average 27.1 µm X 14.2 µm, which is slightly smaller than that reported for other members of this genus (30-45 µm X 10-22 µm).

3) No microconidial production was ever observed in our cultures.

Moreover, the setae adorning the pycnidia are produced under all cultural conditions (including culture on sterilized *Taxus* leaves), thus providing a constant character to be used in classifying this fungus. The small spore size and slightly coiled setae with their ornamentations could provide justification for the establishment of a new species.

Figure 1.1 shows the photomicrographs of the *Spaeriopsis* sp. From upper left to lower right the photos are as follows:

1) UL: Setae of pycnidium on a yew twig from above (Approximately X75).

2) UR: Pycnidium emerging from a yew twig (X200).

3) CL: Setae and pycnidium on a yew twig from the side (X400).

4) CR: Ornamentations on setae (X3000).

5) LL: Cross-section of seta and ornamentations (X14,500).

6) LR: Pycnidiospores (X170).

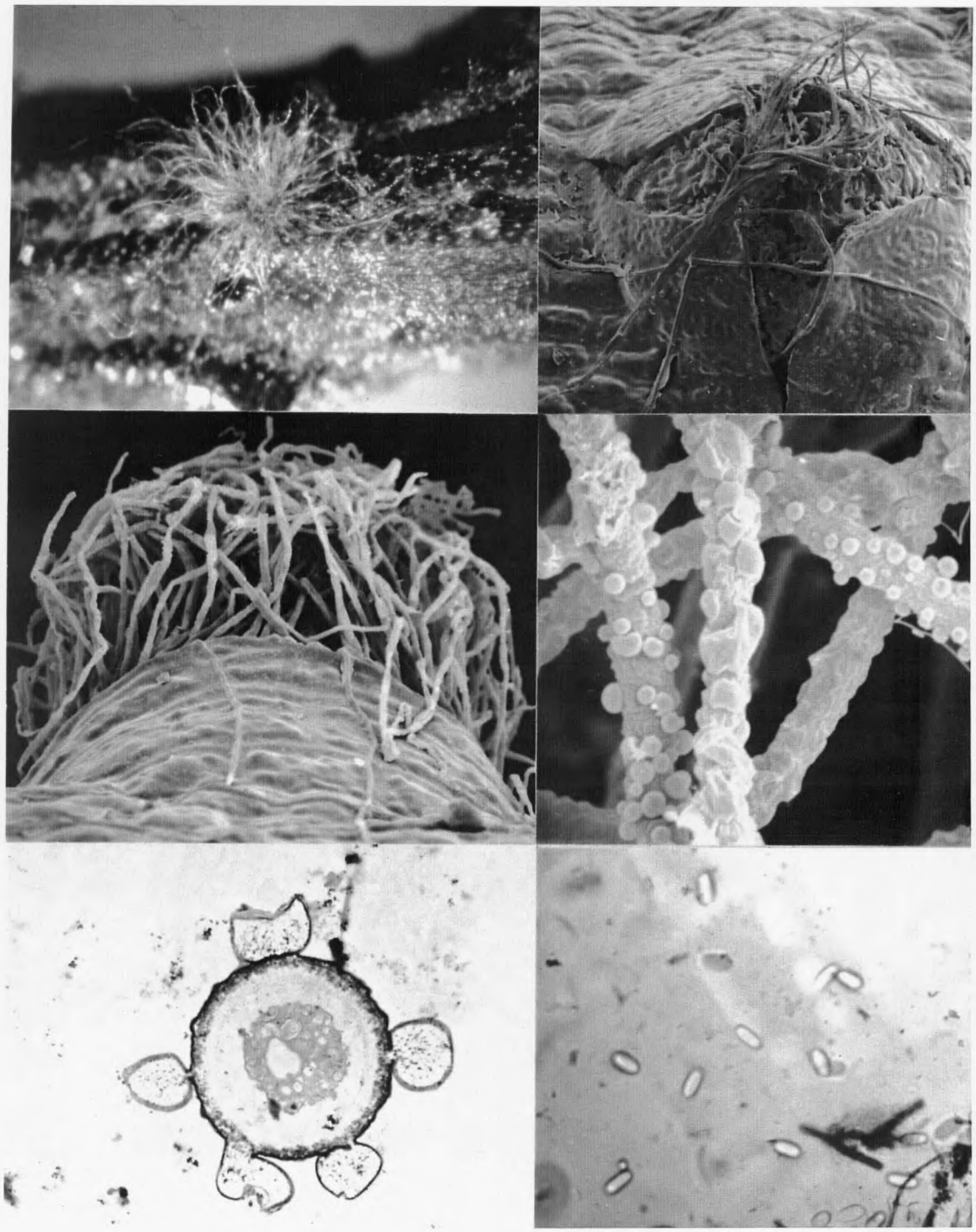


Figure 1.1 Photomicrographs of *Sphaeriopsis* sp.

Chapter 2

GENERAL EXPERIMENTAL METHODS

Throughout the scope of this work, there were many experimental methods which remained constant. This chapter covers the experimental portions for these processes. These are involved throughout the work from the fungal isolation, maintenance, and culturing, to the isolation and structure elucidation of the natural products, and to the determination of the biological activities of the compounds.

Fungal Isolation

The initial fungal isolation was performed by Dr. Bret Niedens. Twig samples of *Taxus globosus*, mexican yew, were surface sterilized with 95% ethanol. After evaporation of the ethanol in a sterile laminar flow hood, the outer bark of the twigs were removed. Small (~1 cm) pieces of inner bark (phloem-cambium and xylem tissues) were removed and placed on water agar plates. After a period of initial growth, hyphal tip transfers of the developing fungi were grown on potato dextrose agar and visually checked for purity.

Fungal Maintenance and Culturing

Microbial samples were maintained on a variety of agars, including Tryptic Soy Agar, Potato Dextrose Agar, M-1-D agar. Agars were prepared from Difco Microbiological agars, or from the corresponding Difco broths and Difco Bacto agar, except M-1-D (shown below). Fungi isolated from yew trees were maintained on the above agars containing 1% yew broth (discussed below).

Fungal culturing was carried out in a variety of synthetic media labled R-1, R-2, medium A, medium B, and medium C. Medium R-1 was the standard medium used in this study and medium R-2 was a high sugar/high nutrient version of medium R-1. Media A, B, and C were a series of trapping media used to limit the amounts of certain nutrients during culturing. The culturing was done in four liter and two liter erlenmeyer flasks, and one liter roux flasks. The volume of medium in each flask was generally chosen to give the largest surface area for growth. Fungi were grown for twenty-one days in still culture, with occasional swirling in some instances.

Yew Broth

Five grams of yew needles and small stems were placed in a beaker with 500 ml of water. The water was boiled for 5 minutes and then allowed to simmer for one hour without heating. The broth was passed through cheesecloth to remove the yew debris and the broth was frozen in ten milliliter portions.

M-1-D Agar

Major salts: $Ca(NO_3)_2$ - (0.28 g/L), KNO_3 - (0.08 g/L), KCl - (0.06 g/L); Minor salts: $FeCl_3 \cdot 6H_2O$ - (2.0 mg/L), $MnSO_4$ - (5.0 mg/L), $ZnSO_4 \cdot 7H_2O$ - (2.5 mg/L), H_3BO_3 - (1.4 mg/L), KI - (0.7 mg/L); $MgSO_4$ anhyd. - (0.36 g/L), $NaH_2PO_4 \cdot H_2O$ - (0.02 g/L), ammonium tartrate - (5.0 g/L), sucrose - (30 g/L), yeast extract (Difco) (0.25 g/L), agar (15 g/L).

R-1 Medium

Bacto soytone (Difco) - (1.0 g/L), sucrose - (10 g/L), 1.0 M $KHPO_4$ buffer (1.0 mL/L).

R-2 Medium

Bacto soytone (Difco) - (5.0 g/L), sucrose - (60 g/L), yeast extract (Difco) - (1.0 g/L), 1.0 M $KHPO_4$ buffer (1.0 mL/L).

Medium A

Bacto soytone (Difco) - (10 g/L), glucose - (40 g/L), $Mg_3(PO_4)_2 \cdot 12H_2O$ - (10 g/L).

Medium B

Bacto soytone (Difco) - (10 g/L), glucose - (40 g/L), $MgCO_3$ - (10 g/L).

Medium C

Bacto soytone (Difco) - (10 g/L), glucose - (40 g/L), $MgSO_4 \cdot 7H_2O$ - (20 g/L).

Extraction and Purifications

All initial mycelia and media extractions were conducted with Fisher Chemical HPLC grade solvents, EM Science HPLC grade solvents or Fisher Chemical ACS grade solvents. Water used for extractions was distilled water from a Wheaton Autostill 1.5 water still.

Low pressure chromatography was performed with various solid supports including Lipophilic Sephadex LH-20 (25-100 µm beads), Toyopearl HW-40F (45 µm beads), BioRad Chelex 100 (50-100 mesh), and J.T. Baker silical gel 40 µm flash chromatography packing. Solvents used were Fisher Chemical HPLC grade solvents and EM Science HPLC grade solvents. Water was distilled with a Wheaton Autostill 1.5 water still. Fractions were collected on a Gilson FC203B fraction collector, and fractions were determined by use of a Gilson 115 variable wavelength UV/VIS detector.

The HPLC system consisted of Waters 510 HPLC pumps, a Waters model 660 Solvent Programmer, a Waters model U6K Injector, and a Waters model 440 Absorbance Detector. Columns used were purchased from Rainin including:

Analytical:

Dynamax 60Å silica column, 4.6 mm ID X 25 cm L, 8 µm packing.

Microsorb silica column, 4.6 mm ID X 25 cm L, 5 µm packing.

Semi-preparative:

Dynamax 60Å silica column, 10 mm ID X 25 cm L, 8 µm packing.

Dynamax 60Å octadecyl column, 10 mm ID X 25 cm L, 8 µm packing.

Preparative:

Dynamax 60Å octadecyl column, 21.4 mm ID X 25 cm L, 8 µm packing.

All columns were protected by Rainin guard columns (5 cm L) of matching diameter and packing material. Solvents used were Fisher Chemical HPLC grade solvents and EM Science HPLC grade solvents. Water was distiilled by a Wheaton Autostill 1.5 water still. All solvents were filtered and degassed with Kontes Brand Ultra-Ware HPLC Resevoir systems through Gelman 5 µm PTFE membrane filters.

Bioassays

In order to establish the presence of bioactive compounds, to follow them through the isolation procedure, and as a final characterization of the purified compounds, bioassay screening methods were used. The screening methods included simple agar-diffusion assays for the antibacterial and antifungal activities, and brine shrimp toxicity bioassays for antitumor bioactivity. Final characterization assays included a paper disc diffusion assay, a mouse thymocyte proliferation assay, an inhibitory concentration against breast adenocarcinoma BT-20 assay, a minimum inhibitory concentration spot test, and a brine shrimp toxicity assay.

Agar-diffusion Assay

In this assay, the fractions to be tested were dissolved in a small amount of solvent and then spotted (10 µL) on an agar medium [Difco Antibiotic Medium 1 (Penassay Seed Agar) for bacteria, and Difco Potato Dextrose Agar for fungi]. The solvent was allowed to evaporate and then the test microbe was inoculated on the plate. The inoculation was accomplished by spraying the plate with an aqueous suspension of the microbe. A positive assay was inhibition of the growth of the microbe in the area where the sample was applied. This method was used to test for antifungal compounds against *Geotrichum candidum* and *Candida albicans* and antibacterial compounds against *Bacillus subtilis*, *Pseudomonas aeruginosa* and *Escherichia coli*.

Brine Shrimp Bioassay

This assay was established as a general bioassay for a broad spectrum of pharmacological activities.[25] It is a convenient tool for isolating antitumor agents. Ferrigni and McLaughlin[26] used this assay to follow activity through fractionations in the isolation of piceatannol, an antileukemic compound, from the seeds of *Euphorbia lagascae*.

In this assay, the sample was dissolved in a small amount of solvent and added (30 µL of methanol) to a test vial of artificial sea water (3.0 ml). Approximately 20 brine shrimp, *Artemia salina*, were added to the vial. The brine shrimp were observed periodically over a twenty-four hour period and the number of dead brine shrimp in each vial were recorded. A positive assay was

the death of all or most of the brine shrimp by the end of the twenty-four hour period.

Paper Disc Diffusion Assay

This assay was a variation of the agar-diffusion assay[27] and was performed in the Stierle's laboratory at Montana Tech in Butte. The difference was the method of application of the sample. A known amount of sample (100 µg, 50µg, or 10 µg) dissolved in solvent was applied to a Difco Bacto Sterile Blank concentration disc, and the solvent was allowed to evaporate. The paper disc wais then placed on the agar and the test microbe was applied. The microbes tested against in this assay were *B. subtillis*, *Staphylococcus aureus*, *E. coli*, *P. aeruginosa*, *Vibrio harveyii*, *C. albicans*, *G. candidum*, *Aspergillus niger*, and *Fusarium oxysporum*.

Mouse Thymocyte Proliferation Assay

The interleukins are a group of glycoproteins which are involved in the regulation of the immune system. Interleukin-1 (IL-1) plays a central role in T-cell activation and has been found to be involved in a number of aspects of inflammation.[28] This assay was used as a general screen for agents that affect IL-1 action.

This assay was performed by Pharmagenesis Pharmaceuticals in Palo Alto, CA and the inhibition of IL-1 mediated thymocyte proliferation was reported.

The procedures followed were the standard, published procedures reported in Laboratory Methods in Immunology.[29]

Minimum Inhibitory Concentration (MIC) Against Breast Adenocarcinoma BT-20

This assay was performed by Cytoclonal Pharmaceuticals, Inc. in Dallas, Texas. The human breast cell cancer lines were exposed to serial dilutions of each derivative. After three days of exposure, the cells were stained with neutral red and the absorbance was measured at 540 nanometers. Samples were dissolved in either methanol or dimethylsulfoxide at negligible levels. The results were reported as inhibitory concentration (IC_{50}) values.

Minimum Inhibitory Concentration (MIC) Spot Test

Extracts were dissolved in methanol or chloroform to final concentrations of 10 µg/µL. Serial dilutions of each sample were made giving final test concentrations of 10, 5 and 2.5 µg/µL. Five microliters of each solution were spotted onto a half-strength YM agar plate (10.5 g/L YM broth (Difco), 15 g/L agar), and allowed to dry. An agar solution, containing *C. albicans,* was prepared to have an $O.D._{590}$ of 0.3. Three millilters of this agar was overlayed on the spotted agar plates and allowed to harden. The plates were incubated for 24 hours at 37 C and observed to determine the MIC as the last dilution where activity was observable.

Organisms for Antibiotic Assays

The microorganisms chosen for the antibiotic assays were chosen to give a broad basis to determine activity. These included the fungi and bacteria (Gram-positive and Gram-negative) indentified below.

Geotrichum Candidum: A member of Fungi Imperfecti that is ubiquitous in soil and dairy products, it is sometimes pathogenic in human respiratory and gastrointestinal tracts.[30]

Candida albicans: A yeast-like fungus, that is found in human and animal digestive tracts which can invade other tissues under certain conditions.[31] Invasive candidiasis is the most common fungal infection in patients with HIV infection, and is associated with increased mortality in bone marrow transplant recipients.[32]

Bacillus subtilis: It is a Gram-positive bacterium that is one of the most common non-pathogenic aerobic spore formers. It is related to *B. anthracis* which is the agent responsible for anthrax in cattle, sheep, and other agriculturally important animals.[33]

Pseudomonas aeruginosa: This is a Gram-negative bacterium and is the only Pseudomonas species that is pathogenic for man and animals.[34] It remains a problem pathogen, becoming resistant to virtually all the clinical antibacterial agents, and is a major challenge in the field of antibiotics.[35]

Escherichia coli: *E. coli*, a Gram-negative bacterium, is the predominant organism in the intestinal canal of man and animals. It can become pathogenic

and may invade the appendix, gall-bladder, peritoneal cavity, kidneys, and the urinary bladder.[36]

Staphylococcus aureus: A Gram-positive, pathogenic, bacterium which man and animals live with from birth until death, it only becomes infectious when their susceptibility is appreciably affected.[37] Its resistance has increased recently and currently vancomycin is the only clinical antibiotic that provides reliable activity against multiply resistant strains.[38]

Vibrio harveyii: This is a Gram-negative marine bacterium which is the primary colonizer in marine fouling. It is related to disease causing Vibrio including *V. choleræ* responsible for cholera.[39]

Aspergillus niger: Aspergillus is a pathogenic filamentous fungus which has a propensity for invasion of blood vessels.[40]

Fusarium oxysporum: The Fusarium are members of the Fungi Imperfecti which include saprophytes and many plant parasites.[41] It is emerging as a human pathogen in immunocompromised patients and often does not repsond to conventional doses of amphotericin B.[42]

Instrumentation for Structure Elucidation

Ultraviolet/Visible Spectrometer: Beckman DU-50 UV/VIS Spectrophotometer.

Polarimeter: Optical rotations were collected on a Perkin-Elmer model 241 MC polarimeter.

Infrared Spectrometer: IR spectra were collected on a Perkin-Elmer model 1600 FTIR.

Mass Spectrometry: Mass spectra were recorded on a VG 10E-HF Mass Spectrometer or a TRIO-2 Electrospray Mass Spectrometer.

TMS derivatives were prepared by reacting the sample with BSA [*N,O*-Bis(trimethylsilyl)acetamide].

Nuclear Magnetic Resonance: NMR spectra were recorded on a Bruker AC300 spectrometer, a Bruker DPX300 spectrometer, a Bruker DRX250 spectrometer, or a Bruker DRX500 spectrometer. Carbon assignments (methyl, methylene, methine, quaternary) were determined via DEPT 90 and DEPT 135 experiments.

Chapter 3

SPHAERIC ACID

One of the most interesting activities from the preliminary bioassays of the crude extracts was the strong activity of the MA17 F/O fraction in the brine shrimp toxicity bioassay (Table 1.2). Activity-guided fractionation of this extract led to the isolation of a novel compound, sphaeric acid (Figure 3.1).

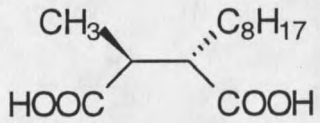
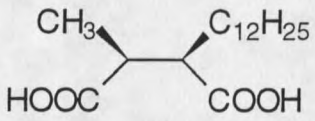


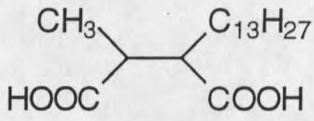
Figure 3.1 Sphaeric acid
(Absolute Stereochemistry unknown)

Sphaeric acid is a member of a class of alkylated succinic acid derivatives, many of which contain more oxidized functional groups than just the two carboxylic acids. Most of these succinic acid derivatives were isolated in non-bioassay guided searches for new compounds. However, there were a series of mono and dialkyl succinic acids sythesized in a search for antitubercular compounds.[43,44]

Roccellic acid and pedicellic acid (Figure 3.2) are the members of this class most similar to sphaeric acid, being 2-alkyl-3-methyl succinic acids. In addition



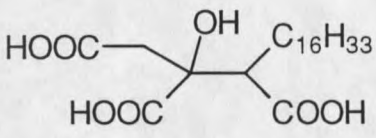
roccellic acid



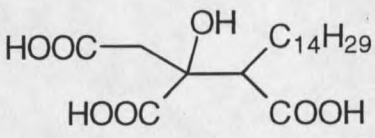
pedicellic acid

Figure 3.2 Roccellic and pedicellic acids

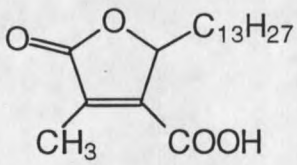
to having a longer alkyl chain, the two alkyl groups are cis in roccellic acid[45] when written as a succinic acid derivative, but in a trans configuration in sphaeric acid. Pedicellic acid is the only example of this skeleton found in higher plants[46], while roccellic acid and the other more oxidized products[47] (Figure 3.3) have been isolated from lichens.



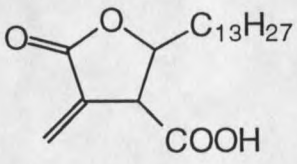
agaricic acid



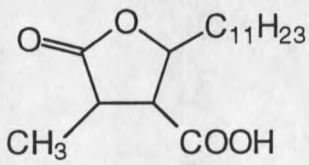
caperatic acid



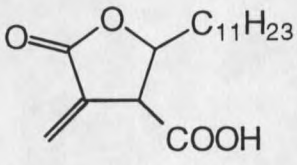
lichesteric acid



protolichesteric acid



nephrosteranic acid



nephrosterinic acid

Figure 3.3 Succinic acid based natural products

Sphaeric acid was isolated because of its activity in the brine shrimp toxicity assay. There was some concern that the brine shrimp toxicity may have been due to the acidity of sphaeric acid. However, the concentrations tested only lowered the pH to approximately 5.6, while a pH as low as 4.0 seemed to show no toxicity to the brine shrimp over the twenty-four hours of testing. The purified diacid was also tested for other activities and gave a positive result in a mouse thymocyte proliferation assay without being cytotoxic at the concentrations tested. This is a general screening assay for agents that affect interleukin-1 (IL-1) action, which plays a central role in T-cell activation of the immune system.[48]

Isolation and Structure Elucidation of Sphaeric Acid

The *Sphaeriopsis* sp. isolated from the inner bark of the mexican yew tree, *Taxus globosa*, was grown in still culture in R-1 media for 21 days. The mycelia were removed by filtration through eight layers of cheesecloth and the filtrate was extracted with methylene chloride. The extract was chromatographed on Sephadex LH-20 and eluted with 1:1 $CHCl_3$:MeOH. The active fractions were subjected to reverse-phase HPLC and eluted with a linear gradient from 70% methanol in water to 100% methanol. The active fraction was submitted to a final separation on a Toyopearl TSK gel and eluted with 2:1 MeOH:H_2O, and pure sphaeric acid was collected.

The high resolution CIMS gave a molecular formula of $C_{19}H_{41}O_4Si_2$ for the protonated TMS derivative of sphaeric acid. Replacing the two TMS groups (C_3H_9Si) with protons gave a molecular formula of $C_{13}H_{24}O_4$. This molecular formula allowed for two sites of unsaturation. The IR signal at 1707 cm^{-1} and the carbon NMR signals at 182.3 and 182.1 ppm suggested the presence of

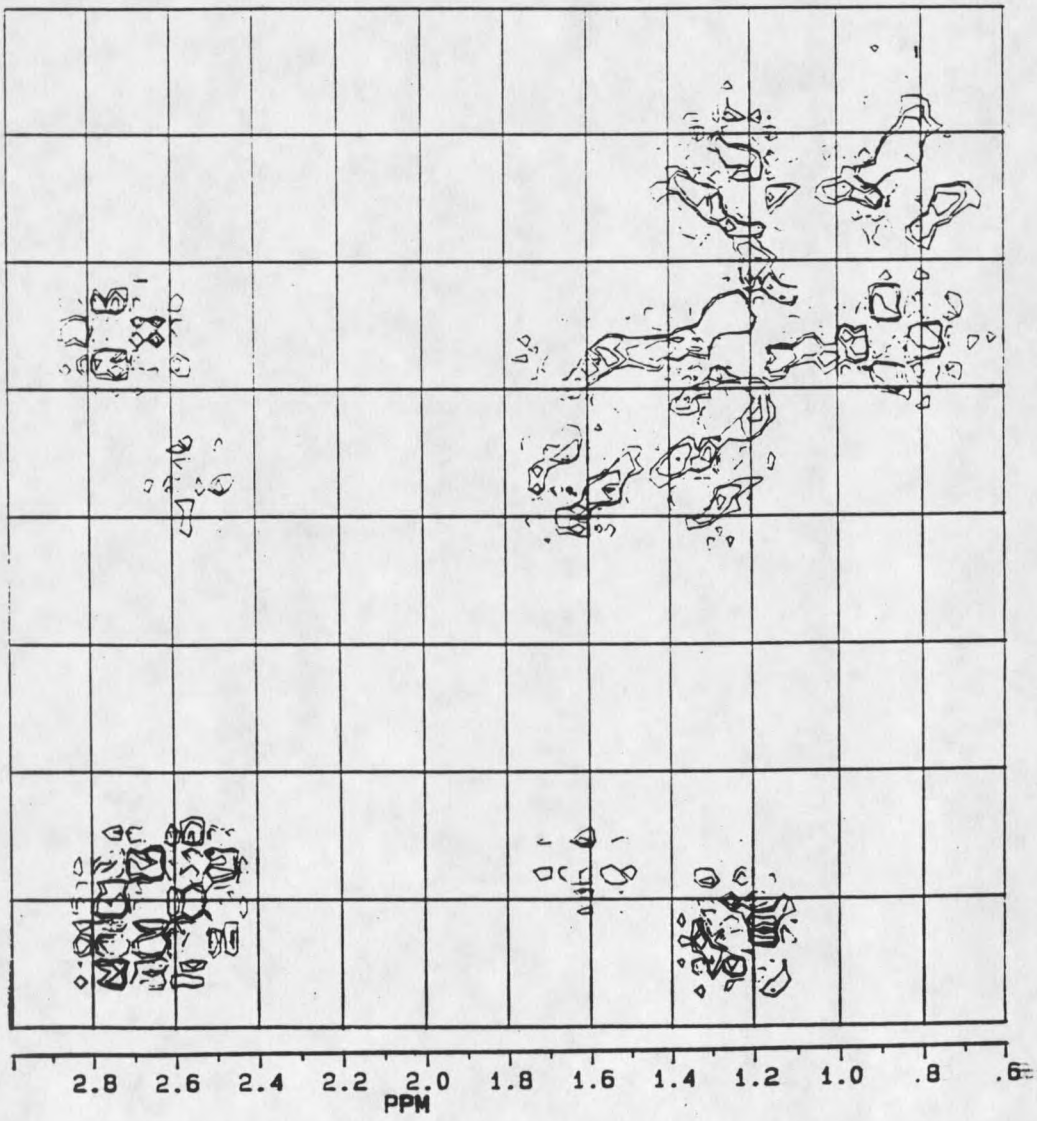


Figure 3.4 COSY of sphaeric acid

two carboxylic acid groups. The two D_2O exchangeable protons at 11.5 ppm in the proton NMR supported the presence of two carboxylic acid groups. This accounted for the two sites of unsaturation, so the rest of the molecule must be saturated.

The carbon NMR data indicated that there were two methyl groups and two methines in the molecule, and the remainder of the signals were methylenes. This, as well as the large proton signal at 1.2 ppm, suggested the presence of a long alkyl chain. The triplet at 0.85 ppm, which integrated to three protons, was typical for a terminal methyl of an alkyl chain.[49] The methine protons were the lowest field protons which each integrated to a single proton. The additional methyl group was included in the signal at 1.2 ppm which accounted for its integration to an odd number of protons (15).

The COSY spectrum of sphaeric acid (Figure 3.4) shows that the two methines at 2.7 ppm and 2.5 ppm coupled to each other. Additionally, the methine at 2.5 ppm coupled to the methylene protons at 1.6 ppm, and the methine at 2.7 ppm coupled to the signal at 1.2 ppm. Since the methine at 2.7 ppm is a doublet of quartets, it must be coupled to the methyl group within the 1.2 ppm signal as well as the other methine. The methine at 2.5 ppm being a doublet of triplets also supported it being coupled to the methylene at 1.6 ppm and the other methine. This gave the basic structure shown in Figure 3.5.

The remaining two sites on the methines must then be the two carboxylic acid groups. This was supported by the absence of other proton signals downfield

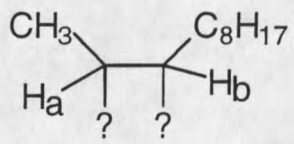


Figure 3.5 Partial Structure of Sphaeric Acid

enough to be adjacent to the carboxylic acid groups, which typically come lower than 2.0 ppm.[50] This gave the final structure for sphaeric acid without stereochemistry as shown in Figure 3.6.

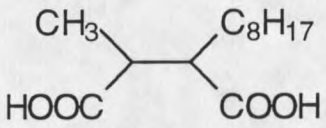
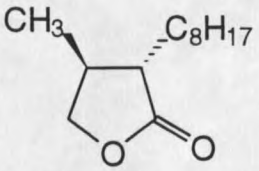
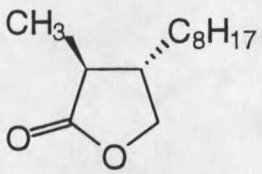


Figure 3.6 Sphaeric Acid (No Stereochemistry)

The relative stereochemistry of the two chiral carbons is based on a comparison to the pair of synthetic lactone derivatives shown in Figure 3.7 (structure elucidation below). Sphaeric acid was reduced with lithium aluminum



Lactone A

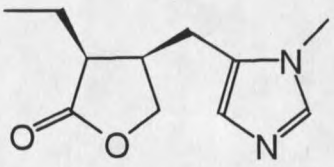


Lactone B

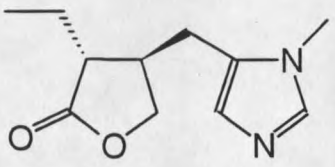
Figure 3.7 Lactone derivatives of sphaeric acid
(Absolute stereochemistry unknown)

hydride to produce the dialcohol. Upon oxidation of the dialcohol with pyridinium chlorochromate (PCC), the lactones A and B were recovered instead of the expected dialdehyde.

The relative stereochemistry was assigned by comparison of the lactones to the known compounds (+)-pilocarpine and (+)-isopilocarpine (Figure 3.8). The chemical shifts of the methylene protons adjacent to the oxygen in the lactone ring were particularly diagnostic. The two isomers, pilocarpine and isopilocarpine, have different chemical shifts for the two protons of the methylene groups (4.05 and 4.15 for pilocarpine and 3.89 and 4.39 for isopilocarpine[51]). The trans stereochemistry was chosen because of the similarity of the methylene protons of the synthesized lactones (4.4/4.3 and 3.8/3.7) to the isopilocarpine signals giving the relative stereochemistry shown in Figure 3.1.



(+)-pilocarpine



(+)-isopilocarpine

Figure 3.8 Pilocarpine and isopilocarpine

Lactone Structure Elucidation

The high resolution GC/EIMS was able to resolve the two lactones, A and B, both of which gave a molecular formula of $C_{13}H_{24}O_2$. The molecular formula

allowed for two sites of unsaturation, but an inspection of the proton NMR shows that the product of the oxidation was not the expected dialdehyde. The two groups of proton signals at 4.3-4.4 ppm and 3.7-3.8 ppm were too low to be the methine protons of the expected dialdehyde (figure 3.9). Additionally, there was no evidence of the aldehyde protons around 9.5-10.0 ppm.[52]

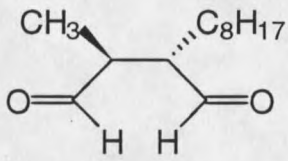


Figure 3.9 Expected dialdehyde of sphaeric acid

The carbon NMR signals at 180.2 and 179.8 ppm suggested the presence of an acid or ester functional group. The ester functionality was also supported by the presence of the oxygen bearing methylenes at 73.0 and 72.1 ppm. Oxidation of one of the hydroxyls followed by cyclization led to the two γ-lactones. The IR peak at 1777 cm^{-1} was supportive of this since the carbonyl of γ-valerolactone[53] (Figure 3.10) absorbs at 1770 cm^{-1}.

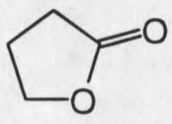


Figure 3.10 γ-Valerolactone

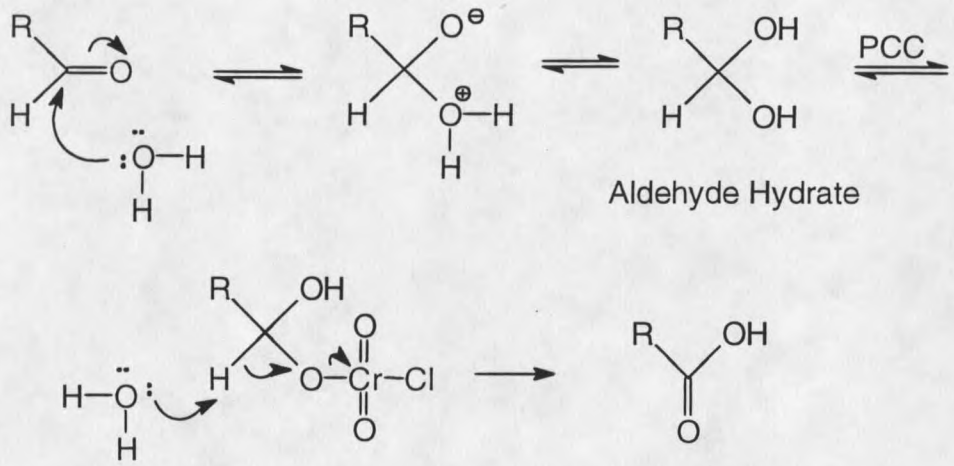


Figure 3.11 Aqueous chromate oxidation to a carboxylic acid

The synthesis of the lactones instead of the dialdehyde can be explained through the formation of an intermediate similar to the aldehyde hydrate formed in an aqueous chromate oxidation of a primary alcohol to a carboxylic acid[54] (Figure 3.11). Instead of attack by the water molecule which leads to the

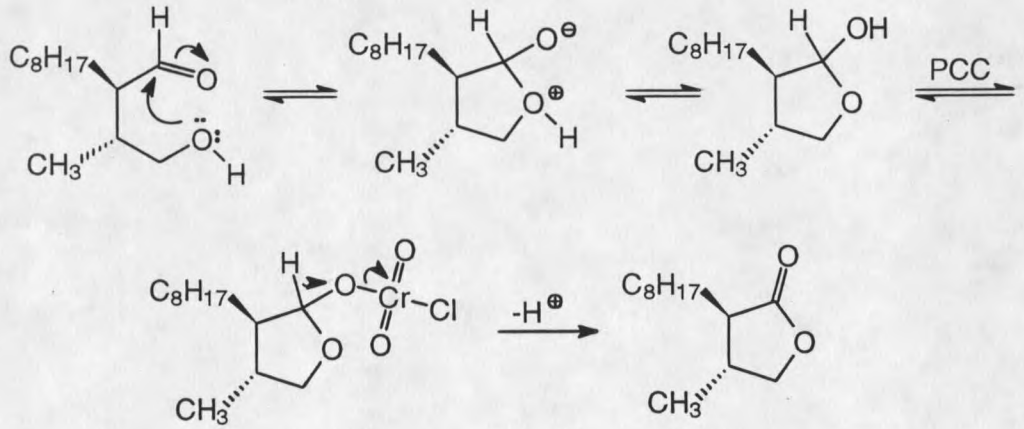


Figure 3.12 Formation of lactones

formation of a carboxylic acid, the neighboring hydroxyl group attacks leading to the formation of the lactone (Figure 3.12).

Mass Spectral and NMR Assignments of Lactones

The two lactones had different retention times in the GC/MS allowing for the identification of each one. As can be seen in Figures 3.16 and 3.17, the two isomers fragment differently. The first one to elute, lactone A, had a base peak of 100, with large peaks at 85 and 113. Lactone B, on the other hand, had a base peak of 55, with large peaks at 70 and 99. The identification of the lactones was based on the base peak of lactone A having a mass of 100. This mass is produced via a γ-H rearrangement to a carbonyl with β-cleavage, or McLafferty rearrangement, shown in Figure 3.13. This is a common rearrangement for carbonyl compounds with a γ proton that goes through a sterically favorable six-membered ring transition state.[55] Lactone B, with the

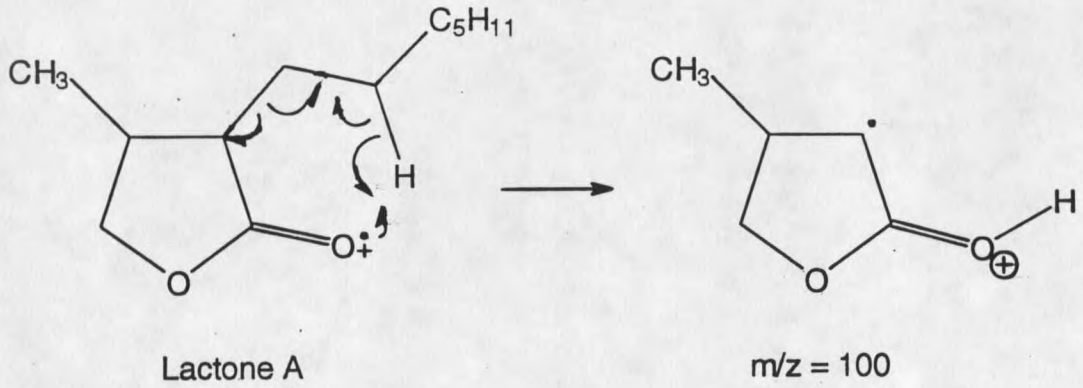


Figure 3.13 Fragmentation of Lactone A

carbonyl on the other side of the ring, cannot undergo this rearrangement, and must fragment by other methods, leading to the smaller mass fragments.

The proton assignments of the protons on the lactone ring can be made using the COSY of the lactones (Figure 3.14). The proton at 2.3 ppm correlated to the methyl signal at 1.1 ppm, as well as the 4.2 ppm/3.7 ppm pair for the methylene adjacent to the oxygen of the ester. This combination can only occur with lactone A. This proton also coupled to the proton at 2.1 ppm. On

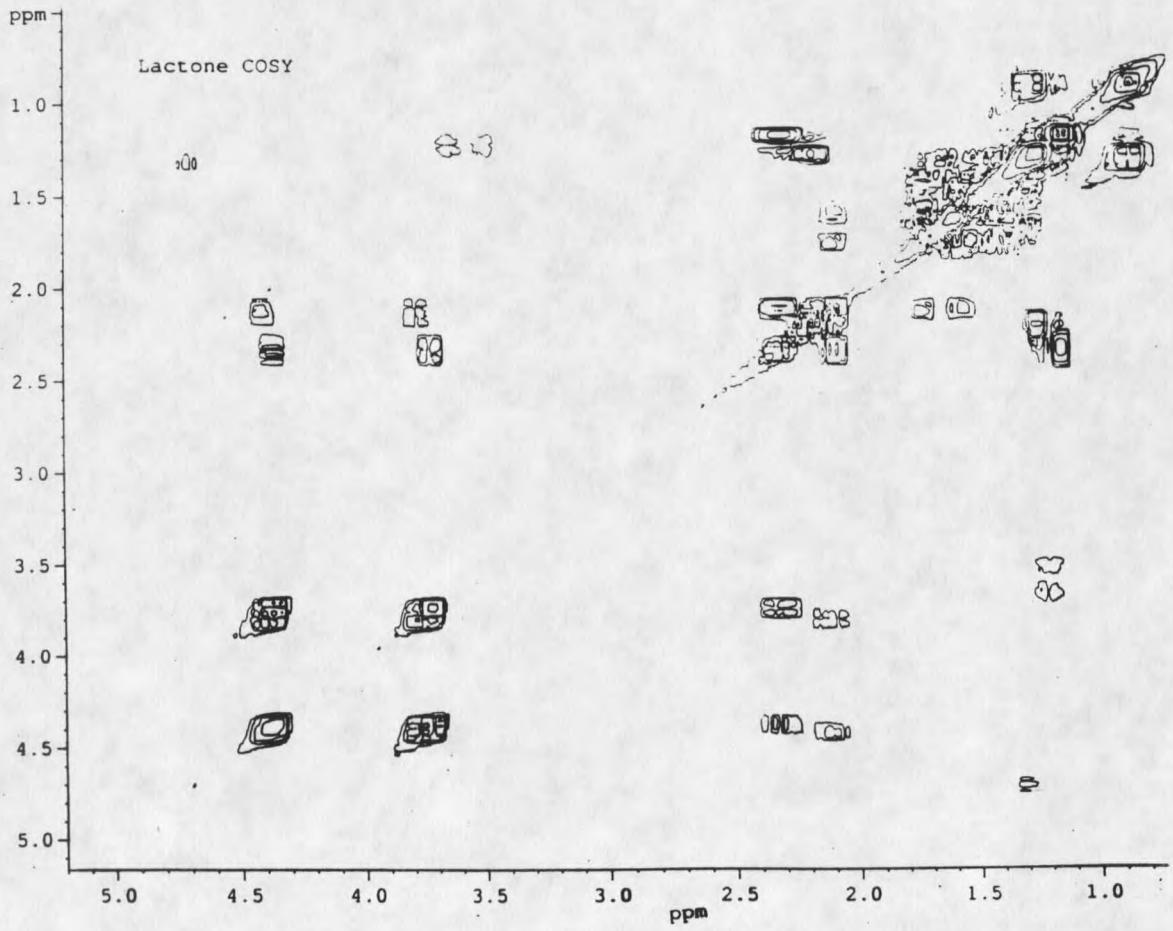


Figure 3.14 COSY of lactones

the other hand, the methyl signal of lactone B at 1.2 ppm coupled to the methine signal at 2.2 ppm. The methylene protons at 4.4 ppm and 3.8 ppm correlated to the methine signal at 2.1 ppm which also coupled to the other methine. This gave the resulting assignments shown in Figure 3.15. This allowed for the determination of the relative amounts of the lactones produced. Using the integration figures of the methyene protons at 3.8 ppm and 3.7 ppm, it appeared that the ratio was approximately 55:45 for the two lactones (A:B).

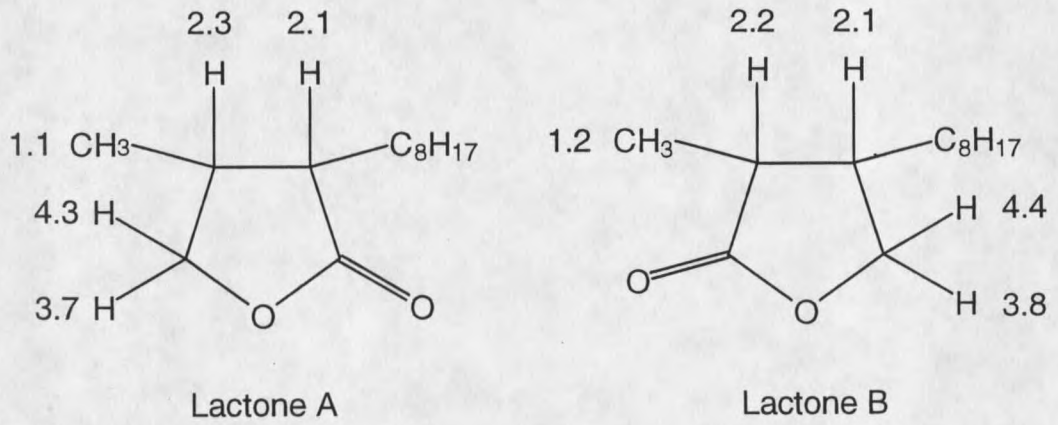


Figure 3.15 Proton assignments of lactones

Experimental

Culture Conditions

The *Sphaeriopsis* sp., labled as MA-17, was maintained on M-1-D agar containing 1% yew broth. MA-17 was grown in still culture in two liter erlenmeyer flasks and 1000 ml roux flasks in R-1 media containing 1% yew

broth for 21 days. Since MA-17 grew on the surface of the culture liquid the amount of liguid used in each flask was chosen to give the largest surface area for growth.

Extraction and Isolation

The fungal mycelia were removed by filtration through eight layers of cheesecloth, and the filtrate was extracted once with 400 mL of methylene chloride. The filtrate was acidified to pH = 1 and extracted with two additional 400 mL portions of methylene chloride. The extracts were combined and evaporated to dryness on a rotoevaporator (23.5 mg/L). The extracts were chromatographed on a Sephadex LH-20 column (2.8 cm X 110 cm), and eluted with 1:1 $CHCl_3$:MeOH. Nine total fractions were collected and isolation of the active compound was carried out by activity-guided fractionation using a brine shrimp toxicity bioassay. Fractions 4 and 5 (19.5 mg), were then chromatographed by reverse phase HPLC on a preparative Rainin Dynamax 60-Å C18 column by gradient elution using a linear gradient from 70% methanol in water to 100% methanol, followed by a methylene chloride wash. Four fractions were collected, with fraction 3 (11.6 mg) exhibiting activity. This fraction was submitted to a final separation on a Toyopearl TSK gel HW-40F column (2.8 cm X 40 cm) and eluted with 2:1 MeOH:H_2O. Sixteen fractions were collected, with fraction 2 giving pure sphaeric acid (7.1 mg).

Reduction to Diol

Sphaeric acid (11 mg, 0.046 mmole) in anhydrous ether (0.5 ml) was added dropwise through the condenser to a solution of lithium aluminum hydride (15 mg, 0.39 mmole) in anhydrous ether (0.5 ml). The solution was refluxed for one hour and cooled. The excess hydride was decomposed by adding 1 ml "wet" ether and refluxing for an additional 15 min. "Wet" ether was prepared by mixing equal parts of ether and water and retaining the upper organic layer. Sulfuric acid (~10 % aq, 4 mL) was added to dissolve the aluminum complex, and the aqueous solution was extracted with three 5 ml portions of chloroform. The combined extracts were dried over anhydrous magnesium sulfate and evaporated to give pure diol (10 mg, 0.046 mmole).

Oxidation to Lactones

Diol (10 mg, 0.046 mmole) in methylene chloride (1.0 ml) was added to pyridinium chlorochromate (22 mg, 0.10 mmole) in methylene chloride (1.0 ml) and the solution stirred for 3 hours. The chromium reagent was removed on a flash silica column (1 cm X 2 cm) and eluted with methylene chloride. The lactones (5 mg, 0.024 mmole) was recovered by overnight evaporation of the methylene chloride.

Physical/Chemical Data for Sphaeric Acid

Light yellow oil; $[\alpha]^{25}D$ +7 (23 mg/ml, CH_3OH); UV (CH_3CN) λ_{max} (ε) 205 (159); IR υ_{max} (cm^{-1}) (neat) 2919 br, 1707, 1460, 1413, 1278, 1225, 931, 720; HRCIMS

m/z [TMS der + H]$^+$ 389.2494, calculated 389.2543 for $C_{19}H_{41}O_4Si_2$; EIMS m/z 181(6), 154(32), 142(18), 111(20), 97(39), 83(21), 69(47), 55(100); ^{1}H NMR δ ($CDCl_3$) 11.5 (2H, br, D_2O exchange), 2.7 (1H, dq, J=9.0, 7.1Hz), 2.5 (1H, ddd, J=9.0, 9.0, 4.7 Hz), 1.6 (2H, m), 1.2 (15 H, br s), 0.85 (3H, t, J=6.5 Hz); ^{13}C NMR δ $CDCl_3$ 182.3 (C), 182.1 (C), 47.8 (CH), 40.9 (CH), 31.8 (CH_2), 29.5 (CH_2), 29.3 (CH_2), 29.2 (CH_2), 29.1 (CH_2), 26.6 (CH_2), 22.6 (CH_2), 15.1 (CH_3), 14.1 (CH_3).

Physical/Chemical Data for Diol

Colorless oil; UV (MeOH) λ_{max} (ε) 210 (290); IR υ_{max} (cm^{-1}) (neat) 3328 br, 2922, 2854, 1715, 1456, 1378, 1035, 757, 721; HRCIMS m/z [M + H]$^+$ 217.2171 calculated 217.2168 for $C_{13}H_{29}O_2$; EIMS m/z 168(20), 154(02), 140(05), 125(08), 111(19), 97(37), 83(47), 69(88), 55(100); ^{1}H NMR δ ($CDCl_3$) 3.7 (2H, m), 3.5 (2H, m), 2.5 (2H, br, D_2O exchange), 1.7 (1H, m), 1.4 (1H, m), 1.2 (14H, br), 0.9 (3H, d, J=7.2 Hz), 0.85 (3H, t, J=6.6 Hz); ^{13}C NMR δ ($CDCl_3$) 65.2 (CH_2), 62.6 (CH_2), 44.1 (CH), 37.5 (CH), 32.3 (CH_2), 30.4 (CH_2), 30.0 (CH_2), 29.7 (CH_2), 29.5 (CH_2), 28.1 (CH_2), 23.1 (CH_2), 15.5 (CH_3), 14.5 (CH_3).

Physical/Chemical Data for Lactones

Light yellow oil; UV (MeOH) λ_{max} (ε) 212 (430); IR υ_{max} (cm^{-1}) (neat) 2923, 2854, 1777, 1456;HREIMS m/z **A**: M$^+$ 212.1775 calculated 212.1776 for $C_{13}H_{24}O_2$; **B**: M$^+$ 212.1770 calculated 212.1776 for $C_{13}H_{24}O_2$; EIMS m/z **A**: 212(02), 197(06), 113(53), 100(100), 85(82), 69(16), 55(33); **B**: 212(01),

181(01), 99(54), 83(32), 70(89), 55(100); ^{1}H NMR δ ($CDCl_3$, 500MHz) 4.4 (0.45H, dd, J=8.7, 7.7 Hz, **B**), 4.3 (0.55H, dd, J=8.5, 8.0 Hz, **A**), 3.8 (0.45H, dd, J=9.2, 8.7 Hz **B**), 3.7 (0.55H, dd, J=8.9, 8.5 Hz, **A**), 2.3 (0.55H, m, **A**), 2.2 (0.45H, m, **B**), 2.1 (1H, m), 1.7 (1H, m), 1.6 (2H, m), 1.5 (1H, m), 1.3 (10H, br), 1.2 (1.5H, d, J=6.5 Hz, **B**), 1.1 (1.5H, d, J=6.6 Hz, **A**), 0.9 (3H, t, J=6.3 Hz); ^{13}C NMR δ ($CDCl_3$,) 180.2 (C), 179.8 (C), 73.0 (CH_2), 72.1 (CH_2), 47.1 (CH), 44.2 (CH), 41.0 (CH), 36.5 (CH), 32.6 (CH_2), 32.2 (CH_2), 30.1 (CH_2), 30.0 (CH_2), 29.8 (CH_2), 29.6 (CH_2), 29.5 (CH_2), 29.4 (CH_2), 27.6 (CH_2), 27.2 (CH_2), 23.0 (CH_2), 17.3 (CH_3), 14.4 (CH_3).

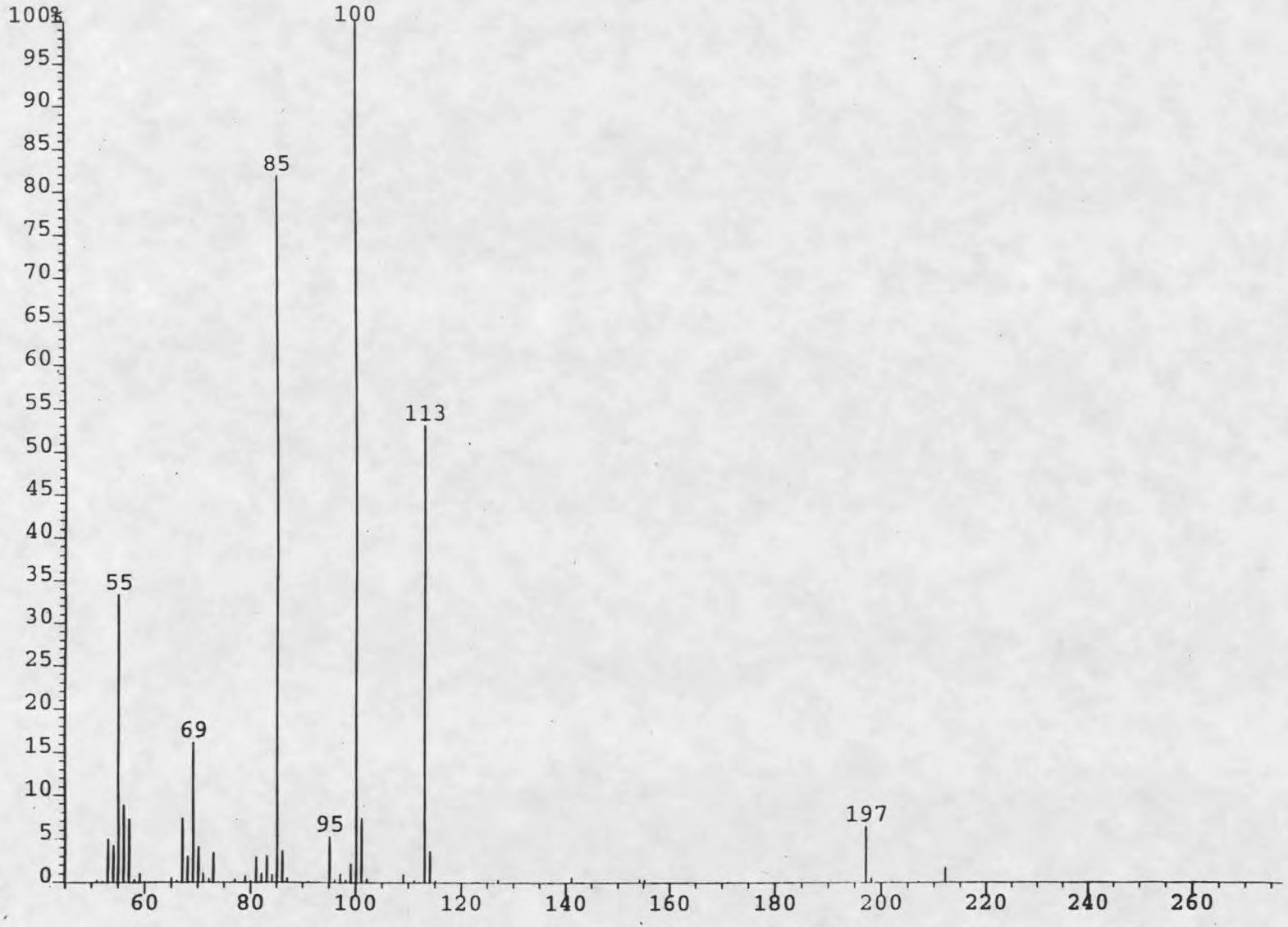


Figure 3.16 EIMS of lactone A

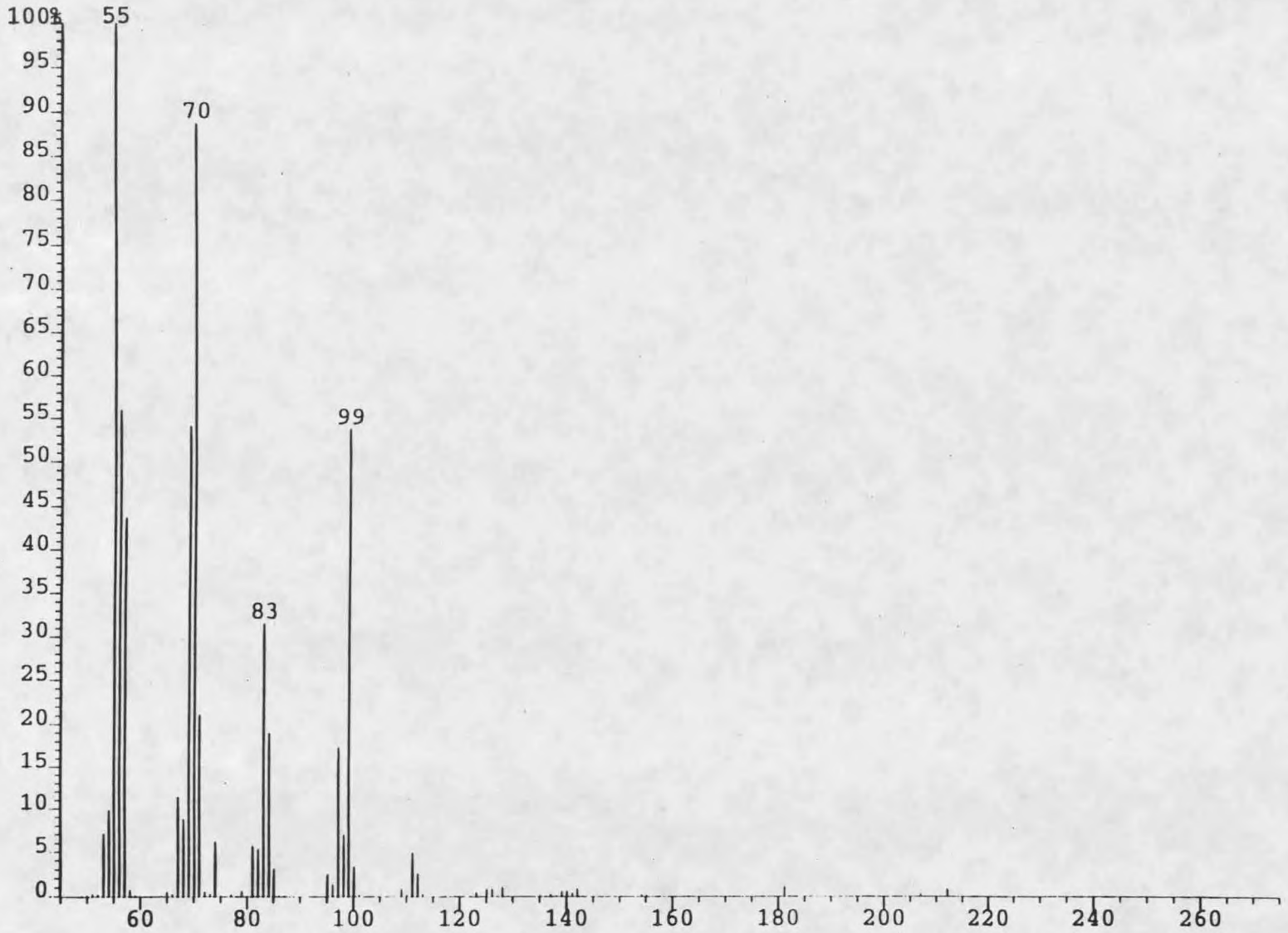


Figure 3.17 EIMS of lactone B

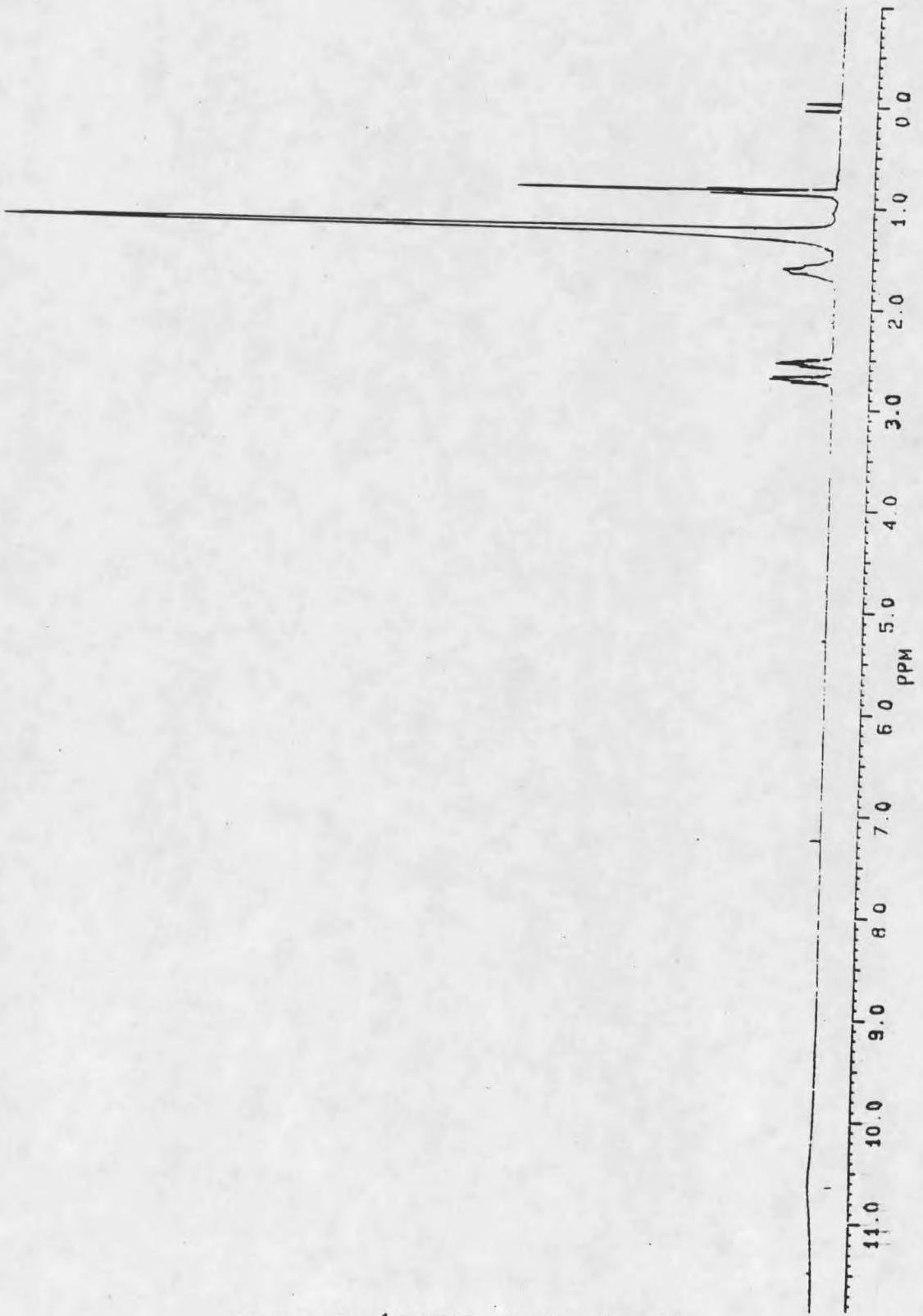


Figure 3.18 ^{1}H NMR of sphaeric acid

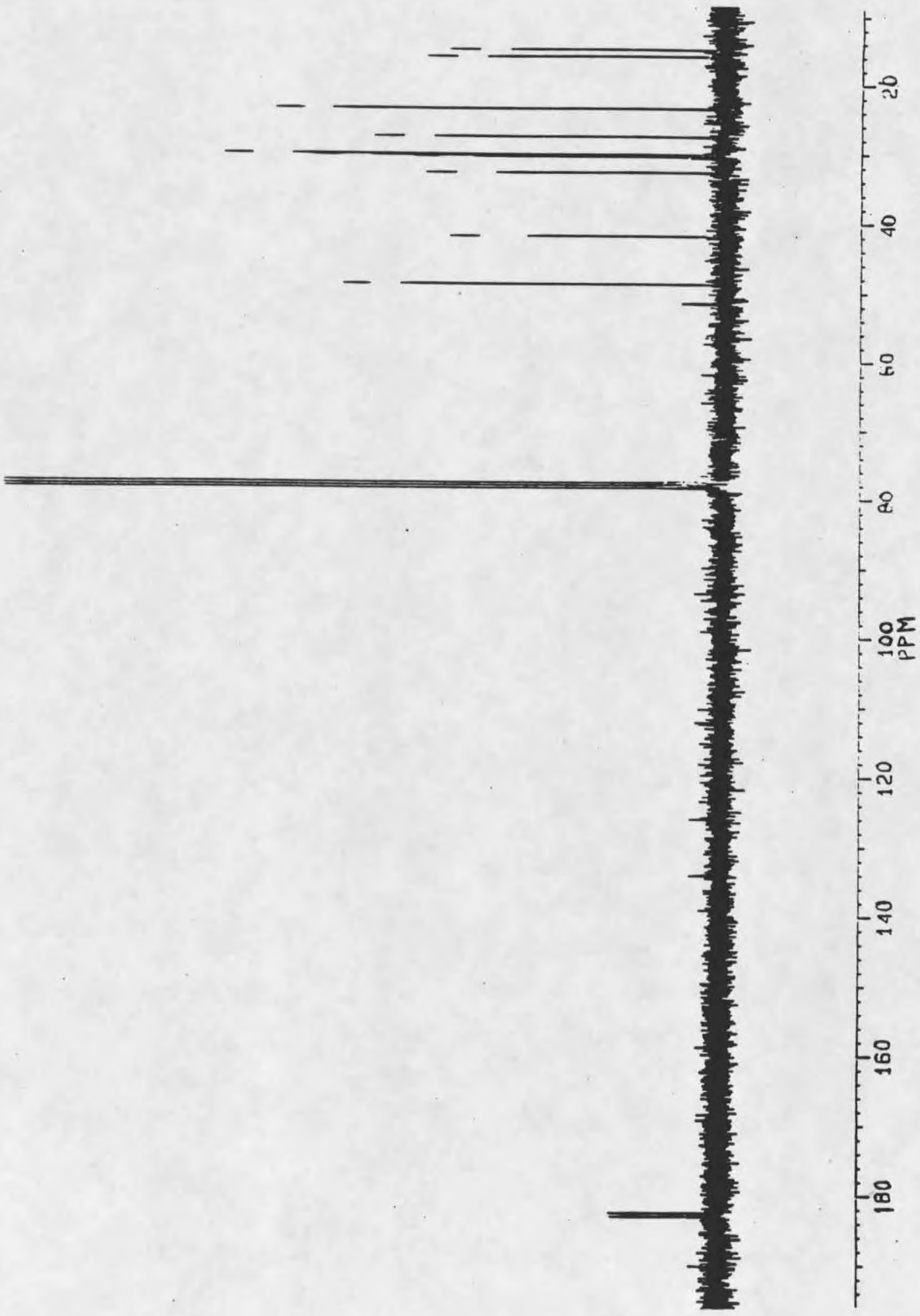


Figure 3.19 ^{13}C NMR of sphaeric acid

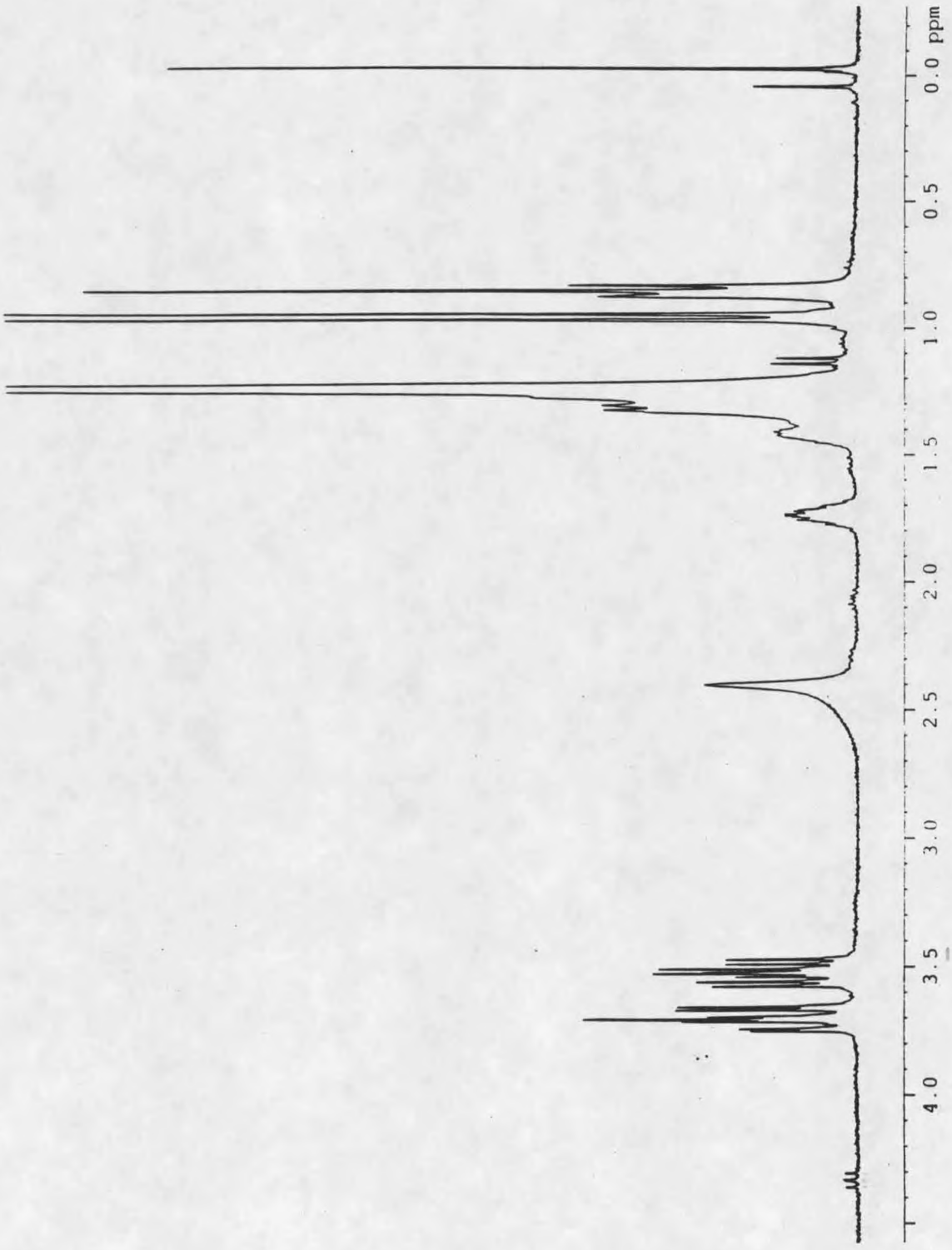


Figure 3.20 ^{1}H NMR of diol

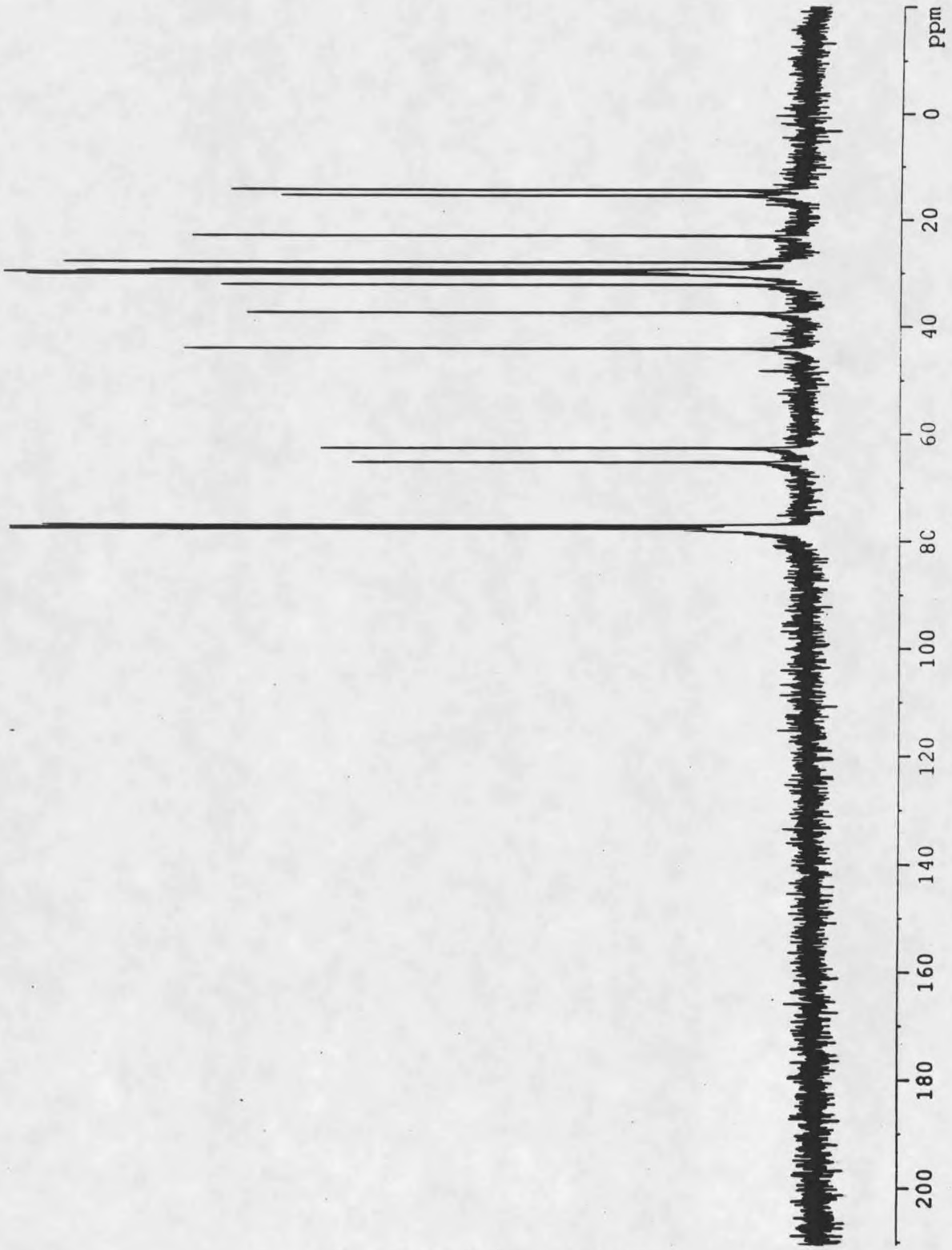


Figure 3.21 ^{13}C NMR of diol

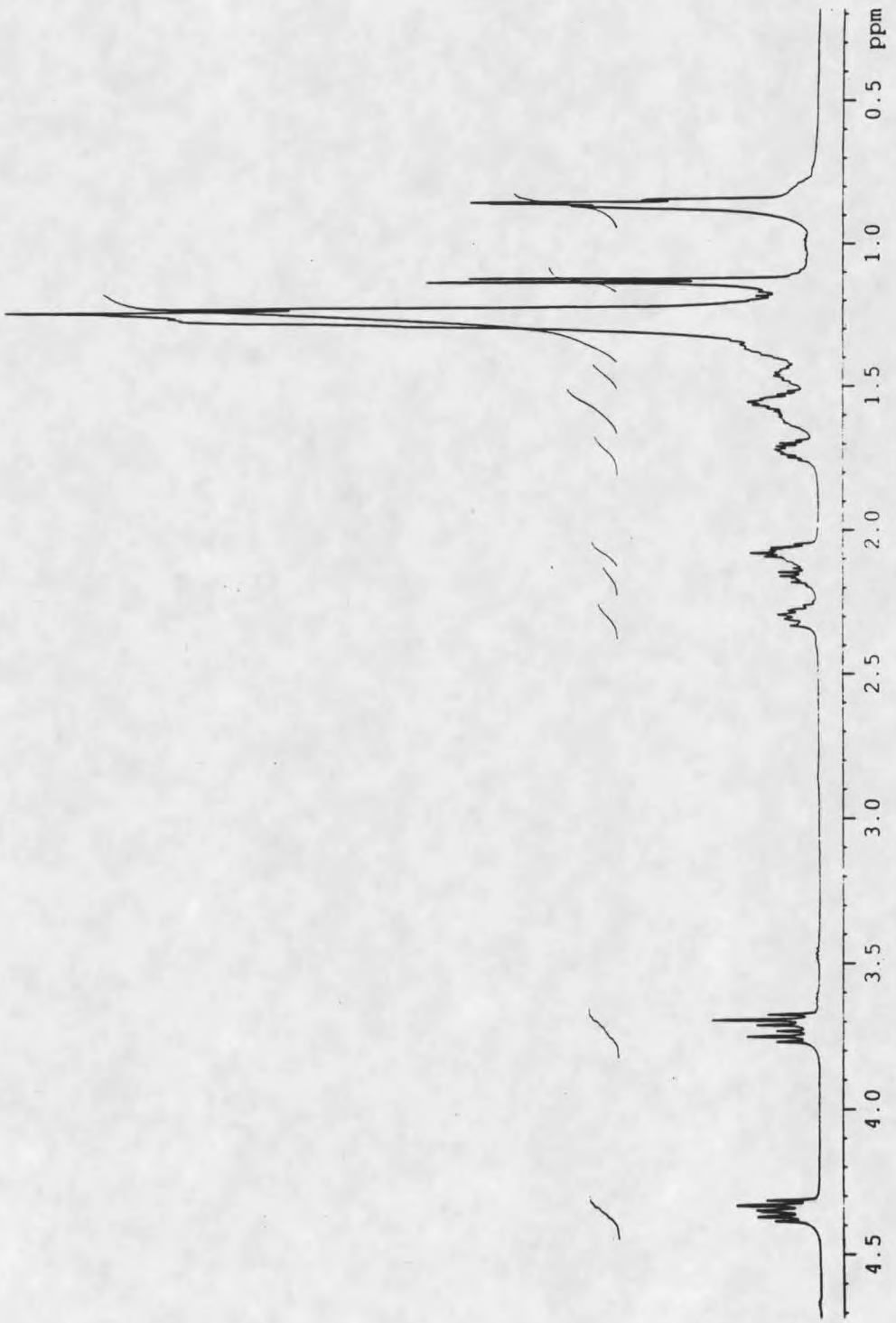


Figure 3.22 ^{1}H NMR of lactones

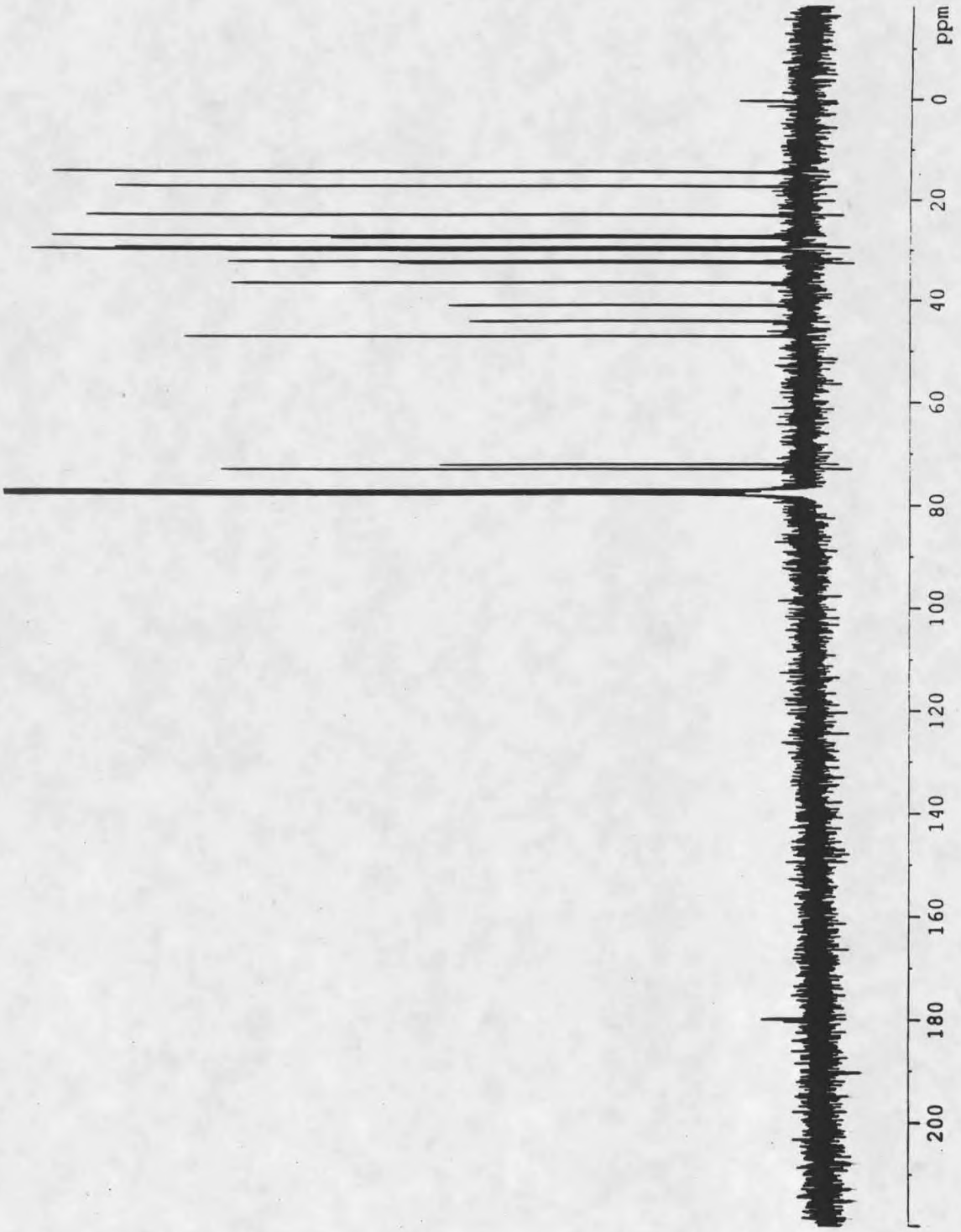
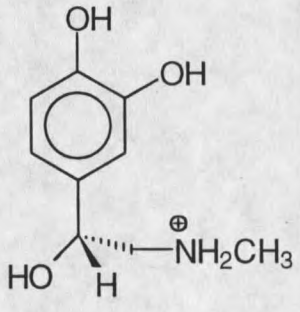


Figure 3.23 ^{13}C NMR of lactones

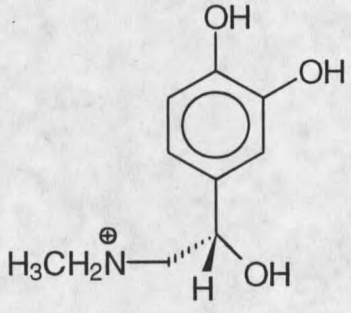
Chapter 4

DERIVATIVES OF SPHAERIC ACID

Many times a change in the structure of a molecule can have a great effect on its activity. This can be as subtle as a change in the configuration of a chiral center. For example, (R)-(-)-epinephrine is an active adrenal hormone, while (S)-(+)-epinephrine (Figure 4.1) does not correctly fit into the receptor enzyme's active site.[56] Other structural changes are also employed to effect the biological activity of compounds. For example, in studying the structure/activity relationship of 4-phenylethylaminoquinoline (Figure 4.2), Dreikorn et. al. synthesized analogues with structural modifications to study six areas:[57]



(R)-(-)-epinephrine



(S)-(+)-epinephrine

Figure 4.1 R and S Epinephrine

1) Positioning on the quinoline for the phenylethylamino group.
2) Varying the length of the alkyl (ethyl) chain.
3) Substitutions on the quinoline rings.
4) Substitutions on or replacement of the phenyl ring.
5) Substitution on the chain nitrogen or the alkyl chain.
6) Replacement of the chain nitrogen with other atoms.

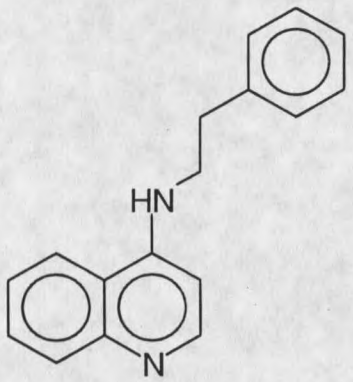


Figure 4.2 4-Phenylethylaminoquinoline

Included in the six categories of modifications were several general alterations. The electronic environment of the aromatic rings were altered by substitution with electron withdrawing groups (F, Cl, Br, I, CF_3) and electron donating groups (OH, OR, R). Steric factors were also considered in the substitutions of the phenyl ring by using increasingly bulky groups (H, Me, Et, i-Pr, t-Bu). The importance of the nitrogen in the alkyl chain was also investigated by replacing it with sulfur, sulfoxide, oxygen, and carbon. This can affect the electronic

environment of the quinoline ring, but could also indicate any hydrogen bonding requirements for activity.

In a similar manner, an investigation of the structure/activity relationship of sphaeric acid was conducted. In doing this, it was important to note its lipophilic properties. While its activity could be a result of interference in key metabolic pathways or enzyme inhibition, the most likely activity is an effect on cellular membranes. Lipid soluble antibiotics often effect the cellular membrane in one of three ways: those which appear to disorganize the membrane structure, those which inhibit a membrane bound protein, and those which change the permeability of the membrane to specific ions.[58]

In an attempt to study the structure/activity relationship of sphaeric acid, several derivatives were synthesized. An attempt was made to vary the polarity and charge of the polar end of sphaeric acid. This was accomplished by various methods including esterifications, amidifications, a reduction, and a subsequent oxidation. A series of bioassays were then conducted on sphaeric acid and the synthetic derivatives to test for any structure/function relationships.

The Derivatives

Methyl esters

The mono-methyl esters (Figure 4.3) were synthesized by two methods. Sphaeric acid was reacted with thionyl chloride in an attempt to make the diacid chloride. However, since the methyl ester singlets of the resulting products, at

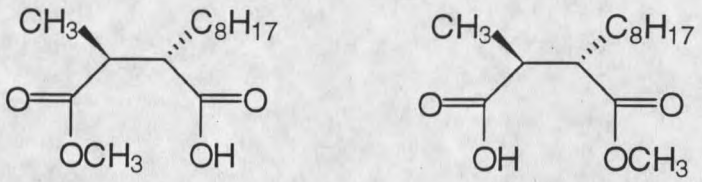


Figure 4.3 Mono-methyl esters of sphaeric acid
(Absolute stereochemistry unknown)

3.65 and 3.66 ppm, only integrated to three protons, it appeared that only the mono-methyl esters were formed. This can be explained by the formation of the five-membered anhydride ring upon reaction with the thionyl chloride instead of the diacid chloride (Figure 4.4). The mono-methyl ester was then formed by nucleophilic attack on one carbonyl by the methanol forming the ester which opened the ring giving the carboxylic acid. Sphaeric acid was also reacted with

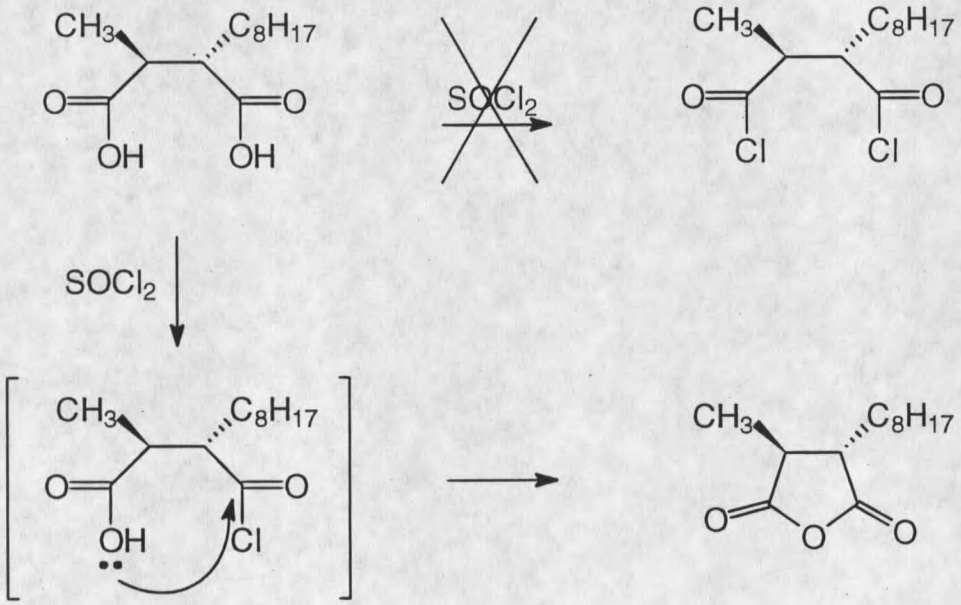


Figure 4.4 Formation of anhydride

diazomethane in an attempt to form the dimethyl ester, but again, the mono-methyl ester product was formed. It is possible, since the first step of the methylation involves the transfer of the proton from the carboxylic acid to the diazomethane[59], that the second proton was held too tightly between the two oxygens to transfer to the diazomethane (Figure 4.5). Another explanation could be that the acid was isolated as the mono-sodium salt, which would only react once to form the mono-ester mono-salt. However, the acid protons generally integrated to more than one proton (at least 1.5 protons) in the ^{1}H NMR, so it seems that at least some of the diester would be observed.

The other spectral data also supported the formation of the mono-methyl esters. The HREIMS analysis of the TMS (trimethylsilane) derivative gave a molecular formula of $C_{16}H_{31}O_4Si$, which corresponded to the TMS derivative of a mono-methyl ester of sphaeric acid with the loss of a methyl group. It is common in TMS derivatives for the molecular ion peak to be missing, but the M-15 peak from the cleavage of one of the silicon methyl bonds is often prominent.[60] The IR data included two carbonyl peaks at 1742 and 1704 cm^{-1}, corresponding to an ester and a carboxylic acid. The ^{13}C NMR data also supported the presence of two mono-methyl esters. There were two methyl signals at 52.2 and 52.0 ppm that correspond to carbons attached to oxygens. Additionally, there were four carbonyl signals at 176.3, 176.1, 175.9, and 175.7 ppm corresponding to the acid and ester carbons of each isomer. The

remaining proton and carbon signals corresponded very well to those of sphaeric acid.

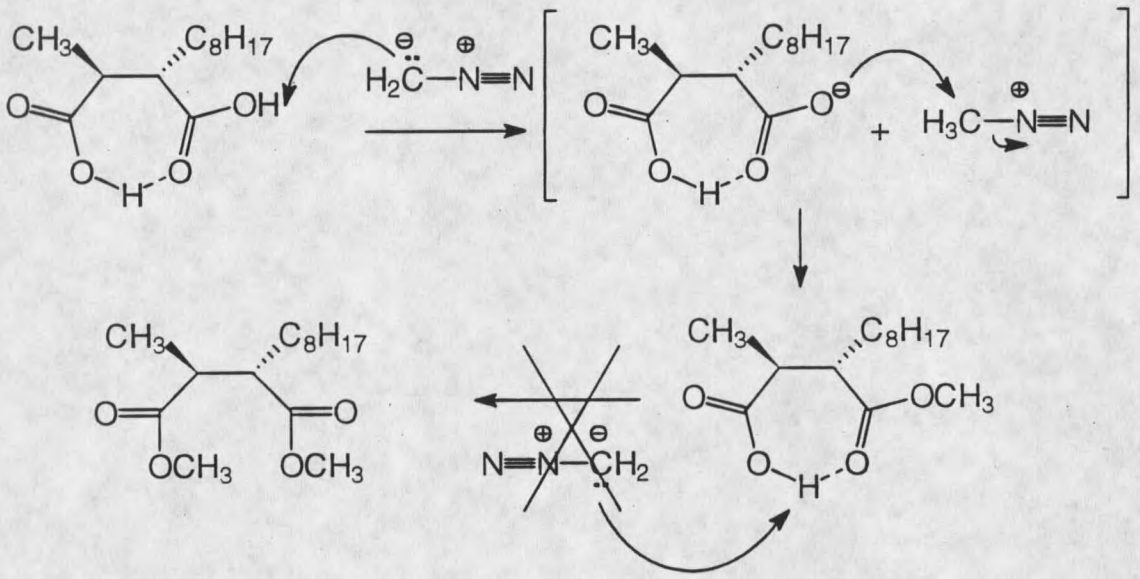


Figure 4.5 Diazomethane synthesis of mono-methyl ester

Ethyl esters

The two isomers of mono-ethyl esters of sphaeric acid (Figure 4.6) were formed in the same manner as the mono-methyl esters, using thionyl chloride to produce the cyclic anhydride. In this case, an attempt was made to try to produce the diacid chloride by using a ten-fold excess of thionyl chloride, but again, only the mono esters were recovered.

The HRCIMS gave a molecular formula of $C_{15}H_{32}NO_4$ which corresponded to the molecular ion plus NH_4 of a mono-ethyl ester of sphaeric acid. The IR data supported the mono-ester structure with two carbonyl signals at 1738 and 1704

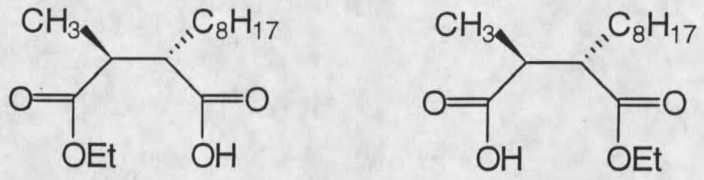


Figure 4.6 Mono-ethyl esters of sphaeric acid
(Absolute stereochemistry unknown)

cm^{-1} corresponding to an ester and a carboxylic acid. The proton NMR spectrum included two quartets at 4.13 and 4.14 ppm, corresponding to the methylenes on the oxygen of the ester and adjacent to a methyl group, which integrated to a total of two protons. The ^{13}C NMR also showed the methylene signals adjacent to an oxygen at 61.2 and 60.9 ppm and an the additional methyl signal at 14.7 ppm which was not present in sphaeric acid or the mono-methyl esters.

Isopropyl esters

The two isomers of mono-isopropyl esters of sphaeric acid (Figure 4.7) were formed in the same manner as the methyl and ethyl esters. The HRCIMS gave a molecular formula of $C_{16}H_{31}O_4$ which corresponds to the molecular ion plus a proton. The IR data supported the mono-ester structure with two carbonyl signals at 1732 and 1704 cm^{-1} corresponding to an ester and a carboxylic acid. The proton NMR spectrum included the septet at 5.0 ppm for the methine proton adjacent to the oxygen of the ester and two identical methyl groups. Additionally there was the doublet at 1.19 ppm of the methyl protons adjacent to

the above methine. The ^{13}C NMR showed the methine carbons adjacent to an oxygen at 68.6 and 68.4 ppm and the additional methyl signals at 22.1 and 22.0 ppm. There were also four carbonyl signals at 180.6, 179.7, 175.4, and 174.9 for the ester and carboxylic acid of each isomer.

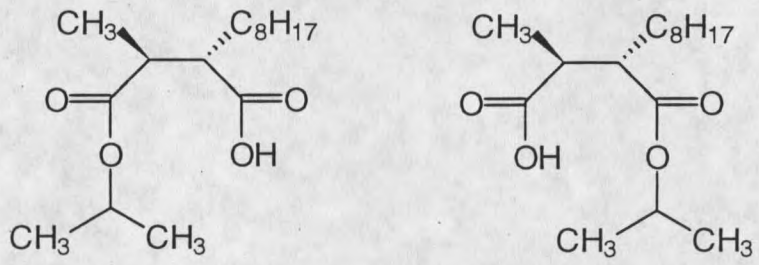


Figure 4.7 Mono-isopropyl esters of sphaeric acid
(Absolute stereochemistry unknown)

2-Chloroethyl esters

The two isomers of mono-2-chloroethyl esters of sphaeric acid (Figure 4.8) were formed in the same manner as the previous mono-esters. The HREIMS of the TMS derivative gave a molecular formula of $C_{17}H_{32}O_4SiCl$ which corresponded to the TMS derivative of a mono-2-chloroethyl ester of sphaeric acid minus a methyl group. The mono-chlorinated product was also supported by the presence of an M+2 peak of one third the intensity of the molecular ion peak. The IR data supported the mono-ester structure with two carbonyl signals at 1739 and 1707 cm^{-1} corresponding to an ester and a carboxylic acid. The proton NMR spectrum contained the two triplets, 4.3 and 3.6 ppm, of the 2-chloroethanol group, and the two methylene carbons are at 64.6 and 41.9 ppm.

in the ^{13}C NMR. The only evidence that both mono-esters were formed is the presence of three carbonyl signals at 180.1, 175.3, and 174.8 ppm instead of the two that would be present if only one isomer were formed.

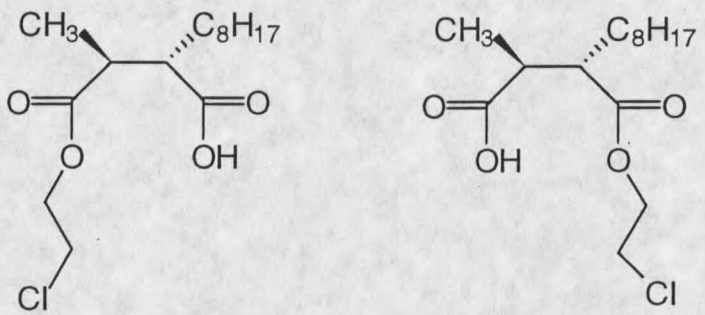


Figure 4.8 Mono-2-chloroethyl esters of sphaeric acid
(Absolute stereochemistry unknown)

2-Bromoethyl esters

The two isomers of mono-2-bromoethyl esters of sphaeric acid (Figure 4.9) were formed in the same manner as the previous esters. The HREIMS of the TMS derivative gave a molecular formula of $C_{17}H_{32}O_4SiBr$ which corresponded to the TMS derivative of a mono-2-bromoethyl ester of sphaeric acid minus a methyl group. The mono-brominated product was also supported by the presence of an M+2 peak of intensity equal to the molecular ion peak. The IR data supported the mono-ester structure with two carbonyl signals at 1738 and 1708 cm^{-1} corresponding to an ester and a carboxylic acid. The proton NMR spectrum contained the two triplets, 4.4 and 3.5 ppm, of the 2-bromoethanol group, and the two methylene carbons are at 64.5 and 64.3 ppm in the ^{13}C

NMR. The ^{13}C NMR data supported the presence of the two isomers with the four carbonyl signals at 180.4, 179.6, 175.2, and 174.6 ppm for the ester and acid of each isomer. Additionally, there were two signals for each of the methines (47.5/47.7 ppm and 40.9/41.0 ppm) and two signals for the methyl group (14.7/14.8 ppm) adjacent to one of the methines.

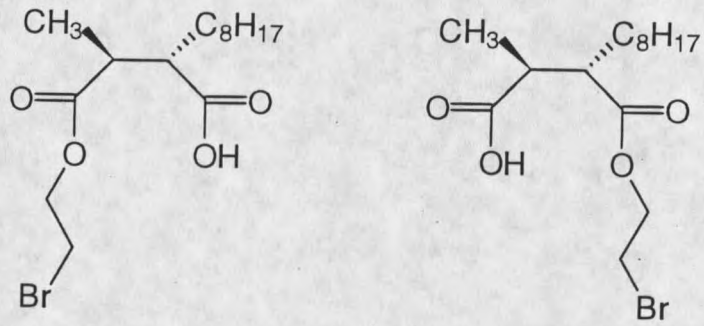


Figure 4.9 Mono-2-bromoethyl esters of sphaeric acid
(Absolute stereochemistry unknown)

Mono-amide

The two isomers of the mono-amide derivative of sphaeric acid (Figure 4.10) were made in a similar method to the mono-esters. The cyclic anhydride was produced by reaction of the diacid with thionyl chloride, and then allowed to react with the ammonia in solution.

The mass spectral data was unavailable for these compounds. The IR data supported the mono-amide structure by showing two carbonyl peaks at 1714 cm^{-1} and a shoulder at 1650 cm^{-1} corresponding to an acid and an amide. The proton NMR, as expected, was very similar to that of sphaeric acid, with no additional peaks. The ^{13}C NMR was also very similar to that of sphaeric acid,

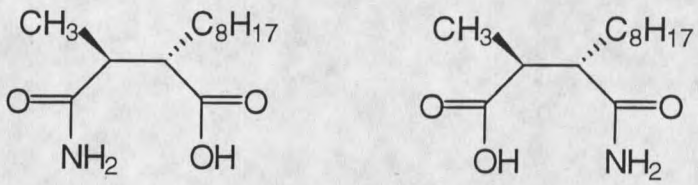


Figure 4.10 Mono-amides of sphaeric acid
(Absolute stereochemistry unknown)

but the presence of the two isomers was suggested by the pairs of signals for the methines at 48.7/49.0 ppm and 42.1/42.4 ppm.

Mono-methyl amide

The two isomers of the mono-methyl amide of sphaeric acid (Figure 4.11) were made in a manner similar to the mono-amide derivatives. The CIMS contained an M+1 peak at 258, and a base peak at 257 which results from the loss of a hydrogen atom α to the nitrogen of the amide (i.e. from the N-methyl). The IR data supported the mono-amide structures with two carbonyl signals at 1650 and 1574 cm^{-1} corresponding to an amide and a carboxylate anion. The proton NMR showed the presence of the two N-methyl signals of the amides at 2.71 and 2.70 ppm. These combined to an integration of only three protons since only the mono-amide was being formed. The ^{13}C NMR also supported the formation of the mono-methyl amides. The two methyls attached to the amide nitrogens appeared at 25.7 and 25.8 ppm. The four carbonyl signals, 183.6, 182.6, 178.8, and 178.1 ppm, corresponded to the acid and amide of each isomer. Additionally, there were pairs of signals for each of the methines

(52.8/51.2 ppm and 45.8/44.1 ppm) as well as for several of the other carbon signals.

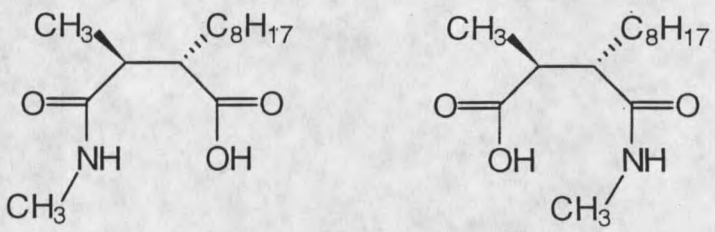


Figure 4.11 Mono-methyl amides of sphaeric acid
(Absolute stereochemistry unknown)

Phenyl imide

The phenyl imide of sphaeric acid (Figure 4.12) was made in the same manner as the other mono-amides. The HRCIMS gave a molecular formula of $C_{19}H_{28}NO_2$ corresponding to the molecular ion plus a proton. The HREIMS gave a molecular formula of $C_{19}H_{27}NO_2$ corresponding to the molecular ion. The broad, strong peak at 3380 cm^{-1} in the IR was believed to come from the O-H stretch of the water molecule produced in the dehydration of the mono-amides (Figure 4.13)

The IR showed only a single carbonyl peak at 1701 cm^{-1}. The proton NMR showed the aromatic protons of the phenyl ring at 7.3-7.5 ppm with the appropriate splitting for a mono-substituted benzene. The ^{13}C NMR was very clean without any of the multiple signals observed in the other derivatives. There were only two carbonyl signals (179.3 and 178.6 ppm) and two signals

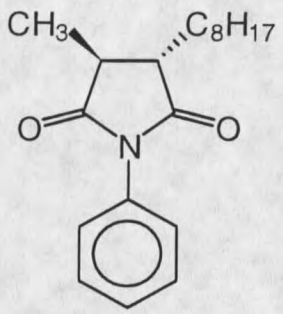


Figure 4.12 Phenyl imide of sphaeric acid

for the methines (48.5 and 41.5 ppm). This suggested that only one isomer, the phenyl imide, was being formed instead of the multiple isomers of the mono-amides observed in the other derivatives.

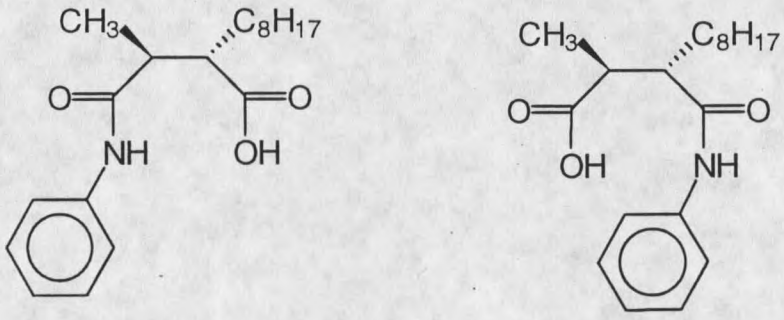


Figure 4.13 Mono-phenyl amides of sphaeric acid

Mono-glucosamides

The two isomers of the mono-glucosamide derivatives of sphaeric acid (Figure 4.14) were made in a manner similar to the other mono-amides. There was also some evidence that a small amount of mono-glucosamine esters were

formed as well with esterification through nucleophilic attack by one of the alcohols instead of the by the amine. For example, the small peaks at 5.3 ppm in the proton NMR were probably due to the methines attached to the esterified oxygens.

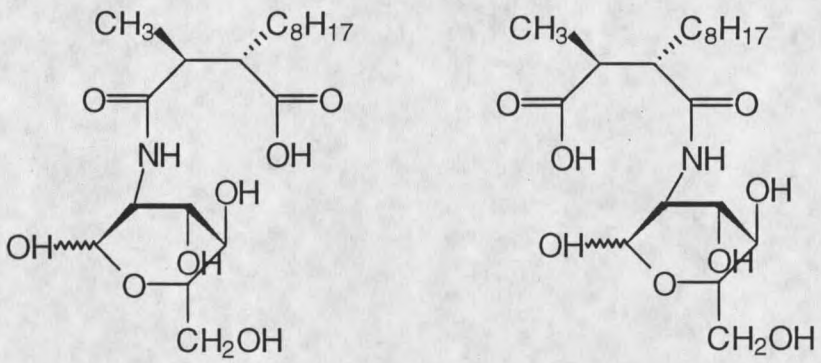


Figure 4.14 Mono-glucosamides of sphaeric acid
(Absolute stereochemistry unknown)

The IR data supported the mono-amide structure with two carbonyl signals at 1724 and 1652 cm^{-1} corresponding to an acid and an amide. The proton NMR showed the methine protons of the sugar attached to the nitrogen of the amide at 5.05 and 5.07 ppm and the methine protons of the sugar on the oxygen bearing carbons at 3.7-3.8 ppm. The doublet at 3.4 ppm was appropriate for the methylene protons of the glucosamine. The ^{13}C NMR contained three carbonyl peaks (177.8, 177.2, and 176.7 ppm) and two signals for the acetal methine carbon (91.7 and 91.6 ppm). The three oxygen bearing methines (72.2, 71.7, and 71.5 ppm), the nitrogen bearing methine (54.8 ppm), and the methylene (61.8) of the glucosamine were also observed.

Diol

See Chapter 3.

Carbonate of diol

The carbonate of the dialcohol (Figure 4.15) of sphaeric acid was prepared by reacting the diol of sphaeric acid with triphosgene. The HRCIMS gave a molecular formulas of $C_{14}H_{27}O_3$ corresponding to the protonated molecular ion and $C_{14}H_{30}NO_3$ corresponding to the molecular ion plus an ammonium ion. The IR gave two carbonyl peaks at 1778 and 1746 cm^{-1}, suggesting that there was at least one other compound formed in this reaction. The proton NMR contained the two methylenes adjacent to the oxygens of the carbonate at 4.2 and 4.0 ppm. The ^{13}C NMR has a single carbonyl peak at 151.1 ppm which would be acceptable for a carbonate. There were two peaks for each of the methylenes adjacent to the carbonate (74.7/72.8 ppm and 70.8/68.6 ppm) and a number of peaks for the methines in the ring (39.2-39.4 ppm and 33.3-33.6 ppm). There were also multiple peaks for the methyl group on the ring (13.0-13.4 ppm). The mass spectral data for the remaining products were not available at this time. It was possible that the dicarbonate was formed or carbonate dimers of sphaeric acid were formed.

Lactones

See Chapter 3.

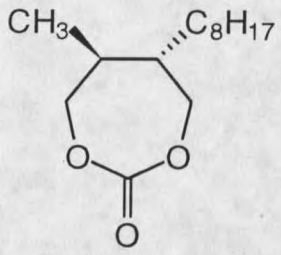


Figure 4.15 Carbonate of diol of sphaeric acid
(Absolute stereochemistry unknown)

Bioassays of Derivatives

Unfortunately, the company that originally tested sphearic acid and reported the mouse thymocyte proliferation activity was unable to perform this assay on the synthetic derivatives. However, several other bioassays were conducted on sphearic acid and the set of derivatives in order to explore any possible structure/function variations among the derivatives. These bioassays included a brine shrimp toxicity assay, a minimum inhibitory concentration spot test against *C. albicans*, some antibiotic disc diffusion assays, and an inhibitory concentration assay against breast adenocarcinoma BT-20.

The brine shrimp toxicity assay gave a wide variety of activity for the derivative series (Table 4.1). The results ranged from the glucosamine derivatives, which were non-toxic, to the carbonate and lactone derivatives which killed all the brine shrimp within two hours. Three other derivatives (diol, mono-amides, and mono-ethyl esters) killed all the brine shrimp within 12 hours,

and two (mono-methyl esters, and mono-chloroethyl esters) killed them all within the 24 hours of testing.

Derivative	t = 0 hr	t = 2 hr	t = 12 hr	t = 24 hr	total #
Diacid	0	0	5	8	14
Me ester	0	0	8	11	11
Et ester	0	26	32	-	32
I-Pr ester	0	0	3	9	17
ClEt ester	0	1	4	17	17
BrEt ester	0	2	2	7	15
Amide	0	8	12	-	12
Me Amide	0	0	0	2	20
Ph Amide	0	0	5	7	14
Glu Amide	0	0	0	0	8
Diol	0	4	9	-	9
Carbonate	0	12	-	-	12
Lactone	0	9	-	-	9

Table 4.1 Brine shrimp bioassay of derivatives
(Number of dead brine shrimp at each time interval)

It appears that the most non-polar derivatives, the carbonate and lactones, had the greatest activities while the most polar, the glucosamine amides, showed no activity. Beyond the extremes however, there is no clear-cut differentiation. The amide derivatives go from the glucosamine amides, which were inactive to the mono-amide which killed all the brine shrimp within 12 hours. The esters are all intermediate in their activities, ranging from killing all

the brine shrimp within 12 hours (the mono-ethyl esters) to killing only half the brine shrimp within 24 hours (the mono-isopropyl esters and mono-bromoethyl esters.

Anti-tumor assays were also conducted on the derivative series. Inhibitory concentration (IC_{50}) values were determined against breast adenocarcinoma BT-20 cells (Table 4.2). Although none of the derivatives were particularly active, there were variations in the activities. The best activities were found for the diol, the mono-bromoethyl esters, the mono-chloroethyl esters, and the mono-isopropyl esters. The least active derivatives were the mono-amides, the mono-methylamides, and the mono-glucosamides.

There was some correlation between the brine shrimp toxicity results and the anti-tumor assay, but there were also some differences. Both the diol and the lactones were among the most active compounds in each assay, while the mono-methylamides and mono-glucosamides were among the least active. The major differences between the assays was the carbonate derivative. The carbonate was one of the two most active compounds in the brine shrimp bioassay, but showed only moderate to low activity against the BT-20 cells. On the other hand, the mono-isopropyl esters, the mono-chloroethyl esters, and the mono-bromoethyl esters showed good activity against the BT-20 cells, but only had intermediate activity against the brine shrimp.

The disc diffusion assay showed activities only against *S. aureus*, *B. subtilis*, and *V. harveyii* (Table 4.3). The derivative series was also tested against *E.*

Derivative	stock (mg/ml)	IC_{50} (µg/ml)	IC_{50} (µM)
Diacid	40	>40	>160
Me ester	40	>40	>150
Et ester	40	>40	>150
I-Pr ester	40	35	120
ClEt ester	40	35	110
BrEt ester	40	30	86
Amide	5	>125	>514
Me amide	5	>125	>486
Ph amide	5	91	290
Glu amide	5	>125	>308
Diol	40	25	120
Carbonate	40	>40	>170
Lactone	40	40	190

Table 4.2 Inhibitory concentrations (IC_{50}) against BT-20

coli, *P. aeruginosa*, *C. albicans*, *G. candidum*, *A. niger*, and *F. oxysporum*, with no observed inhibition at tested levels. Activity was limited to antibacterial activity against both gram-positive and gram-negative bacteria. Two of the four gram-negative bacteria were inhibited, while the only gram-positive organism tested was inhibited. There was no evidence of anti-fungal activity against the four fungi at the levels tested.

There were a few interesting trends in the anti-biotic activities observed. The mono-chloroethyl ester seemed to be the most inhibitory compound against all

Derivative	*S. aureus*	*B. subtilis*	*V. harveyii*
Diacid	10	12	
Me ester	8	15	
Et ester		8	
I-Pr ester	10	10	
ClEt ester	12	12	10
BrEt ester	8	8	
Amide		8	
Me amide			
Ph amide			
Glu amide		10	
Diol	10		8
Carbonate			
Lactone	8	8	8

Table 4.3 Antibiotic disc diffusion assay (inhibition zone in mm)

three microorganisms at the levels tested. The diol appeared to be active only against the gram-negative bacteria, and not against the gram-positive *B. subtilis*. On the other hand, the mono-ethyl ester, the mono-amide, and the mono-glucosamide only inhibited the gram-positive bacterium. The least active derivatives, the mono-methyl and mono-phenyl amides and the carbonate derivative, showed no inhibition against any of the test organisms.

A minimal inhibitory concentration spot test was also conducted against *C. albicans* at three concentrations. Only one active spot was observed at the highest concentration (0.1 mg/10 µL) for the carbonate derivative.

Experimental

Synthesis of Derivatives

Synthesis of methyl esters: Method I Sphaeric acid (5.0 mg, 0.020 mmole) in methylene chloride (50 µL) was placed in a three milliliter conical vial. Thionyl chloride (3.5 µL; 0.048 mmole) was added, and the mixture was heated on an approximately 125°C sand bath for thirty minutes. The reaction was allowed to cool to room temperature and then excess methanol was added. Method II Sphaeric acid (5.0 mg, 0.020 mmole) in ethyl ether (3.0 mL) was placed in the outer tube of an Aldrich MNNG-diazomethane kit. MNNG (1-methyl-3-nitro-1-nitrosoguanidine) (100. mg, 0.68 mmole) was placed in the inner tube with water (0.5 mL). The apparatus was immersed in an ice bath and 5M NaOH (0.5 mL, 2.5 mmole) was added dropwise to produce the diazomethane. The solution was allowed to sit for three hours to go to completion. Method III Sphaeric acid (8.7 mg, 0.036 mmole) were reacted with previously distilled diazomethane. The diazomethane was added dropwise until there was no apparent additional reaction. The products were purified in each method by the evaporation of the remaining reactants.

Synthesis of ethyl esters Sphaeric acid (5.0 mg, 0.020 mmole) in methylene chloride (50 µL) was placed in a three milliliter conical vial. Thionyl chloride (30. µL, 0.41 mmole) was added, and the mixture was heated on an approximately 125°C sand bath for thirty minutes. The reaction was allowed to cool to room temperature and then ethanol (500. µL, 8.5 mmole) was added. The products (3.15 mg, 0.0116 mmole) were purified by the evaporation of the remaining reactants.

Synthesis of iso-propyl esters Sphaeric acid (5.0 mg, 0.020 mmole) in chloroform (100. µL) was placed in a three milliliter conical vial. Thionyl chloride (3.5 µL, 0.048 mmole) was added, and the mixture was heated on an approximately 90°C sand bath for thirty minutes. The reaction was allowed to cool to room temperature and then isopropanol (100. µL, 1.3 mmole) was added. The products (4.10 mg, 0.0143 mmole) were purified by evaporation of the remaining reactants.

Synthesis of 2-chloroethyl esters Sphaeric acid (5.0 mg, 0.020 mmole) in methylene chloride (50. µL) was placed in a three milliliter conical vial. Thionyl chloride (3.5 µL, 0.048 mmole) was added, and the mixture was heated on an approximately 65°C sand bath for thirty minutes. The reaction was allowed to cool to room temperature and then 2-chloroethanol (50 µL, 0.75 mmole) was added. The products were purified by flash reverse-phase chromatography (Baker SPE C_{18} columns) and eluted with water, methanol, and methylene

chloride. The products (5.10 mg, 0.0167 mmole) were present in the methanol fraction.

Synthesis of 2-bromoethyl esters Sphaeric acid (5.0 mg, 0.020 mmole) in methylene chloride (50. µL) was placed in a three milliliter conical vial. Thionyl chloride (3.5 µL, 0.048 mmole) was added, and the mixture was heated on an approximately 65°C sand bath for thirty minutes. The reaction was allowed to cool to room temperature and then 2-bromoethanol (50. µL, 0.71 mmole) was added. The products were purified by flash reverse-phase chromatography (Baker SPE C_{18} columns) and eluted with water, methanol, and methylene chloride. The products (7.55 mg, 0.0200 mmole) were present in the methanol fraction.

Synthesis of monoamide derivatives Sphaeric acid (5.0 mg, 0.020 mmole) in methylene chloride (50. µL) was placed in a three milliliter conical vial. Thionyl chloride (3.5 µL, 0.048 mmole) was added, and the mixture was heated on an approximately 90°C sand bath for thirty minutes. The reaction was allowed to cool to room temperature and then excess 6M ammonium hydroxide was added. The products (6.25 mg) were purified by evaporation of the remaining reactants.

Synthesis of mono-methylamide derivatives Sphaeric acid (5.0 mg, 0.020 mmole) in methylene chloride (50. µL) was placed in a three milliliter conical vial. Thionyl chloride (3.5 µL, 0.048 mmole) was added, and the mixture was

heated on an approximately 65°C sand bath for thirty minutes. The reaction was allowed to cool to room temperature and then flushed with air to evaporate any remaining thionyl chloride. Two drops of a 6M methylamine solution (405 mg methylamine hydrochloride, 240 mg NaOH, 1 mL water) was added. The products were purified by flash reverse-phase chromatography (Baker SPE C_{18} columns) and eluted with water and methanol. The products (6.85 mg) were present in the methanol fraction.

Synthesis of mono-phenylamide derivative Sphaeric acid (5.0 mg, 0.020 mmole) in methylene chloride (50. µL) was placed in a three milliliter conical vial. Thionyl chloride (3.5 µL, 0.048 mmole) was added, and the mixture was heated on an approximately 65°C sand bath for thirty minutes. The reaction was allowed to cool to room temperature and then aniline (50. µL, 0.55 mmole) was added. The product (3.75 mg, 0.0117 mmole) was purified by evaporation of the remaining reactants.

Synthesis of glucosamine derivatives Sphaeric acid (5.0 mg, 0.020 mmole) in methylene chloride (50. µL) was placed in a three milliliter conical vial. Thionyl chloride (3.5 µL, 0.048 mmole) was added, and the mixture was heated on an approximately 90°C sand bath for thirty minutes. The reaction was cooled in an ice bath and then 2 M glucosamine solution (215 mg D-(+)-glucosamine hydrochloride, 40 mg NaOH, 0.50 mL water) (50. µL, 0.10 mmole) was added. The products were purified by size exclusion chromatography (LH-20, 1 X 30

cm column) and eluted with methanol. The products (5.20 mg, 0.0128 mmole) were present in tubes 7 and 8.

Synthesis of diol See Chapter 3.

Synthesis of carbonate of diol Triphosgene [bis(trichloromethyl) carbonate] (9.0 mg, 0.030 mmole) was placed in a three milliliter conical vial with methylene chloride (0.3 mL). Diol of sphaeric acid (6.35 mg, 0.029 mmole) in methylene chloride (0.5 mL) was added dropwise and allowed to stand for 30 minutes.

At this point the reaction was not quite complete, so the procedure was repeated with triphosgene (3.0 mg, 0.010 mmole) in the vial and the product from above was added to the solution dropwise. The products (5.60 mg) were purified by evaporation of the remaining reactants.

Synthesis of lactones See Chapter 3.

Physical/Chemical Data of Derivatives

Physical/Chemical data for methyl esters Colorless oil; UV (CH_3OH) λ_{max} (ε) 206 (570); IR υ_{max} (cm^{-1}) (neat) 3200 br, 2924, 2855, 1742, 1704, 1456, 1200; HREIMS m/z [TMS derivative - Me]$^+$ 315.1993, calculated 315.1992 for $C_{16}H_{31}O_4Si$; EIMS m/z 241(22), 185(81), 170(16), 160(78), 128(100), 101(46), 88(58), 69(73), 55(89); ^{1}H NMR δ ($CDCl_3$) {3.66, 3.65 (3H, s)}, 2.8 (1H, m), 2.7 (1H, m), 1.6 (2H, m), 1.2 (12H, br s), 1.1 (3H, d, J=6.9 Hz), 0.85 (3H, t, J=6.6

Hz); ^{13}C NMR δ ($CDCl_3$) {176.3 (C), 176.1 (C)}, {175.9 (C), 175.7 (C)}, {52.2 (CH_3), 52.0 (CH_3)}, 47.9 (CH), 41.2 (CH), 32.2 (CH_2), 29.9 (CH_2), 29.8 (CH_2), 29.6 (CH_2), 29.1 (CH_2), 27.3 (CH_2), 23.0 (CH_2), 14.6 (CH_3), 14.5 (CH_3).

Physical/Chemical data for ethyl esters Colorless oil; UV (CH_3OH) λ_{max} (ε) 207 (550); IR υ_{max} (cm^{-1}) (neat) 3100 br, 2925, 2856, 1738, 1704, 1463, 1378, 1184; HRCIMS m/z $[M + NH_4]^+$ 290.2319, calculated 290.2331 for $C_{15}H_{32}NO_4$; EIMS m/z 227(08), 199(04), 181(07), 160(16), 154(27), 142(27), 114(20), 97(37), 87(27), 69(50), 55(100); ^1H NMR δ ($CDCl_3$) {4.14, 4.13 (2H, q, J=7.2 Hz)}, 2.7 (2H, m), 1.6 (2H, m), 1.2 (15H, br s), 1.18 (3H, d, J=6.9 Hz), 0.85 (3H, t, J=6.6 Hz); ^{13}C NMR δ ($CDCl_3$) 179.9 (C), 175.8 (C), {61.2 (CH_2), 60.9 (CH_2)}, {47.9 (CH), 47.7 (CH)}, {41.3 (CH), 41.0 (CH)}, 32.2 (CH_2), 29.9 (CH_2), 29.8 (CH_2), 29.6 (CH_2), 28.9 (CH_2), 27.2 (CH_2), 23.0 (CH_2), 14.7 (CH_3), 14.6 (CH_3), 14.5 (CH_3).

Physical/Chemical data for isopropyl esters Colorless oil; UV (CH_3OH) λ_{max} (ε) 206 (460); IR υ_{max} (cm^{-1}) (neat) 3100 br, 2925, 2856, 1732, 1704, 1456, 1374, 1182, 1108; HRCIMS m/z $[M + H]^+$ 287.2210, calculated 287.2222 for $C_{16}H_{31}O_4$; EIMS m/z 227(35), 200(09), 174(19), 154(22), 132(26), 114(38), 97(38), 87(70), 69(58), 55(100); ^1H NMR δ ($CDCl_3$) 5.0 (1H, septet, J=6.3 Hz), 2.7 (2H, m), 1.6 (2H, m), 1.2 (12H, br s), 1.19 (6H, d, J=6.3 Hz), 1.16 (3H, d, J=6.9 Hz), 0.85 (3H, t, J=6.6 Hz); ^{13}C NMR δ ($CDCl_3$) {180.6 (C), 179.7 (C)}, {175.4 (C), 174.9 (C)}, {68.6 (CH), 68.4 (CH)}, 47.8 (CH), 41.2 (CH), 32.2 (CH_2),

30.0 (CH_2), 29.7 (CH_2), 29.6 (CH_2), 29.0 (CH_2), 27.2 (CH_2), 23.0 (CH_2), 22.1 (CH_3), 22.0 (CH_3), 14.7 (CH_3), 14.5 (CH_3).

Physical/Chemical data for 2-chloroethyl esters Colorless oil; UV (CH_3OH) λ_{max} (ε) 209 (470); IR υ_{max} (cm^{-1}) (neat) 3000 br, 2925, 2855, 1739, 1707, 1457, 1169; HREIMS m/z [TMS derivative - Me]$^+$ 363.1750, calculated 363.1758 for $C_{17}H_{32}O_4SiCl$; EIMS m/z 289(07), 233(07), 226(07), 176(13), 154(21), 142(12), 127(20), 113(100), 97(34), 83(21), 69(62), 55(98); ^{1}H NMR δ ($CDCl_3$) 4.3 (2H, t, J=5.6 Hz), 3.6 (2H, t, J=5.6 Hz), 2.8 (1H, t, J=7.5 Hz), 2.7 (1H, q, J=7.0 Hz), 1.6 (2H, m), 1.2 (12H, br s), 1.19 (3H, d, J=7.5 Hz), 0.85 (3H, t, J=6.6 Hz); ^{13}C NMR δ ($CDCl_3$) 180.1 (C), {175.3 (C), 174. 8 (C)}, 64.6 (CH_2), 47.7 (CH), 41.9 (CH_2), 40.8 (CH), 32.2 (CH_2), 30.0 (CH_2), 29.7 (CH_2), 29.6 (CH_2), 28.9 (CH_2), 27.1 (CH_2), 23.0 (CH_2), 14.7 (CH_3), 14.5 (CH_3).

Physical/Chemical data for 2-bromoethyl esters Colorless oil; UV (CH_3OH) λ_{max} (ε) 209 (740); IR υ_{max} (cm^{-1}) (neat) 3100 br, 2925, 2855, 1738, 1708, 1456, 1386, 1278, 1162; HREIMS m/z [TMS derivative - Me]$^+$ 407.1249, calculated 407.1253 for $C_{17}H_{32}O_4SiBr$; EIMS m/z 346(15), 333(17), 277(19), 222(30), 109(100), 69(70), 55(77); ^{1}H NMR δ ($CDCl_3$) 4.4 (2H, t, J=6.0 Hz), 3.5 (2H, t, J=6.0 Hz), 2.8 (1H, m), 2.7 (1H, m), 1.6 (2H, m), 1.2 (12H, br s), 1.18 (3H, d, J=6.9 Hz), 0.85 (3H, t, J=6.6 Hz); ^{13}C NMR δ ($CDCl_3$) {180.4 (C), 179.6 (C)}, {175.2 (C), 174.6 (C)}, 64.5 (CH_2), 64.3 (CH_2), {47.7 (CH), 47.5 (CH)}, {41.0

(CH), 40.9 (CH)}, 32.2 (CH_2), 30.0 (CH_2), 29.8 (CH_2), 29.6 (CH_2), 29.1 (CH_2) 27.2 (CH_2), 23.0 (CH_2), {14.8 (CH_3), 14.7 (CH_3)}, 14.5 (CH_3).

Physical/Chemical data for mono-amides White solid; UV (CH_3OH) λ_{max} (ε) 205 (450); IR υ_{max} (cm^{-1}) (neat) 3200 br, 2920, 1731, 1714, 1384, 1250; HREIMS m/z n/a; EIMS m/z n/a; ^1H NMR δ (CD_3OD) 2.7 (1H, m), 2.6 (1H, m), 1.6 (2H, m), 1.2 (12H, br s), 1.15 (3H, d, J=6.9 Hz), 0.85 (3H, t, J=6.6 Hz); ^{13}C NMR δ (CD_3OD) 179.6 (C), 179.0 (C), {49.0 (CH), 48.7 (CH) DEPT 135}, {42.4 (CH), 42.1 (CH)}, 33.1 (CH_2), 30.8 (CH_2), 30.6 (CH_2), 30.4 (CH_2), 29.9 (CH_2), 28.2 (CH_2), 23.8 (CH_2), {14.9 (CH_3), 14.7 (CH_3)}, 14.5 (CH_3).

Physical/Chemical data for mono-methylamides White solid; UV (CH_3OH) λ_{max} (ε) 207 (770); IR υ_{max} (cm^{-1}) (neat) 3296 br, 2925, 2854, 1650, 1645, 1574, 1568, 1455, 1414, 1162; HREIMS m/z n/a; CIMS m/z 258(16), 257(100), 240(29), 224(19), 208(21); ^1H NMR δ (CD_3OD) {2.71, 2.70 (3H, s)}, 2.4 (1H, m), 2.3 (1H, m), 1.5 (2H, m), 1.2 (12H, br s), 1.1 (3H, d, J=6.9 Hz), 0.85 (3H, t, J=6.6 Hz); ^{13}C NMR δ (CD_3OD) {183.6 (C), 182.6 (C)}, {178.8 (C), 178.1 (C)}, {52.8 (CH), 51.2 (CH)}, {45.8 (CH), 44.1 (CH)}, {31.9 (CH_2), 31.8 (CH_2)}, 30.5 (CH_2), 30.0 (CH_2), {29.6 (CH_2), 29.5 (CH_2)}, {29.3 (CH_2), 29.2 (CH_2)}, {27.7 (CH_2), 27.5 (CH_2)}, {25.8 (CH_3), 25.7 (CH_3)}, 22.6 (CH_2), 16.1 (CH_3), 14.5 (CH_3).

Physical/Chemical data for phenylimide White solid; UV (CH_3OH) λ_{max} (ε) 205 (6100); IR υ_{max} (cm^{-1}) (neat) 3380 br, 2923, 1701, 1182, 1025; HRCIMS m/z

$[M + H]^+$ 302.2118, calculated 302.2120 for $C_{19}H_{28}NO_2$; HREIMS m/z M^+ 301.2027, calculated 301.2042 for $C_{19}H_{27}NO_2$; EIMS m/z 301(08), 202(27), 189(100), 174(12), 161(08), 119(12), 93(13), 69(20), 55(28); CIMS m/z 319(100), 302(14), 286(07); ^{1}H NMR δ ($CDCl_3$) 7.5 (2H, t, J=7.8 Hz), 7.4 (1H, dd, J=10.2, 7.8 Hz), 7.3 (2H, d, J=10.2 Hz), 2.7 (1H, m), 2.5 (1H, m), 2.0 (2H, m), 1.65 (2H, m), 1.4 (3H, d, J=7.2 Hz), 1.3 (10H, br s), 0.85 (3H, t, J=6.6 Hz); ^{13}C NMR δ ($CDCl_3$) 179.3 (C), 178.6 (C), 132.4 (C), 129.5 (CH), 128.9 (CH), 126.8 (CH), 48.5 (CH), 41.5 (CH), 32.2 (CH_2), 31.3 (CH_2), 29.9 (CH_2), 29.8 (CH_2), 29.6 (CH_2), 27.2 (CH_2), 23.0 (CH_2), 17.1 (CH_3), 14.5 (CH_3).

Physical/Chemical data for glucosamine derivatives White solid; UV (CH_3OH) λ_{max} (ε) 204 (2200); IR υ_{max} (cm^{-1}) (neat) 3352 br, 2921, 1732, 1724, 1698, 1556, 1538, 1455; HREIMS m/z n/a; EIMS m/z 308(08), 280(15), 252(14), 226(09), 179(12), 155(21), 140(20), 126(27), 113(35), 69(78), 55(100); ^{1}H NMR δ (CD_3OD) {5.07, 5.05 (1H, d, J=3.3 Hz)}, 3.8 - 3.7 (4H, m), 3.4 (2H, d, J=9 Hz), 2.7 (1H, m), 2.6 (1H, m), 1.6 (2H, m), 1.3 (12H, br s), 1.1 (3H, d, J=7.5 Hz), 0.85 (3H, t, J=6.6 Hz); ^{13}C NMR δ (CD_3OD) {177.8 (C), 177.2 (C)}, 176.7 (C), {91.7 (CH), 91.6 (CH)}, 72.2 (CH), 71.7 (CH), 71.5 (CH), 61.8 (CH_2), 54.8 (CH), ~48 (CH, under CD_3OD), 42.1 (CH), 32.0 (CH_2), 29.9 (CH_2), 29.6 (CH_2), 29.5 (CH_2), 29.3 (CH_2), 27.1 (CH_2), 23.2 (CH_2), 15.0 (CH_3), 13.4 (CH_3).

Physical/Chemical data for diol See Chapter 3.

Physical/Chemical data for carbonate of diol Colorless oil; UV (Et_2O) λ_{max} (ε) 211 (460); IR υ_{max} (cm^{-1}) (neat) 2925, 2855, 1778, 1746, 1467, 1379, 1240, 1159, 1086, 959, 799; HRCIMS m/z $[M + H]^+$ 243.1950, calculated 243.1960 for $C_{14}H_{30}O_3$, $[M + NH_4]^+$ 260.2206, calculated 260.2226 for $C_{14}H_{30}NO_3$; EIMS m/z 180(13), 154(06), 137(09), 124(09), 109(30), 95(46), 82(85), 69(85), 55(100); ^1H NMR δ ($CDCl_3$) 4.2 (2H, m), 4.0 (2H, m), 2.1 (1H, m), 1.8 (1H, m), 1.6 (2H, m), 1.2 (12H, br s), 0.95 (3H, m), 0.85 (3H, t, J=6.6 Hz); ^{13}C NMR δ ($CDCl_3$) 151.1 (C), {74.7 (CH_2), 72.8 (CH_2)}, {70.8 (CH_2), 68.6 (CH_2)}, {39.4 - 39.2 (CH)}, {33.6 - 33.3 (CH)}, 32.2 (CH_2), 30.2 (CH_2), 29.8 (CH_2), 29.6 (CH_2), 27.9 (CH_2), 23.0 (CH_2), 14.5 (CH_3), {13.4 - 13.0 (CH_3)}.

Physical/Chemical data for lactones See Chapter 3.

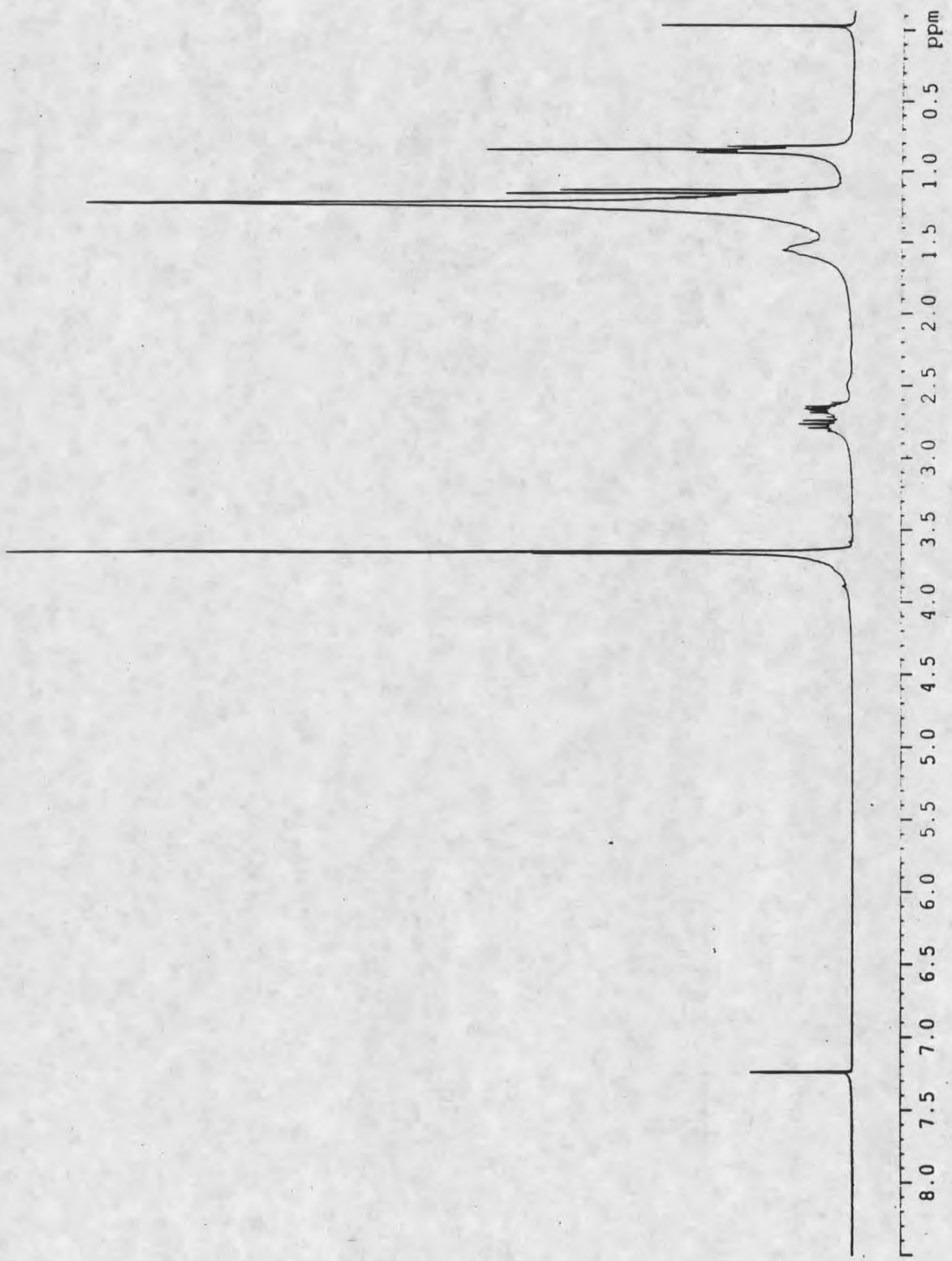


Figure 4.16 ^{1}H NMR of mono-methyl esters of sphearic acid

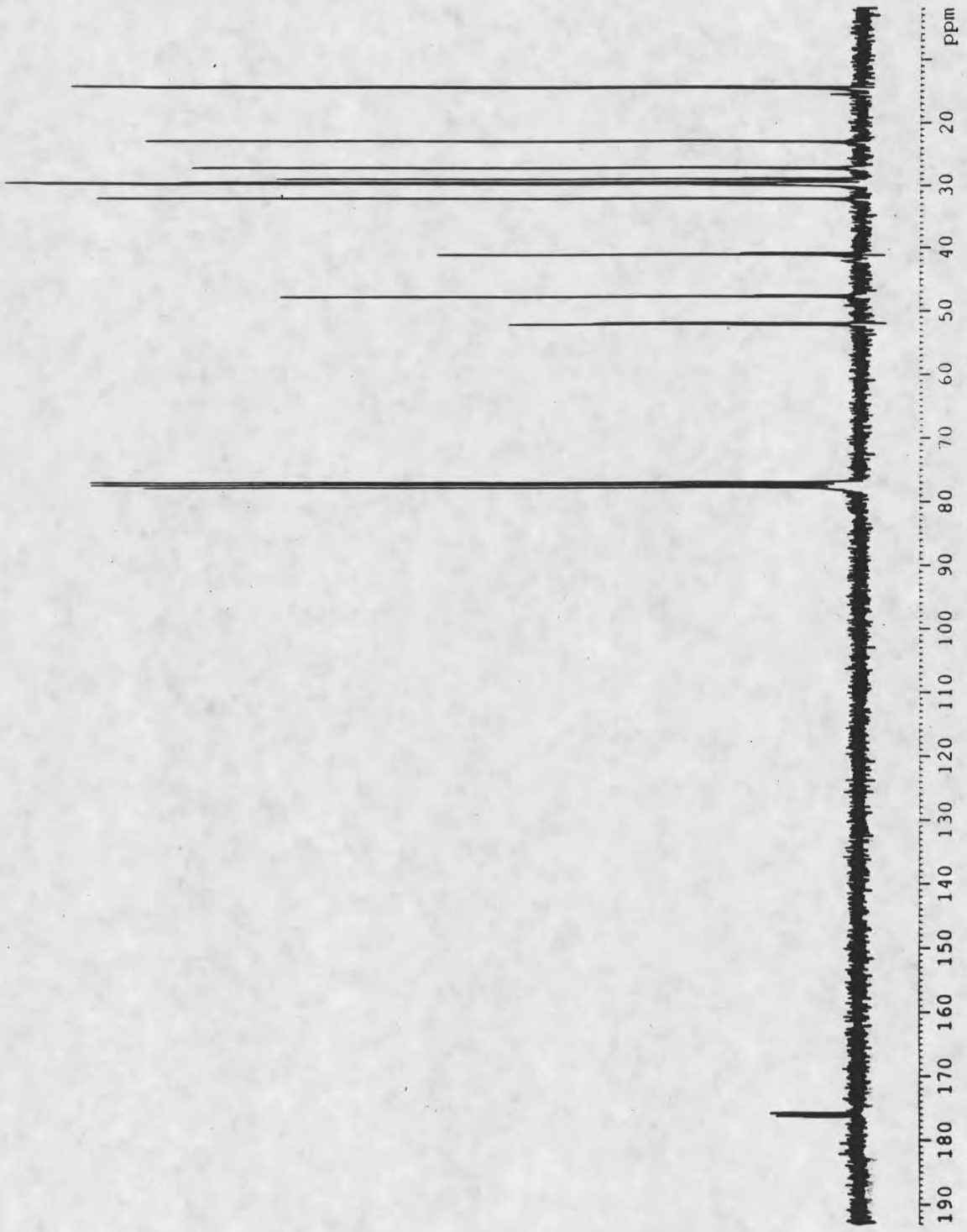


Figure 4.17 ^{13}C NMR of mono-methyl esters of sphaeric acid

Figure 4.18 ^{1}H NMR of mono-ethyl esters of sphaeric acid

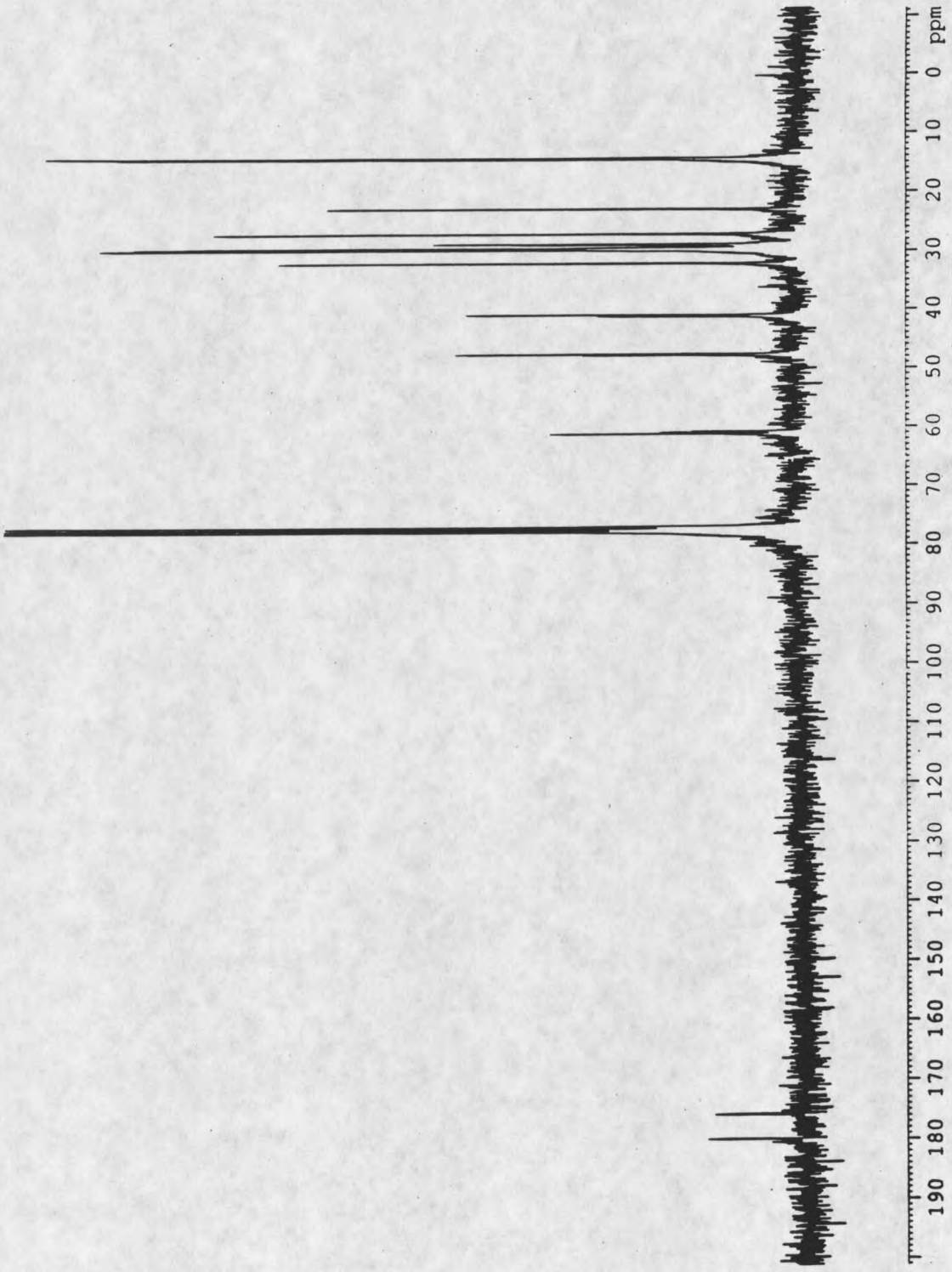


Figure 4.19 ^{13}C NMR of mono-ethyl esters of sphaeric acid

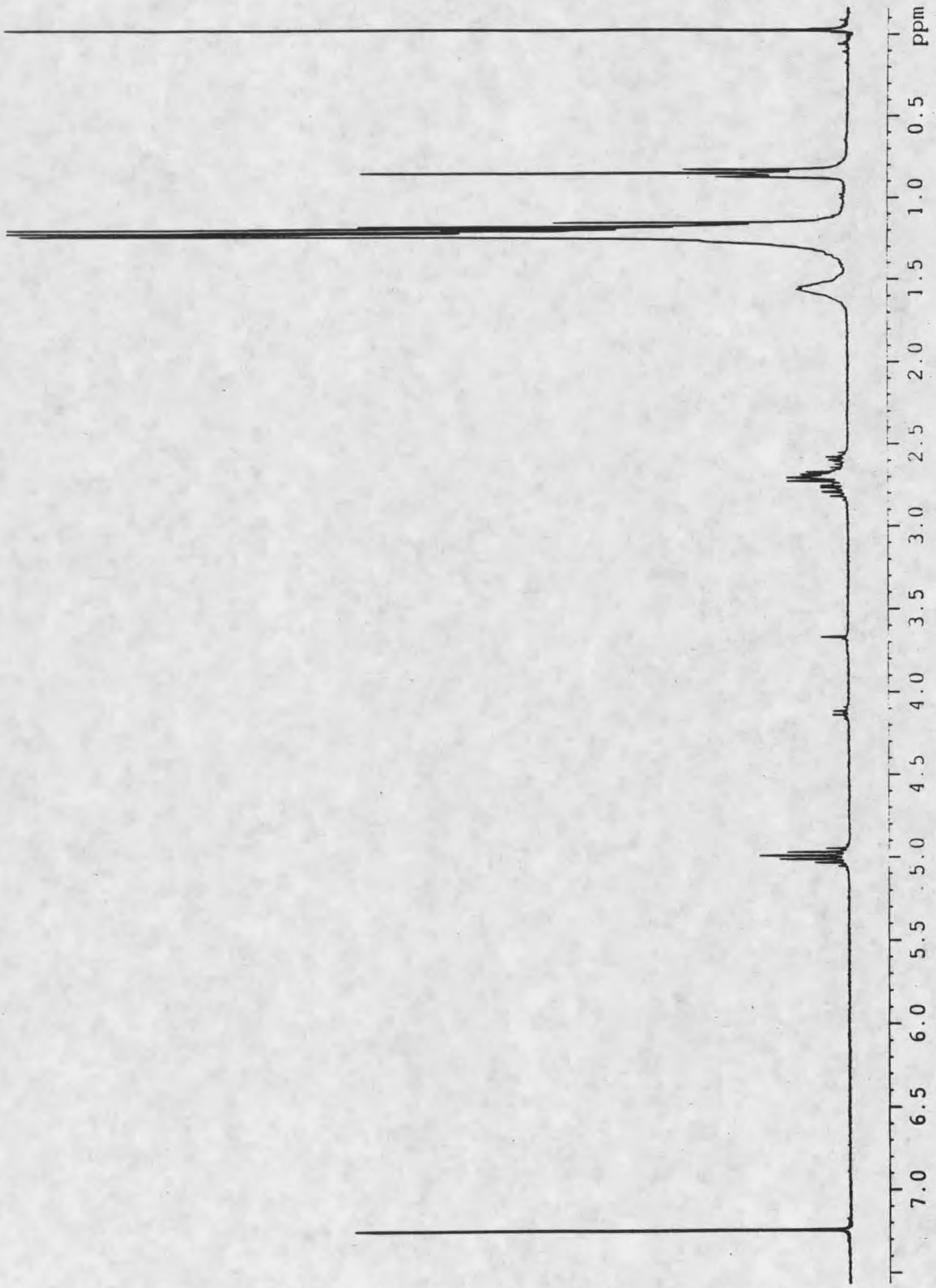


Figure 4.20 ^{1}H NMR of mono-isopropyl esters of sphaeric acid

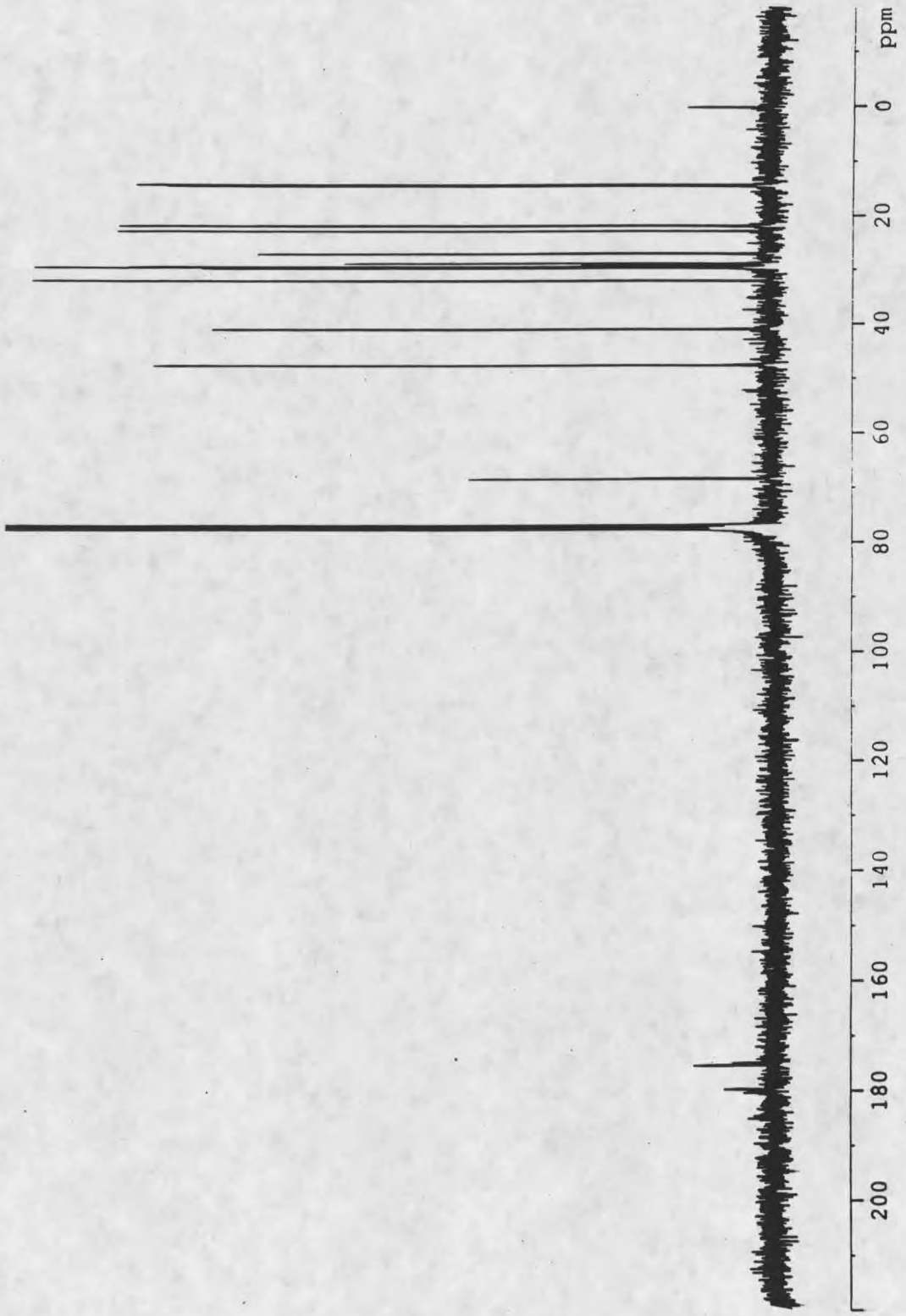


Figure 4.21 ^{13}C NMR of mono-isopropyl esters of sphaeric acid

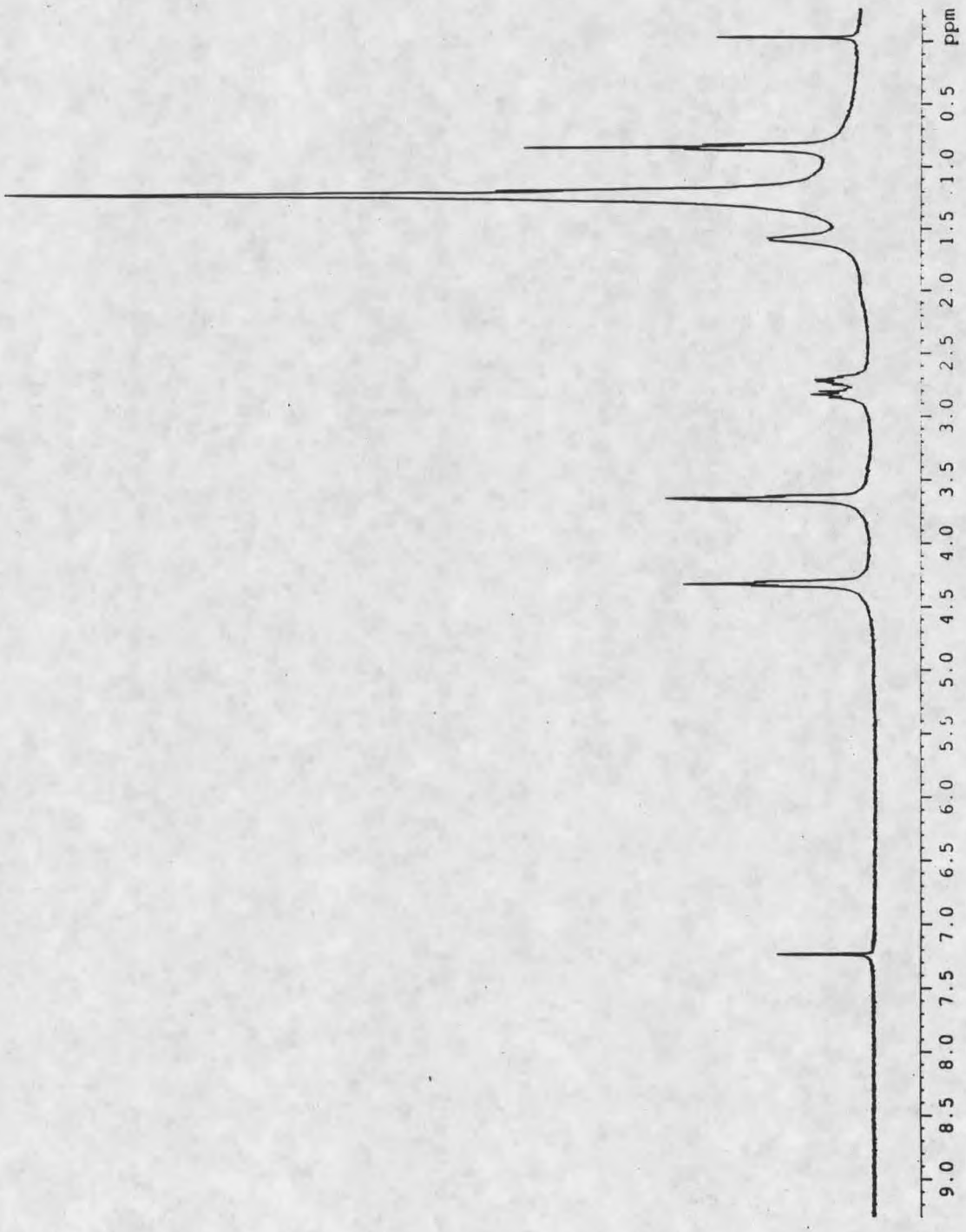


Figure 4.22 ^{1}H **NMR of mono-2-chloroethyl esters of sphaeric acid**

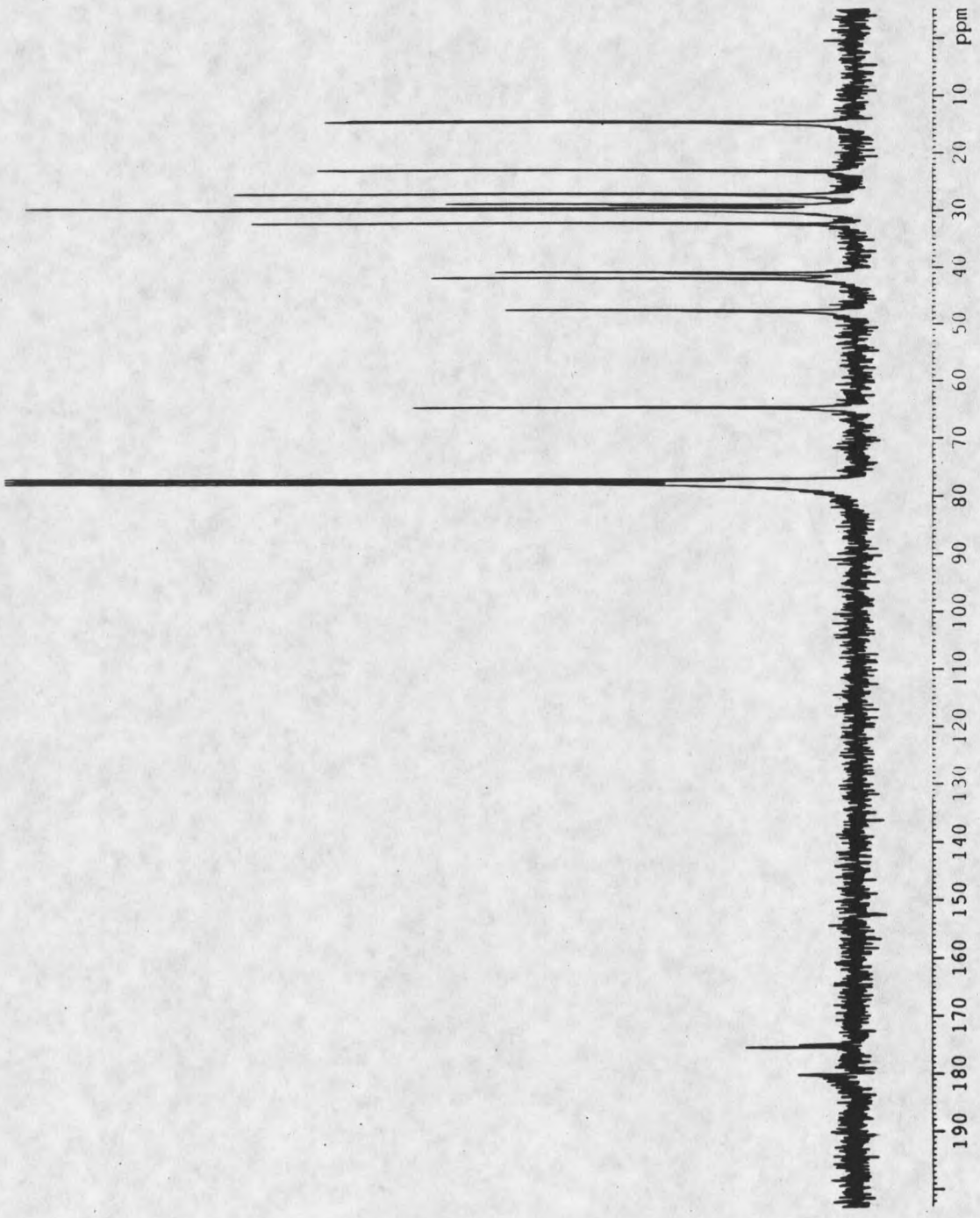


Figure 4.23 ^{13}C **NMR of mono-2-chloroethyl esters of sphaeric acid**

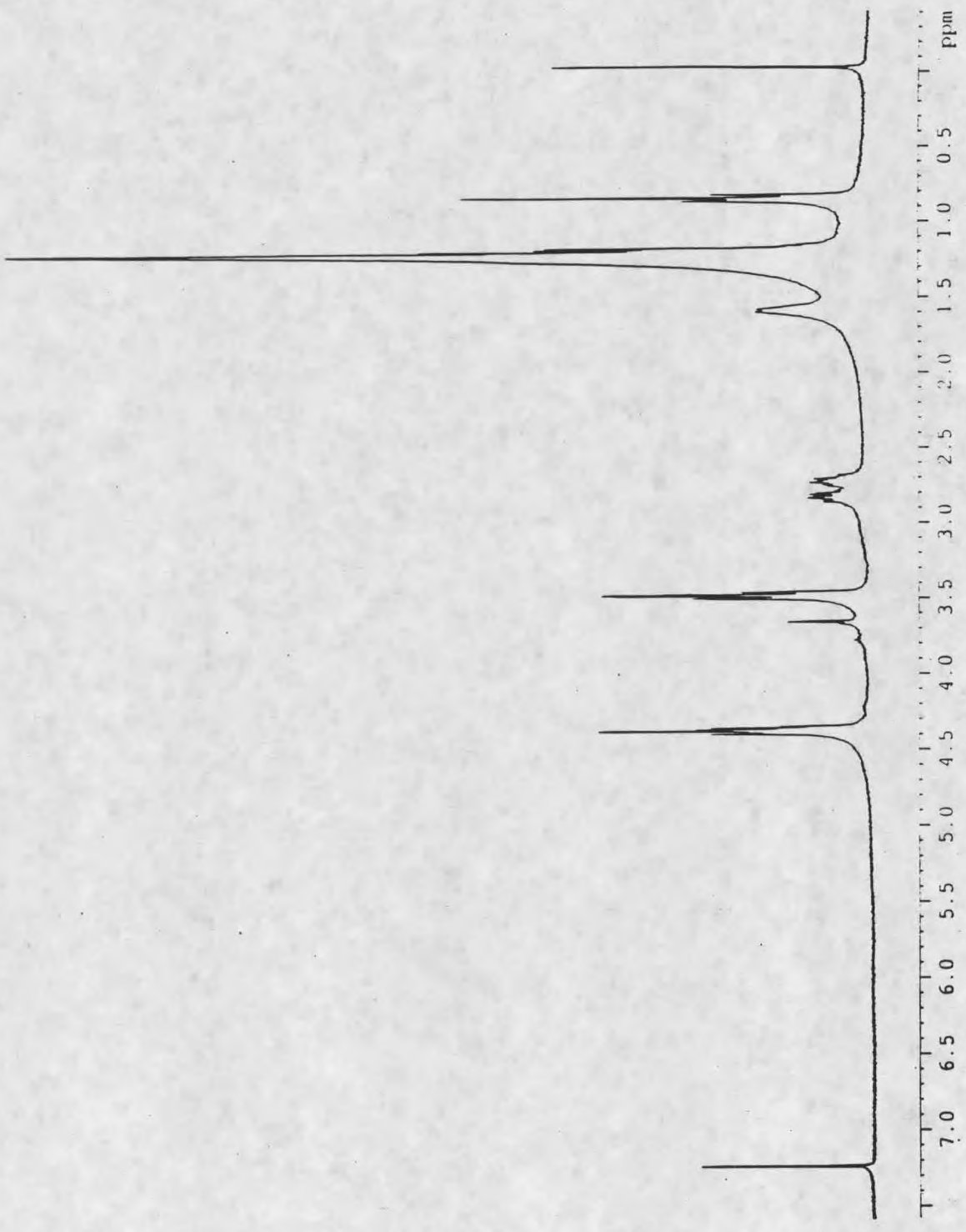


Figure 4.24 ^{1}H **NMR of mono-2-bromoethyl esters of sphaeric acid**

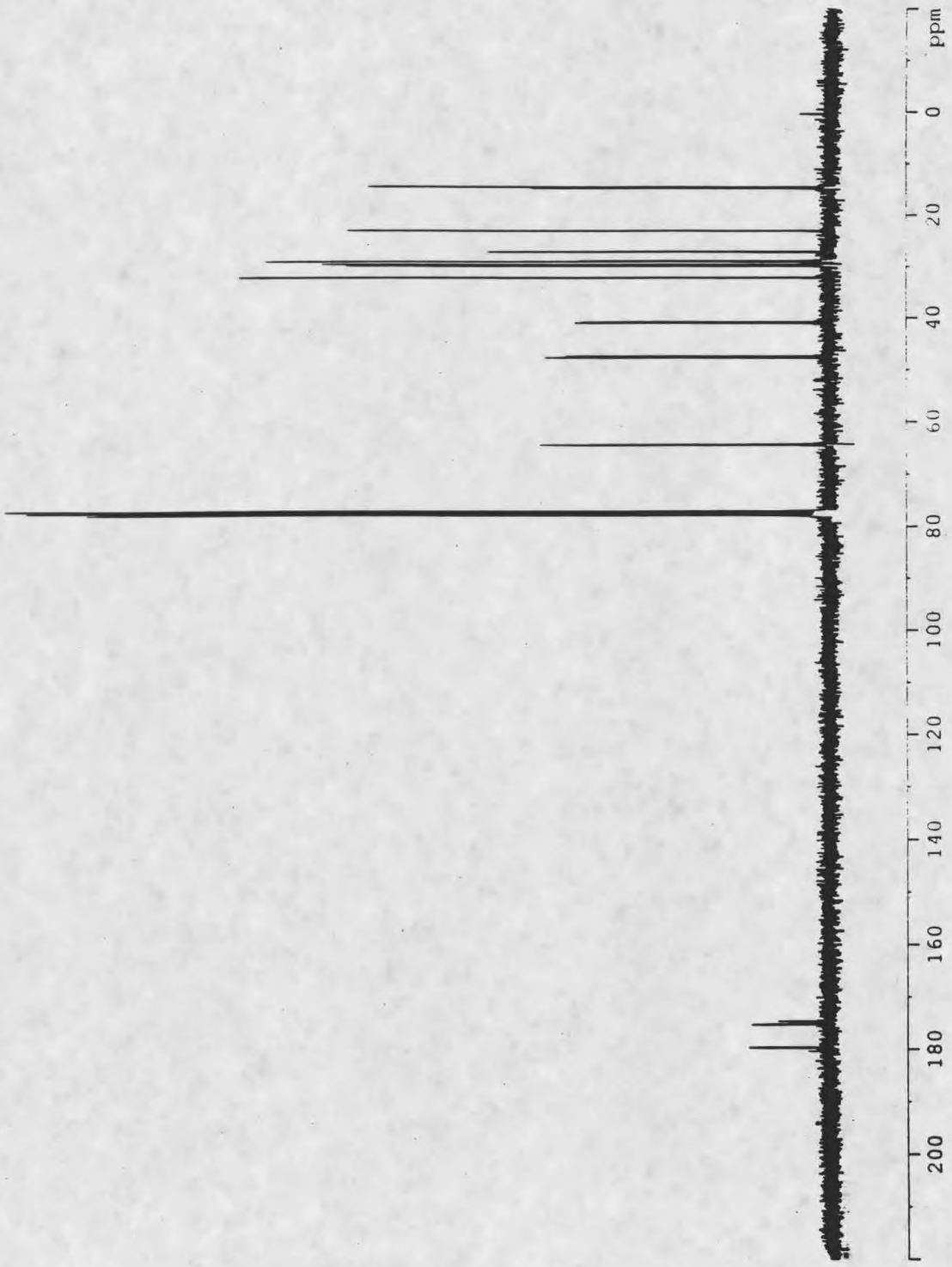


Figure 4.25 ^{13}C NMR of mono-2-bromoethyl esters of sphaeric acid

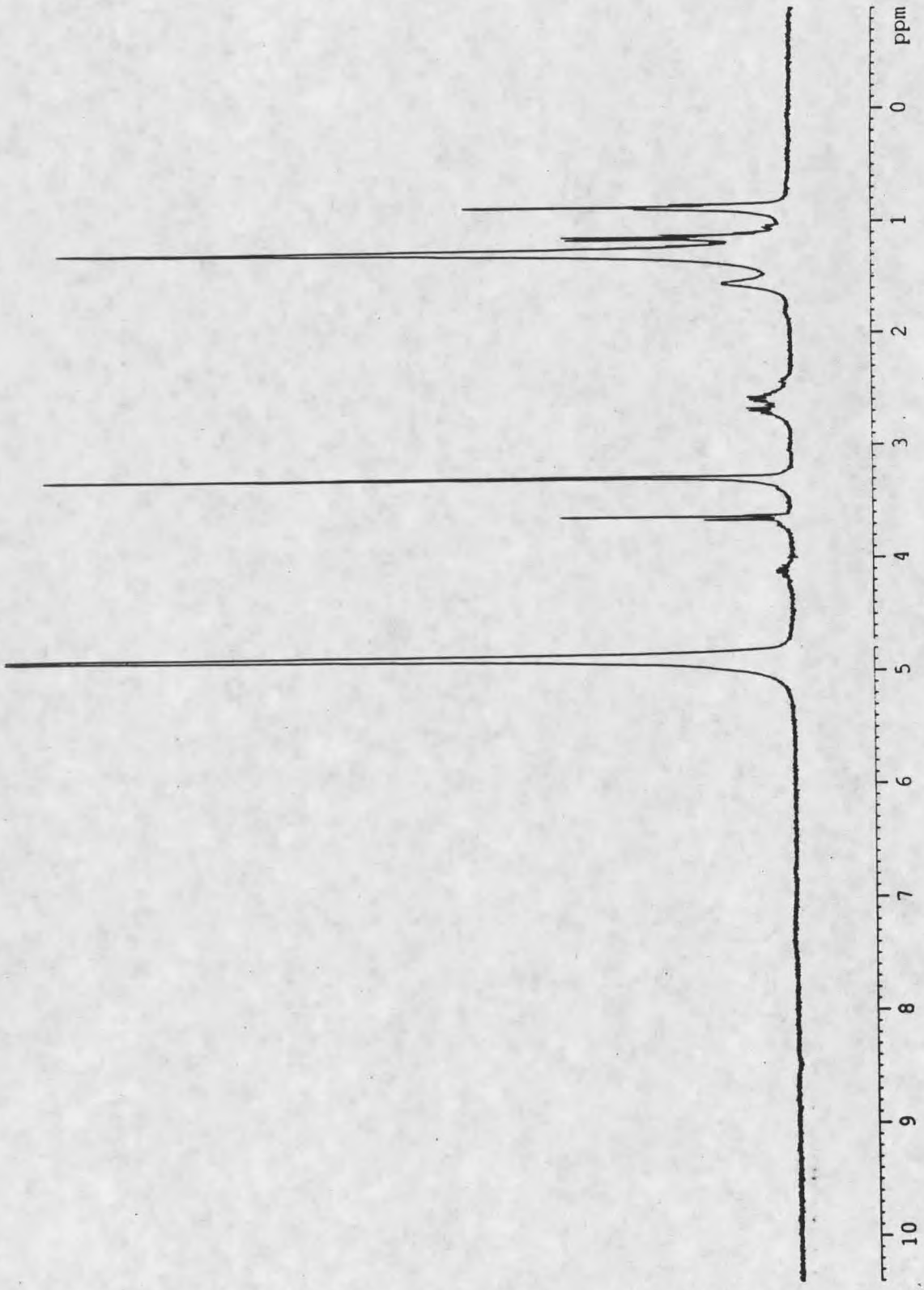


Figure 4.26 ^{1}H NMR of mono-amides of sphaeric acid

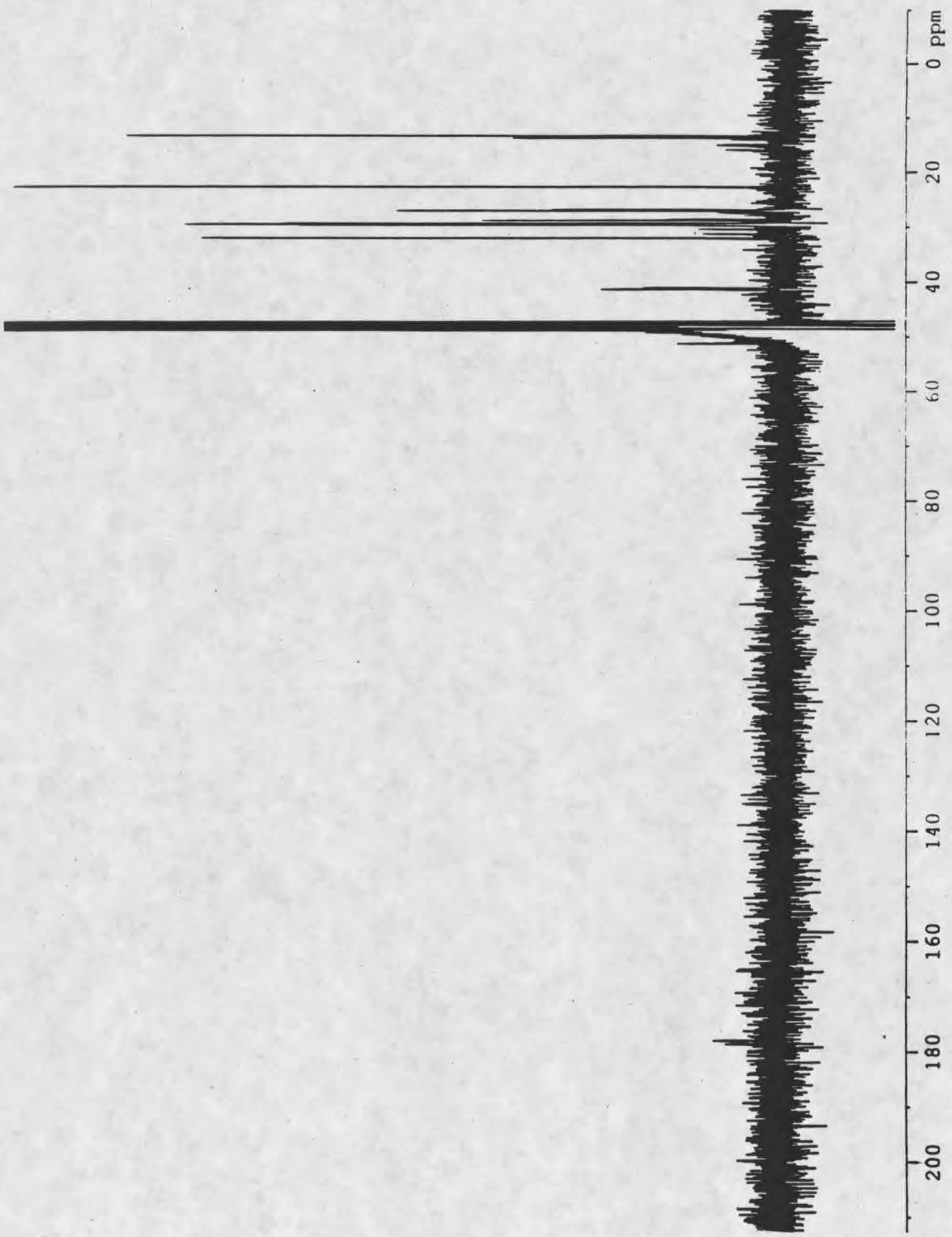


Figure 4.27 ^{13}C NMR of mono-amides of sphaeric acid

Figure 4.28 ^{1}H NMR of mono-methyl amides of sphaeric acid

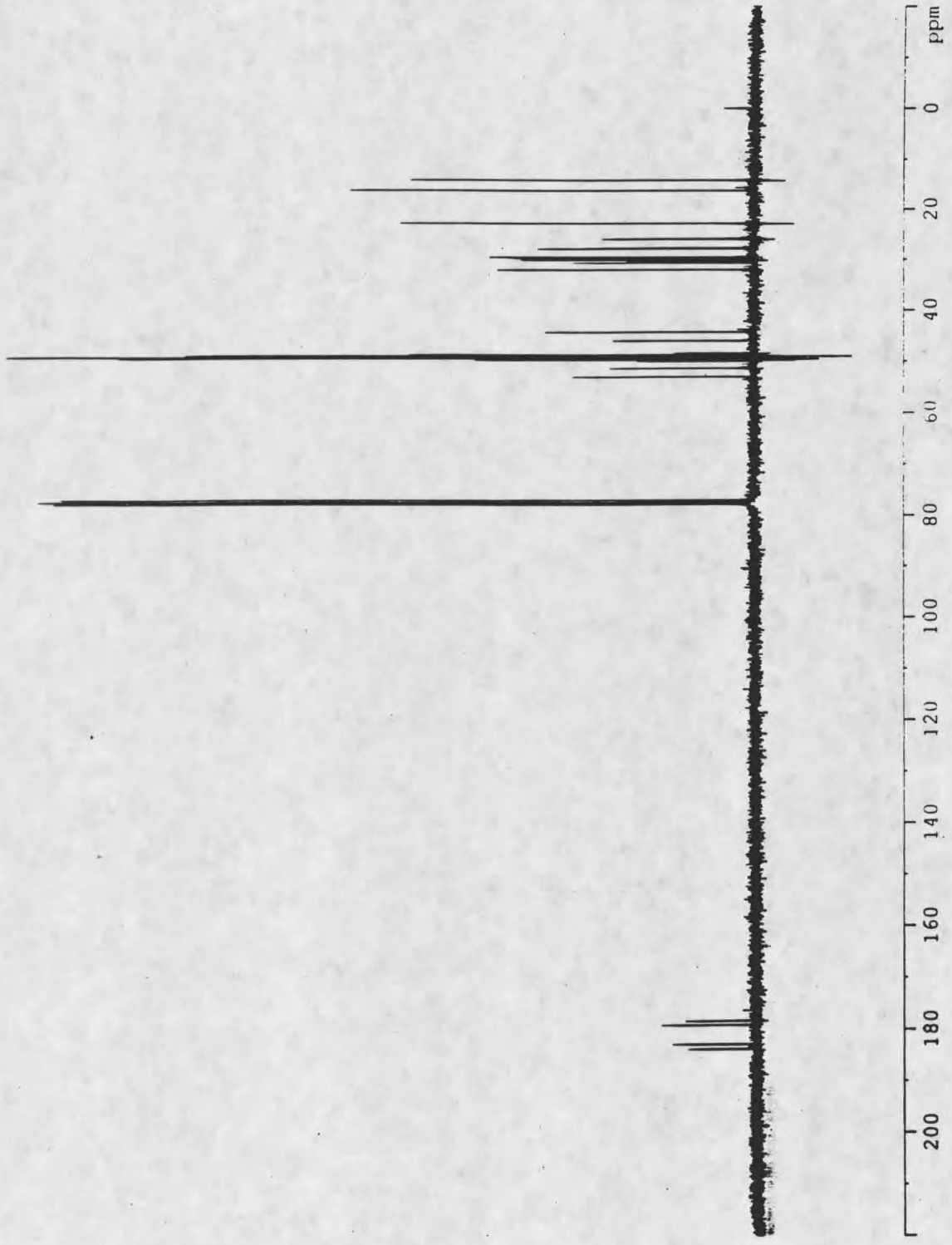


Figure 4.29 ^{13}C NMR of mono-methyl amides of sphaeric acid

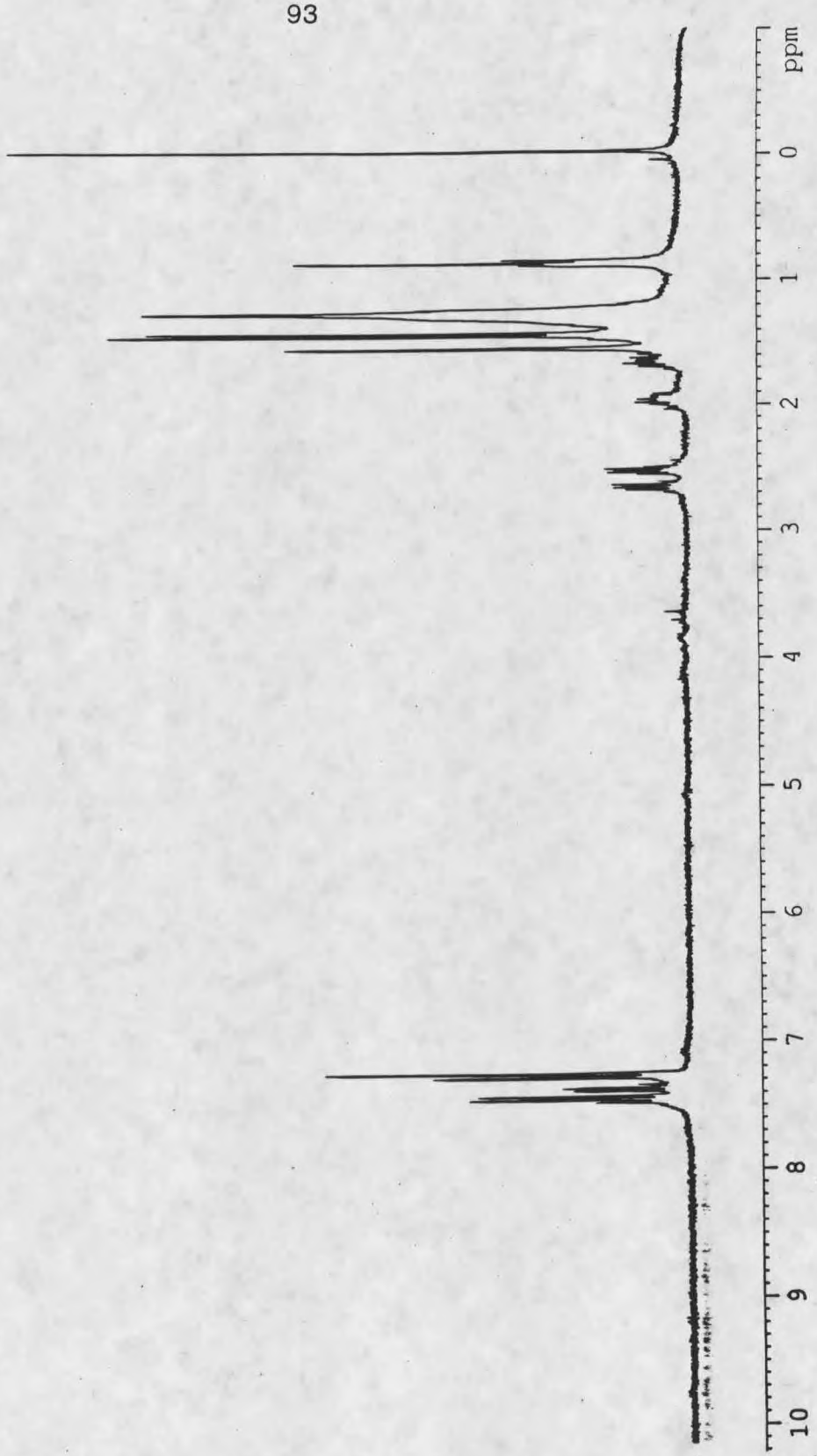


Figure 4.30 ^{1}H NMR of phenyl imide of sphaeric acid

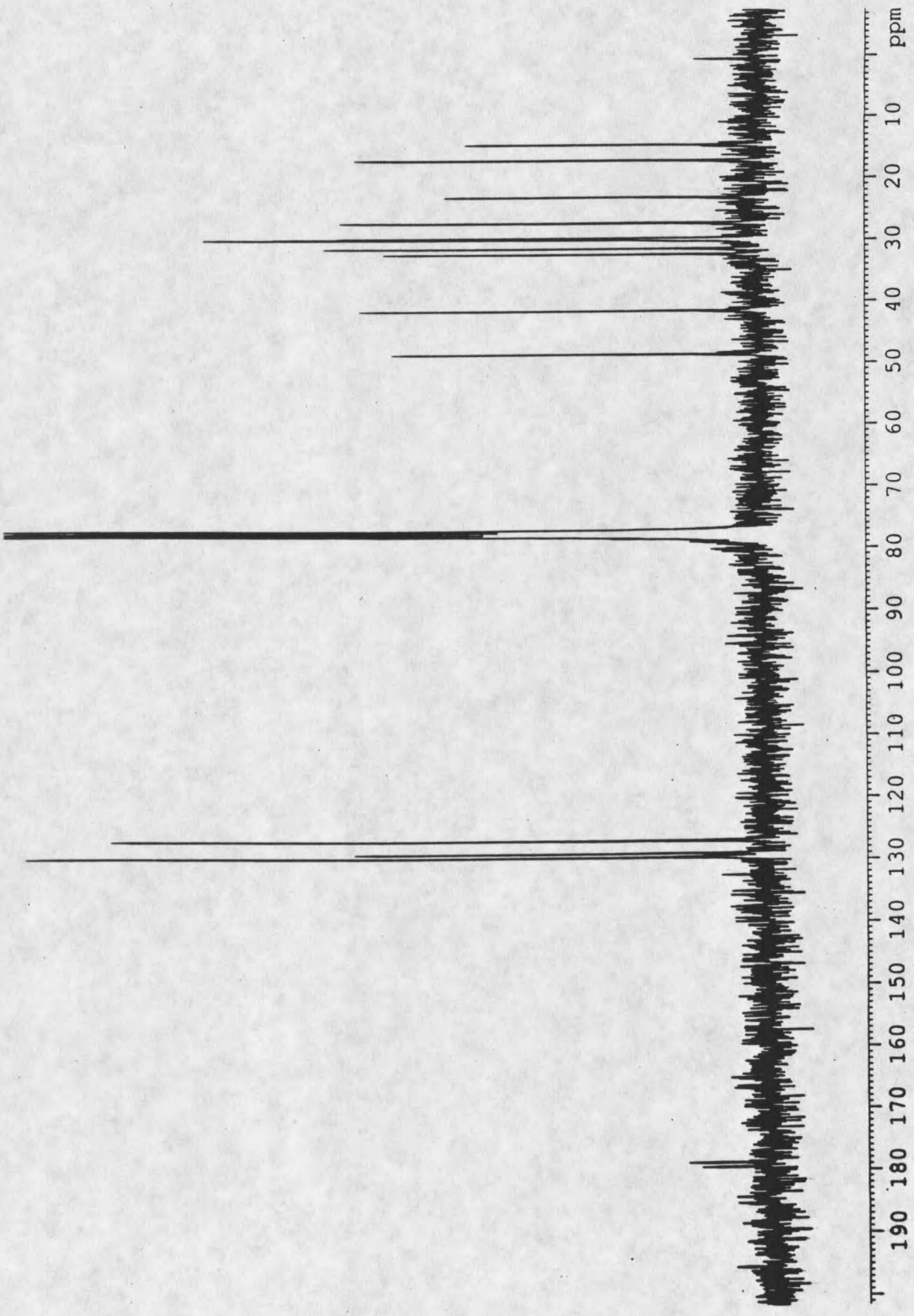


Figure 4.31 ^{13}C NMR of phenyl imide of sphaeric acid

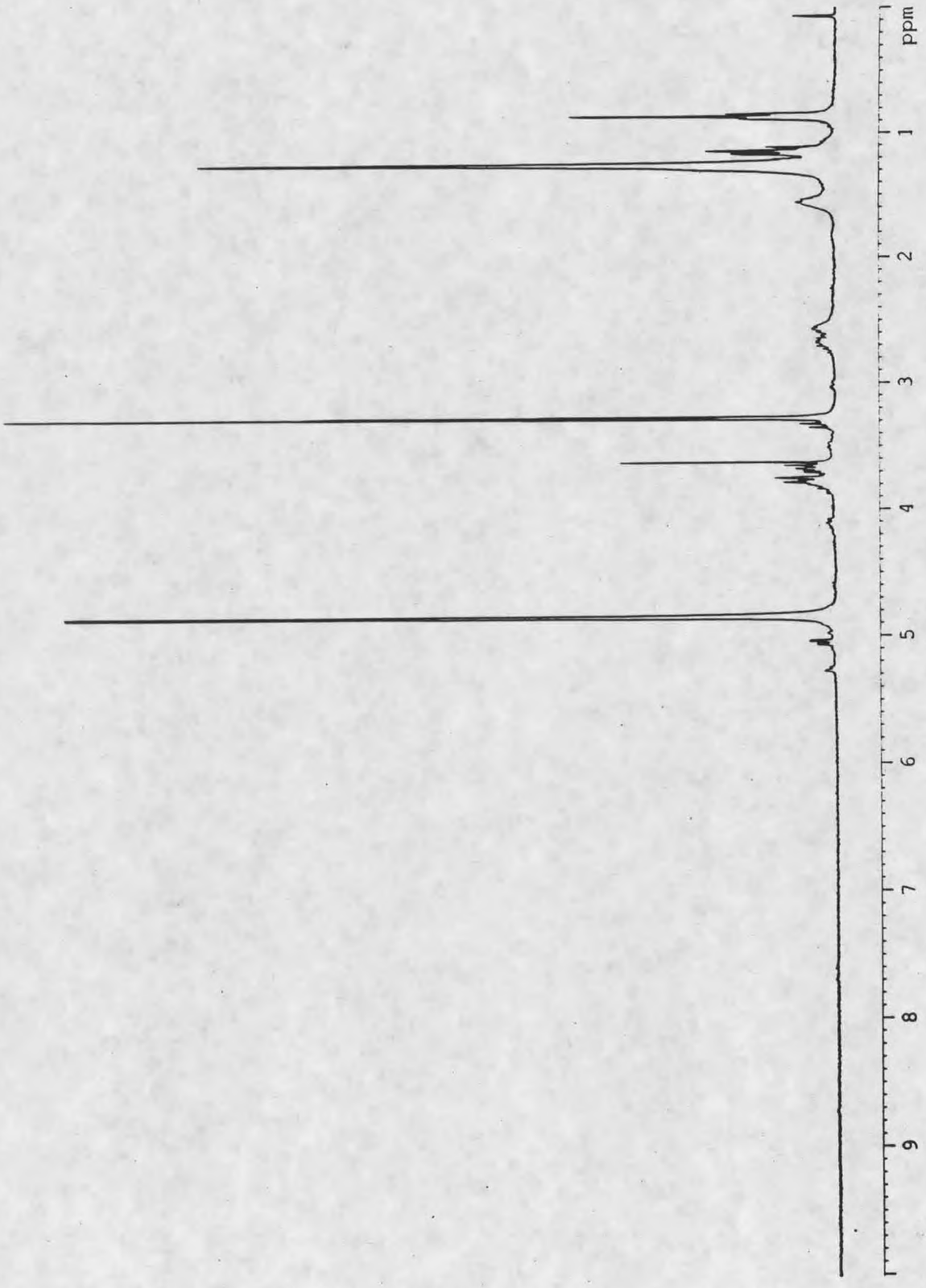


Figure 4.32 ^{1}H **NMR of mono-glucosamides of sphaeric acid**

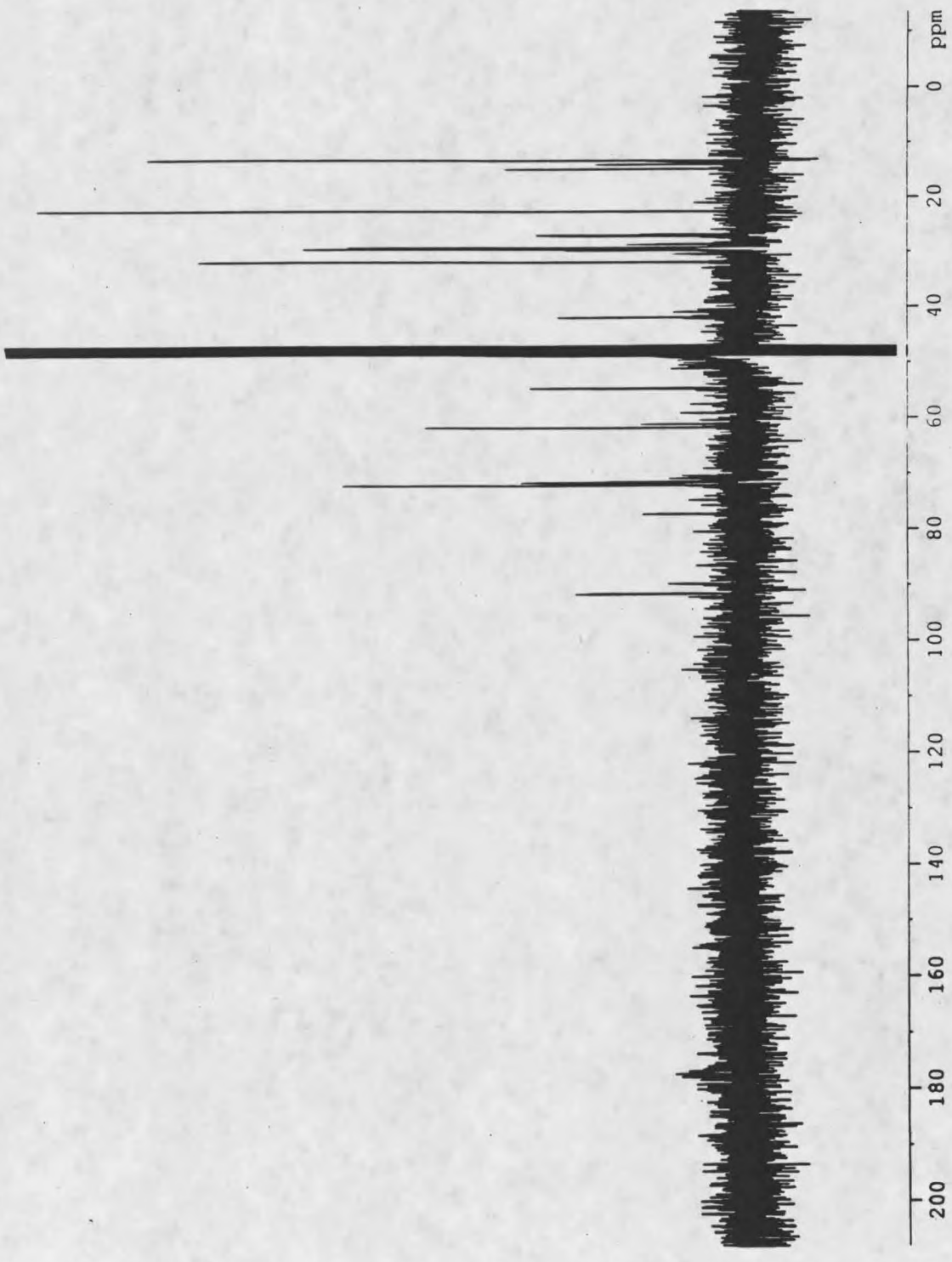


Figure 4.33 ^{13}C **NMR of mono-glucosamides of sphaeric acid**

Figure 4.34 ^{1}H NMR of carbonate of diol

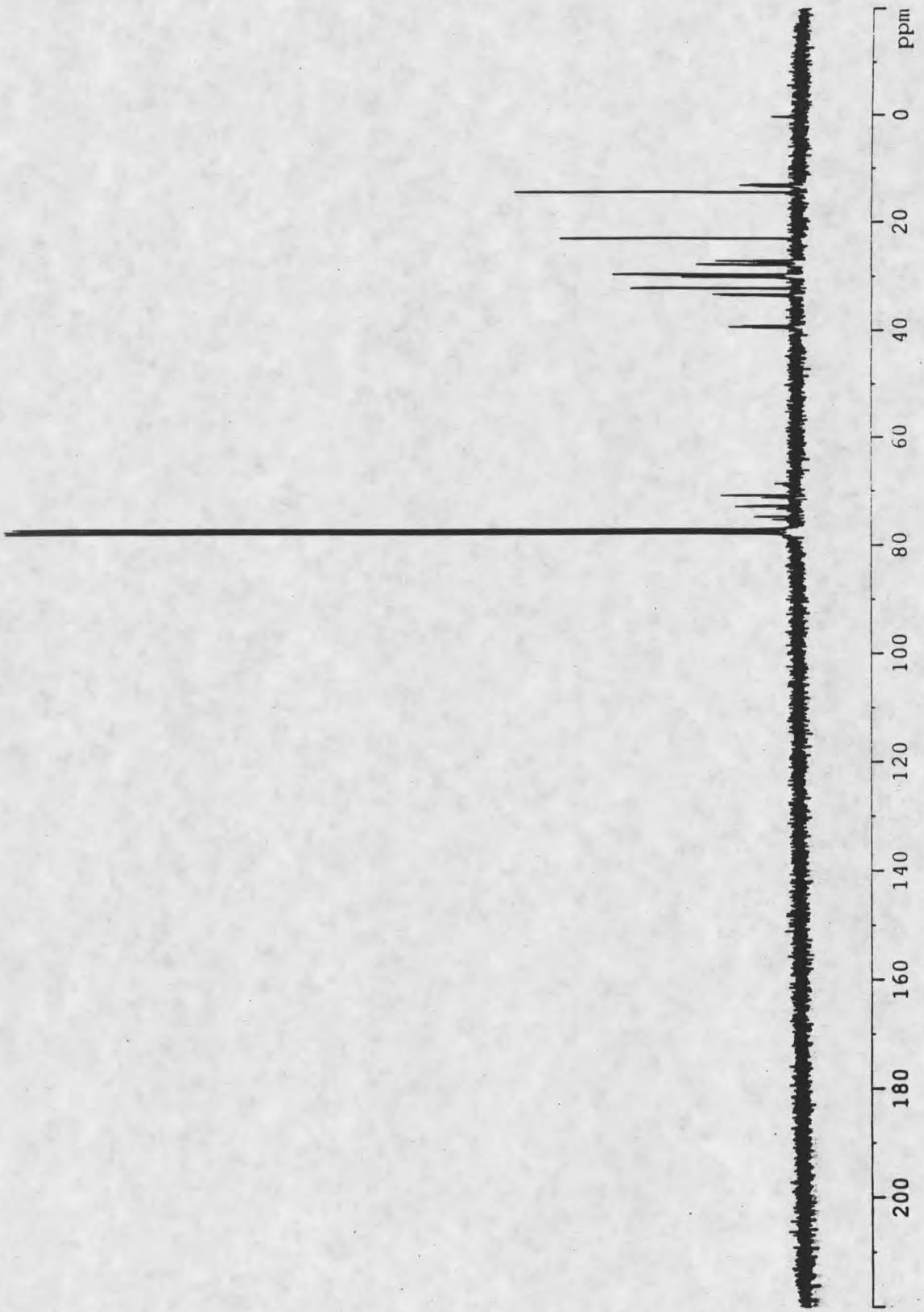


Figure 4.35 ^{13}C NMR of carbonate of diol

Chapter 5

MEDIA EFFECTS

There are many factors that affect screening for bioactive compounds from microorganisms. One of the most important of these is the fermentation process.[61] Fermentation factors that can change the production of secondary metabolites include both chemical/nutritional factors and physicochemical factors.

The physicochemical factors include pH, temperature, aeration, agitation, "knit ball" formation, and length of fermentation. Variations of pH and temperature within the growth-permissible ranges can alter the production of secondary metabolites. The amount of dissolved oxygen is also important for secondary metabolite production and fungal growth. One way to increase dissolved oxygen levels is by agitating the culture flasks on a rotary shaker. However, this also induces the formation of "knit balls", intertwined pellets of the filamentous mycelia of fungi. The size and density of the resulting "knit balls" can then change the amount of oxygen transferred into the cells within them. Finally, the length of fermentation can change the amount and type of secondary metabolites produced.[62] There is an initial log-phase growth stage in which the mycelial mass increases quickly, but few secondary metabolites are

produced in this stage. Most secondary metabolites are produced during the lag-phase following this, when growth rate slows down and production of secondary metabolites increases.

The chemical/nutritional factors influencing secondary metabolite production include glucose, ammonia, inorganic phosphate, metal ions, small molecule precursors, and host organism metabolites. Antibiotic production has been found to be reduced or eliminated when levels of glucose, amino acids, and other carbon and nitrogen sources exceed certain thresholds.[63] Ammonia and inorganic phosphate often suppress antibiotic production, while various metal ions can either promote or inhibit secondary metabolite production. The presence of small molecule precursors, such as amino acids, short chain fatty acids, and short chain alcohols, can enhance antibiotic production of metabolites containing these precursors or variations of them. Finally, host organism metabolites are important for stimulating antibiotic production. Especially with endophytic microbes which often cease production of secondary metabolites when removed from their host organisms.[64]

In order to increase the production and variety of bioactive compounds produced, five different media types were utilized for fermentation of the *Sphaeriopsis* sp., labled as MA-17, and the *Epicoccum* sp. labled as ME-19. The first two media differed in nutrient concentrations. Medium R-1 contained low nutrient concentrations, while medium R-2 contained much higer nutrient levels. Three additional media variations were also used and labled as A, B,

and C. An ammonium-ion depressed fermentation was carried out using medium A containing magnesium phosphate.[65] Medium B, containing magnesium carbonate, was used for a phosphate-ion depressed fermentation[66], and medium C, containing magnesium sulfate, was used as a metal ion spiked medium.

The fungi were grown in each of the five media types for 21 days and then subjected to the standard extraction scheme resulting in the five fractions shown below.

1) CH_2Cl_2: The methylene chloride extract of the aqueous media.
2) EtOAc: The ethyl acetate extract of the aqueous media.
3) H_2O: The remaining aqueous media after extractions.
4) m-CH_2Cl_2: The methylene chloride extract of the mycelia.
5) m-H_2O: The aqueous extract of the mycelia.

The results of the screening bioassays for each of these fractions for each fungus in each of the five media types are shown in Tables 5.1 to 5.4.

There were few variations in activity for the *Epicoccum* sp., ME-19, among the various media. The nutrient levels seemed to have little effect on antibiotic production. There are only a couple of differences between the low and high nutrient levels. The media/methylene chloride fraction of the low nutrient media showed some *E. coli* inhibition, while the ethyl acetate fraction of the high nutrient media had some *C. albicans* activity. The only major difference among

Media	Fraction	G. cand.	C. alb.	B. sub.	E. coli	P. aerug.	B.Shr.
R-1	CH_2Cl_2	++++	++++	+++++	+++	-	++*
R-1	EtOAc	-	-	+++	+	-	n/a
R-1	H_2O	-	-	+	-	-	-
R-1	m-org.	++	++	++	-	-	-
R-1	m-H_2O	-	-	-	-	-	-
R-2	CH_2Cl_2	+++	++++	++++	-	n/a	n/a
R-2	EtOAc	-	+++	+++	-	n/a	n/a
R-2	H_2O	n/a	n/a	n/a	n/a	n/a	n/a
R-2	m-CH_2Cl_2	+++	+++	++++	-	n/a	n/a
R-2	m-H_2O	-	-	-	-	n/a	n/a

Table 5.1 ME-19 High and Low Sugar Media
* Combined CH_2Cl_2 and EtOAc fractions

Media	Fraction	G. cand.	C. alb.	B. sub.	E. coli	P. aerug.	B.Shr.
A	CH_2Cl_2	+++	+++	+++++	++	+	-
A	EtOAc	-	-	+	-	-	-
A	H_2O	-	-	-	-	-	-
A	m-CH_2Cl_2	-	-	++	-	-	-
A	m-H_2O	-	-	-	-	-	-
B	CH_2Cl_2	+	+	+++	-	-	-
B	EtOAc	-	-	++	-	-	-
B	H_2O	-	-	-	-	-	-
B	m-CH_2Cl_2	-	-	+	-	-	-
B	m-H_2O	-	-	-	-	-	-
C	CH_2Cl_2	+++	++	+++++	+	-	-
C	EtOAc	-	-	+++	-	-	-
C	H_2O	-	+	-	-	-	-
C	m-CH_2Cl_2	-	-	+	-	-	-
C	m-H_2O	-	-	-	-	-	-

Table 5.2 ME-19 Trapping Media

Media	Fraction	G. cand.	C. alb.	B. sub.	E. coli	P. aerug.	B.Shr.
R-1	CH_2Cl_2*	-	+	+++	-	-	+++++
R-1	H_2O	-	-	-	-	-	-
R-1	m-CH_2Cl_2	-	-	+	-	-	-
R-1	m-H_2O	-	+	-	-	-	-
R-2	CH_2Cl_2	-	+	+++	-	n/a	n/a
R-2	H_2O	-	-	-	-	n/a	n/a
R-2	m-CH_2Cl_2	+	++	+++++	-	n/a	n/a
R-2	m-H_2O	-	-	+	-	n/a	n/a

Table 5.3 MA-17 Low and High Sugar Media
*Combined CH_2Cl_2 and EtOAc fractions

Media	Fraction	G. cand.	C. alb.	B. sub.	E. coli	P. aerug.	B. Shr.
A	CH_2Cl_2	+++	+++	+++++	++	++	++
A	EtOAc	+	+++++	+++++	+	++	+++++
A	H_2O	-	-	-	-	-	+++
A	m-CH_2Cl_2	++	++	++	-	+	-
A	m-H_2O	-	-	+	-	-	-
B	CH_2Cl_2	-	-	++	-	-	-
B	EtOAc	-	++	++	-	+	-
B	H_2O	-	-	-	-	-	-
B	m-CH_2Cl_2	-	+	-	-	-	-
B	m-H_2O	-	-	+++	-	-	+
C	CH_2Cl_2	++	+++	+++++	++	++	+
C	EtOAc	-	+	++	+++	+++	+++++
C	H_2O	-	+++	+	-	-	++++
C	m-CH_2Cl_2	-	+	++++	-	-	-
C	m-H_2O	-	-	-	-	-	-

Table 5.4 MA-17 Trapping Media

the other three media was a decrease in activity with medium B, the phosphate-ion depressed medium.

The *Sphaeriopsis* sp., MA-17, showed much more variety in activity for the various media. The high nutrient medium, R-2, had increased activities in the mycelial methylene chloride fraction compared to the low nutrient medium. Among the other three media, there are several differences. Again, there was a major decrease in activity levels with the phosphate-ion depressed medium B. The ethyl acetate fraction of medium A showed greater anti-fungal activity (*G. cand.* & *C. alb.*) than any of the other fractions. The organic fractions of medium C and medium A showed anti-bacterial (gram negative) activity (*E. coli* & *P. aerug.*) that wasn't present in either R-1 or R-2, with the highest activity being in the ethyl acetate fraction of medium C. There was additional brine shrimp activity in the aqueous fraction of A and C that wasn't present in the other media. Finally, there was better overall activity of the mycelial methylene chloride extracts in media R-2, A, and C, than in medium R-1.

Two compounds were isolated from the various media fractions. Succinic acid was isolated as the active agent in the brine shrimp bioassay of the aqueous fraction from MA-17 grown in medium A. A group of triacylglycerols were also isolated from the methylene chloride extract of the mycelia of MA-17 grown in R-2 medium.

Isolation and Structure Elucidation of Triacylglycerols

The *Sphaeriopsis* sp. labled as MA-17 was isolated from the inner bark of the mexican yew tree, *taxus globosa*. It was grown in still culture in R-2 medium for 21 days. The mycelia was removed by filtration through eight layers of cheesecloth, pulverized in an omni-mixer, and lyophilized to dryness. The mycelia were then soaked overnight in methylene chloride to extract any organic soluble compounds. The extract was chromatographed on Sephadex LH-20 and eluted with 1:1 $CHCl_3$:MeOH. Even though it was inactive, fraction two containing the triacylglycerols was studied due to its similarities, both physical and spectroscopic to sphaeric acid. The triacylglycerols, as well as sphaeric acid, were found in the second fraction of the initial Sephadex LH-20 separation of the crude extract and contained a large portion of the initial extract. Additionally, both fractions were pale yellow oils at room temperature. A proton NMR spectrum of the triacylglycerols also showed similarities to sphaeric acid. Both had large methylene envelopes at approximately 1.3 ppm, as well as some downfield peaks (2.0 and 2.3 for triacylglycerols and 2.5 and 2.7 for sphaeric acid). There was an additional peak at 5.3 in the spectrum of the triacylglycerols that suggested some unsaturation in the structure.

Initial EIMS results showed a molecular mass of over 500, much higher than the 244 molecular weight of sphaeric acid. Inspection of the carbon NMR then suggested the presence of a triacylglycerol. The peaks at 173 and 174 ppm were indicative of acid, amide, or ester functionalities. The methine peak at 70

ppm and the methylene at 63 ppm accounted for the oxygen bearing signals of the glycerol backbone. The only remaining signals in the carbon NMR were the methine carbons from 128-130 ppm due to unsaturation in the fatty acids, the terminal methyls at 15 ppm, and the methylene carbons ranging from 23 ppm to 35 ppm.

In order to determine the fatty acids present in the triacylglycerols, the fat was hydrolyzed to its component free fatty acids and the glycerol backbone. The hydrolysis solution was acidified and extracted with methylene chloride to remove the fatty acids, and the glycerol backbone remained in the aqueous solution.

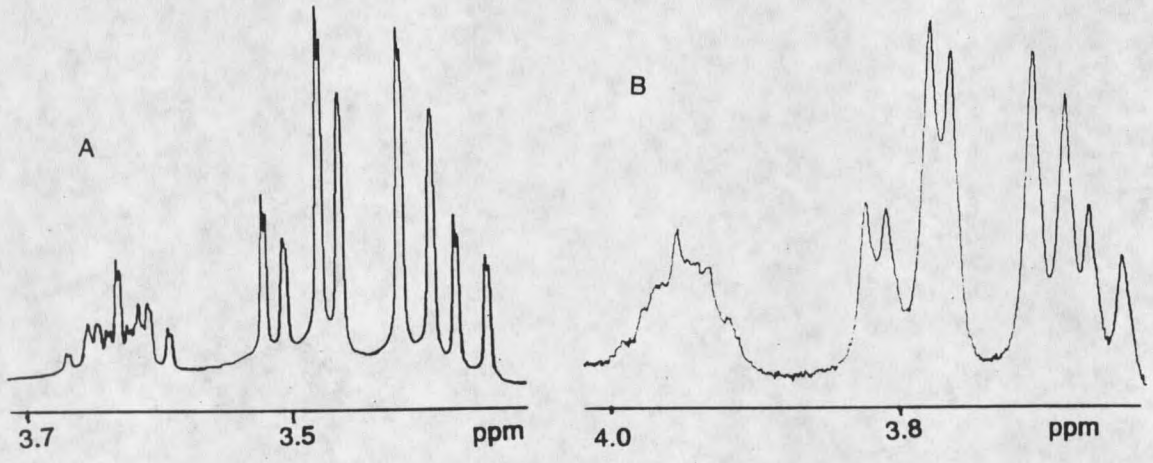


Figure 5.1 Proton NMR of Glycerine (A) and Glycerol Backbone (B)

The aqueous fraction containing the glycerol backbone had only two signals in the carbon NMR, a methine at 73 ppm and a methylene at 63 ppm. These

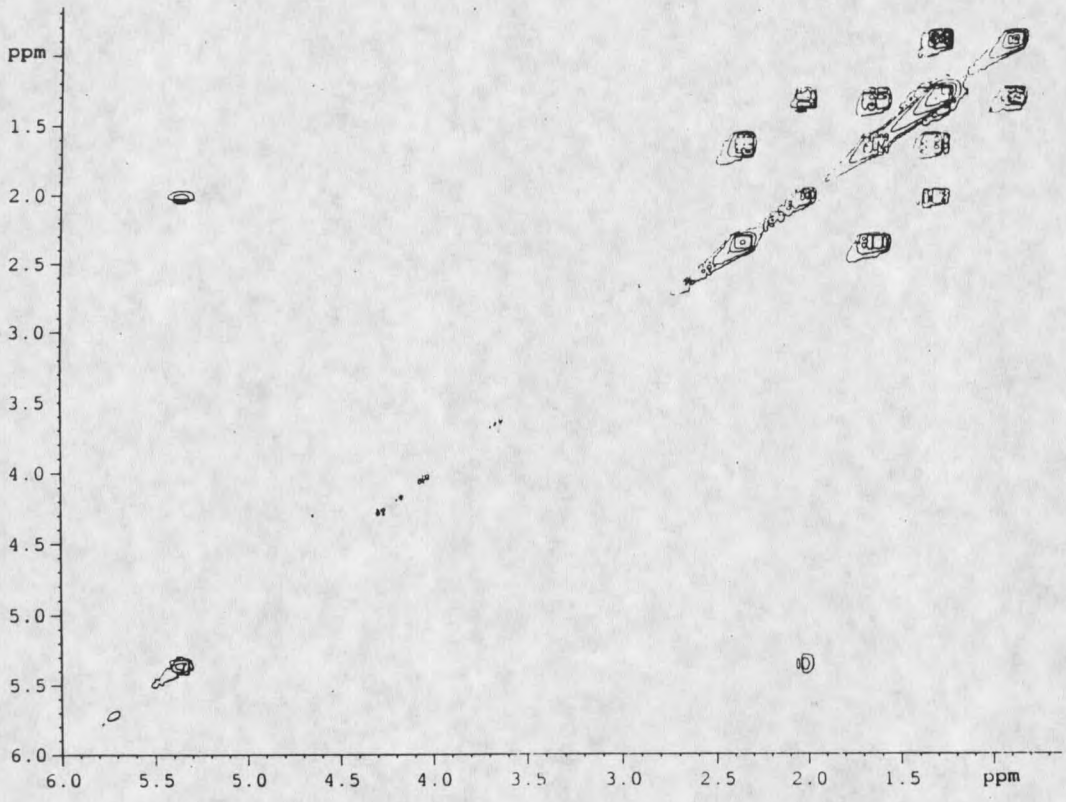


Figure 5.2 COSY of Hydrolized Fatty Acids

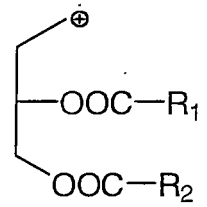
signals are typical of methine and methylene signals attached to an electron withdrawing group such as an oxygen. The proton NMR spectrum was identical to the spectrum of a glycerine sample (Figure 5.1).

The organic fraction, containing the free fatty acids, was subjected to diazomethane methylation, and subjected to GC/MS analysis. The mass spectral library search of the GC/MS analysis showed the presence of two major fatty acids, palmitic (C_{16}) and oleic (cis-C_{18}, ω-9). There was also some evidence for other acids, but in only small amounts, including myristic (C_{14}),

palmitoleic (cis-C_{16}, ω-9), stearic (C_{18}), 9,10-epoxyoctadecanoic acid, eicosanoic (C_{20}), docosanoic (C_{22}), and tetracosanoic (C_{24}). There was even a small amount of partially hydrolized product with only one oleic acid group attached to the glycerol backbone.

A COSY spectrum (Figure 5.2) of the hydrolized fatty acids allowed for the assignment of the signals in the proton NMR. The four protons at 5.3 ppm were due to the double bond protons in oleic acid. These were coupled to the eight adjacent protons at 2.0 ppm. The triplet at 2.3 ppm, integrating to six protons, was appropriate for the methylenes adjacent to the three ester carbonyls[67], and were coupled to the adjacent six methylene protons at 1.6 ppm. Finally, the nine terminal methyl protons, coupled to the methylene envelope, resonated at 0.8 ppm, typical for terminal methyls of alkyl chains.[68] The remaining peaks in the triacylglycerol proton spectrum from the glycerol backbone may be assigned. The methylene protons adjacent to the oxygen were the doublets of doublets at 4.1 and 4.3 ppm, and the methine proton was the multiplet at 5.1 ppm.[69] The additional triplet at 2.7 ppm, which integrated to 1.3 protons, did not appear to couple to any other signals in the fatty acids, and was assumed to be some impurity which was not separated from the triacylglycerol.

The integrations of the proton NMR suggested that there were approximately two double bonds (four olefinic protons) per glycerol. This suggested a ratio of two oleic acid groups to one palmitic acid group for each molecule of glycerol.



I. R_1, $R_2 = (CH_2)_{14}CH_3$

2.$R_1 = (CH_2)_7CH{=}CH(CH_2)_7CH_3$, $R_2 = (CH_2)_{14}CH_3$

3. R_1, $R_2 = (CH_2)_7CH{=}CH(CH_2)_7CH_3$

Figure 5.3 Possible FABMS ions

However, the FABMS spectrum of the original triacylglycerol showed peaks for each of the three possible combinations resulting from the loss of one fatty acid shown in Figure 5.3.

So, even though there was the 2:1:1 ratio, it appears that at least the two possible combinations for the triacylglycerol shown in Figure 5.4 are present as well as possibly the two versions with all palmitic acid groups or all oleic acid groups.

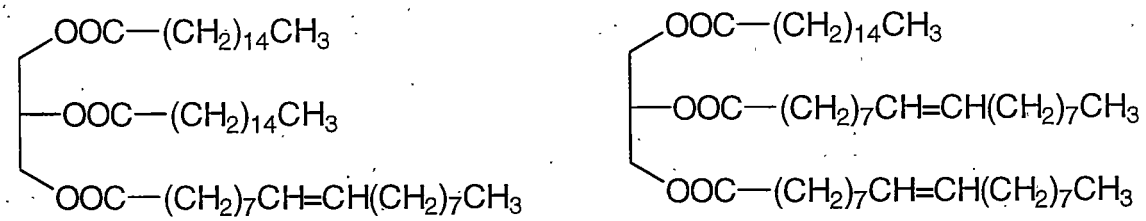


Figure 5.4 Possible triacylglycerol structures

Isolation and Structure Elucidation of Succinic Acid

The *Sphaeriopsis* sp. labled as MA-17 was isolated from the inner bark of the mexican yew tree, *taxus globosa*. It was grown in still culture in medium A for 21 days. The mycelia was removed by filtration through eight layers of cheesecloth and the filtrate was extracted with methylene chloride and ethyl acetate. The remaining aqueous layer was lyophilized to dryness and triturated with methanol. The methanol fraction was chromatographed on Sephadex LH-20 and eluted with 9:1 H_2O:MeOH. The active fractions were chromatographed on Toyopearl TSK gel HW-40 F and eluted with 2:1 MeOH:H_2O. The water insoluble compounds of the active fraction were removed by filtration, and the aqueous fraction was then subjected to reverse phase HPLC and eluted with a step gradient from 9:1 H_2O:MeOH to MeOH. The active fraction was then subjected to reverse phase HPLC and eluted with a stepwise gradient from water through 50% methanol in water and pure succinic acid was collected.

The structure of succinic acid was elucidated solely by NMR spectroscopy. The carbon NMR signal at 175 ppm suggested the presence of a carboxylic acid group and the methylene signal at 29 ppm was typical of a methylene carbon adjacent to a carboxylic acid (27.2 for the methylene carbon of methyl propionate[70]). The presence of only one singlet in the proton NMR at 2.5 suggested a symmetric molecule. This suggested that the isolated compound

was either succinic acid or malonic acid. Since the methylene signal adjacent to two carboxylic acid groups would have a chemical shift of 3.3 ppm[71], and one adjacent to only one would be at 2.3 ppm[72], the isolated acid was most likely succinic acid. This was supported by the base peak of the CIMS of 119 which coincides with the molecular weight plus a proton. However, there were some weaker high mass peaks due to impurities.

Experimental

Culture conditions for triacylglycerols

MA-17 was sustained on M-1-D agar containing 1% yew broth. MA-17 was grown in still culture in two liter erlenmeyer flasks and 1000 milliliter Roux flasks in R-2 media containing 1% yew broth for 21 days. Since MA-17 grew on the surface of the culture medium, the amount of liquid used in each flask was chosen to give the largest surface area for growth.

Culture conditions for succinic acid

MA-17 was sustained on M-1-D agar containing 1% yew broth. MA-17 was grown in still culture in two liter erlenmeyer flasks and 1000 milliliter Roux flasks in medium A for 21 days. Since MA-17 grew on the surface of the culture medium, the amount of liquid used in each flask was chosen to give the largest surface area for growth.

Extraction and Isolation of triacylglycerols

The fungal mycelia were removed by filtration through eight layers of cheesecloth, pulverized in an omni-mixer, and lyophilized to dryness. The mycelia were then soaked overnight in methylene chloride to extract any organic soluble compounds. The extract was chromatographed on a Sephadex LH-20 column (2.8 X 110 cm), and eluted with 1:1 $CHCl_3$:MeOH. Eight fractions were collected and fraction two (25 mg/L) containing the triacylglycerols was studied due to its similarities, both physical and ^1H NMR, to sphaeric acid.

Extraction and Isolation of succinic acid

The fungal mycelia were removed by filtration through eight layers of cheesecloth, and the filtrate was extracted three times with 400 mL portions of methylene chloride and two times with 400 mL portions of ethyl acetate. The remaining aqueous layer was frozen and lyophilized to dryness, and triturated with 200 mL of methanol to remove any methanol soluble compounds. The methanol fraction was chromatographed on a Sephadex LH-20 column (2.5 X 55 cm) and eluted with 9:1 H_2O:MeOH. Nine fractions were collected and isolation of succinic acid was carried out by activity-guided fractionation using the brine shrimp toxicity bioassay. Fractions two and three (78 mg/L) were chromatographed on a Toyopearl TSK gel HW-40 F column (2.5 X 40 cm) and eluted with 2:1 MeOH:H_2O. Four fractions were collected with the succinic acid

contained in fraction three (39 mg/L). The water insoluble compounds were removed by filtration through a J.T. Baker SPE disposable filtration column with a 20 µm frit. The aqueous fraction (38 mg/L) was then subjected to HPLC through a Rainin Dynamax 60 Å C-18 column (21.4mm X 25 cm) and eluted sequentially with 1) 9:1 H_2O:MeOH, 2) 1:1 H_2O:MeOH, 3) MeOH, and 4) methylene chloride. Five fractions were collected with the succinic acid contained in fractions one and two (21 mg/L). These were then subjected to HPLC through a Rainin Dynamax 60 Å C-18 column (21.4mm X 25 cm) and eluted with a stepwise gradient from water through 2%, 4%, 6%, 10%, and 50% methanol in water and rinsed with methanol. Thirteen fractions were collected with pure succinic acid collected in fractions three and four (8 mg/L).

Hydrolysis of triacylglycerols

Triacylglycerol (28 mg) in MeOH (0.1 mL) was placed in a 3 mL conical vial with NaOH (0.1 g) dissolved in H_2O (1.0 ml). The solution was allowed to reflux for 22 hours, then cooled, acidified, and extracted with five 5 mL portions of methylene chloride. The free fatty acids were recovered by rotoevaporation of the solvent and the glycerol was recovered by lyophilization of the aqueous phase.

Methylation of fatty acids

The isolated fatty acids (28 mg) were dissolved in anhydrous diethyl ether (3 mL) and placed in the bottom of an Aldrich MNNG-diazomethane kit. MNNG

(1-methyl-3-nitro-1-nitrosoguanidine) (29 mg) was placed in the upper tube. The apparatus was immersed in an ice bath and 5 M NaOH (0.6 mL) was added dropwise to produce the diazomethane. The reaction was allowed to sit for one hour to go to completion.

Physical/Chemical Data for triacylglycerols

Pale yellow oil; IR ν_{max} (cm^{-1}, neat) 2919, 2849, 1743, 1461; CIMS m/z 849 (01), 766 (66), 738 (47), 712 (67), 612 (100); FABMS m/z 603.4 (37), 577.4 (100), 551.4 (33); ^1H NMR δ ($CDCl_3$) 5.3 (4H, m), 5.2 (1H, m), 4.3 (2H, dd, J = 11.9, 4.3 Hz), 4.1 (2H, dd, J = 5.9, 11.9 Hz), 2.3 (6H, t, J = 5.9 Hz), 2.0 (8H, m), 1.6 (6H, m), 1.2 (64H, br s), 0.8 (9H, m); ^{13}C NMR δ ($CDCl_3$): 173.9 (C), 173.8 (C), 173.4 (C), 133.8 (CH), 130.9 (CH), 130.7 (CH), 130.4 (CH), 128.8 (CH), 128.5 (CH), 69.7 (CH), 62.8(CH_2), 34.9 (CH_2), 34.7 (CH_2), 32.6 (CH_2), 32.2 (CH_2), 30.5 (CH_2), 30.4 (CH_2), 30.3 (CH_2), 30.2 (CH_2), 30.0 (CH_2), 29.9 (CH_2), 29.8 (CH_2), 29.7 (CH_2), 27.9 (CH_2), 27.8 (CH_2), 26.3 (CH_2), 25.6 (CH_2), 25.5 (CH_2), 23.3 (CH_2), 23.2 (CH_2), 14.7(CH_3).

Physical/Chemical Data for succinic acid

White solid;CIMS m/z $[M+H]^+$ 119 (100); ^1H NMR δ (CD_3OD) 2.5 (s); ^{13}C NMR δ (CD_3OD) 175 (C), 29 (CH_2).

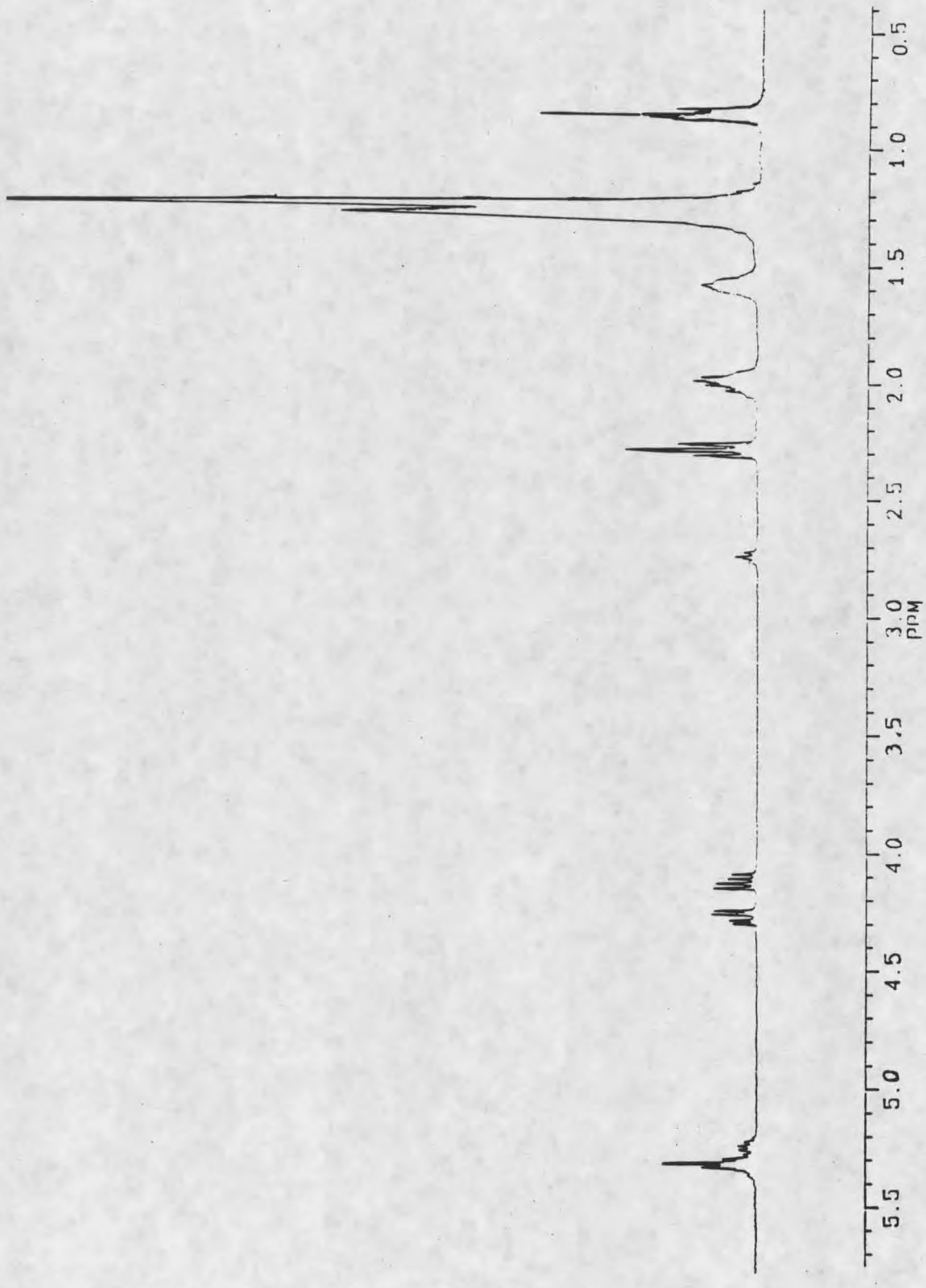


Figure 5.5 ^{1}H NMR of Triacylglycerols

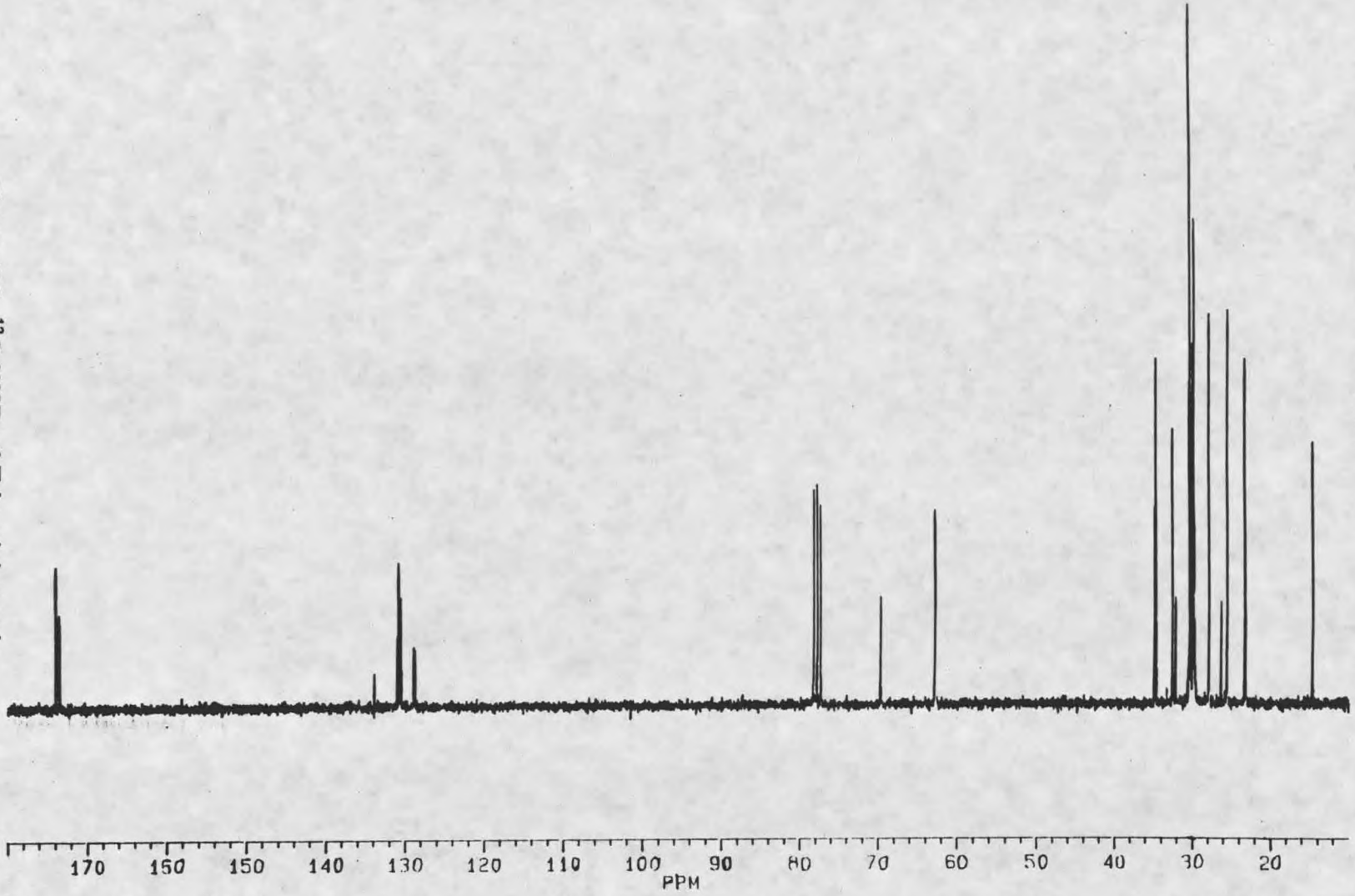


Figure 5.6 ^{13}C NMR of Triacylglycerols

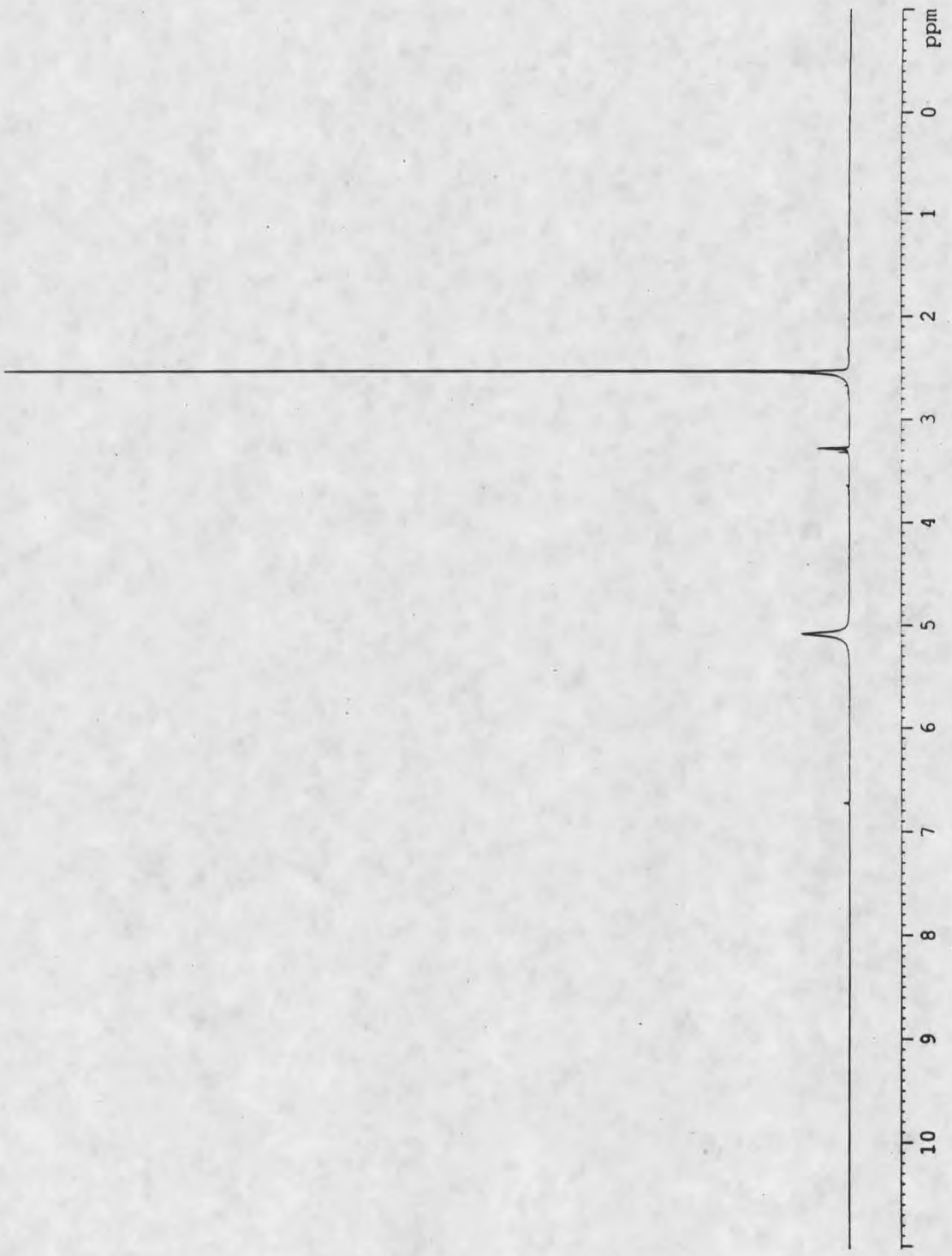


Figure 5.7 ^{1}H NMR of Succinic Acid

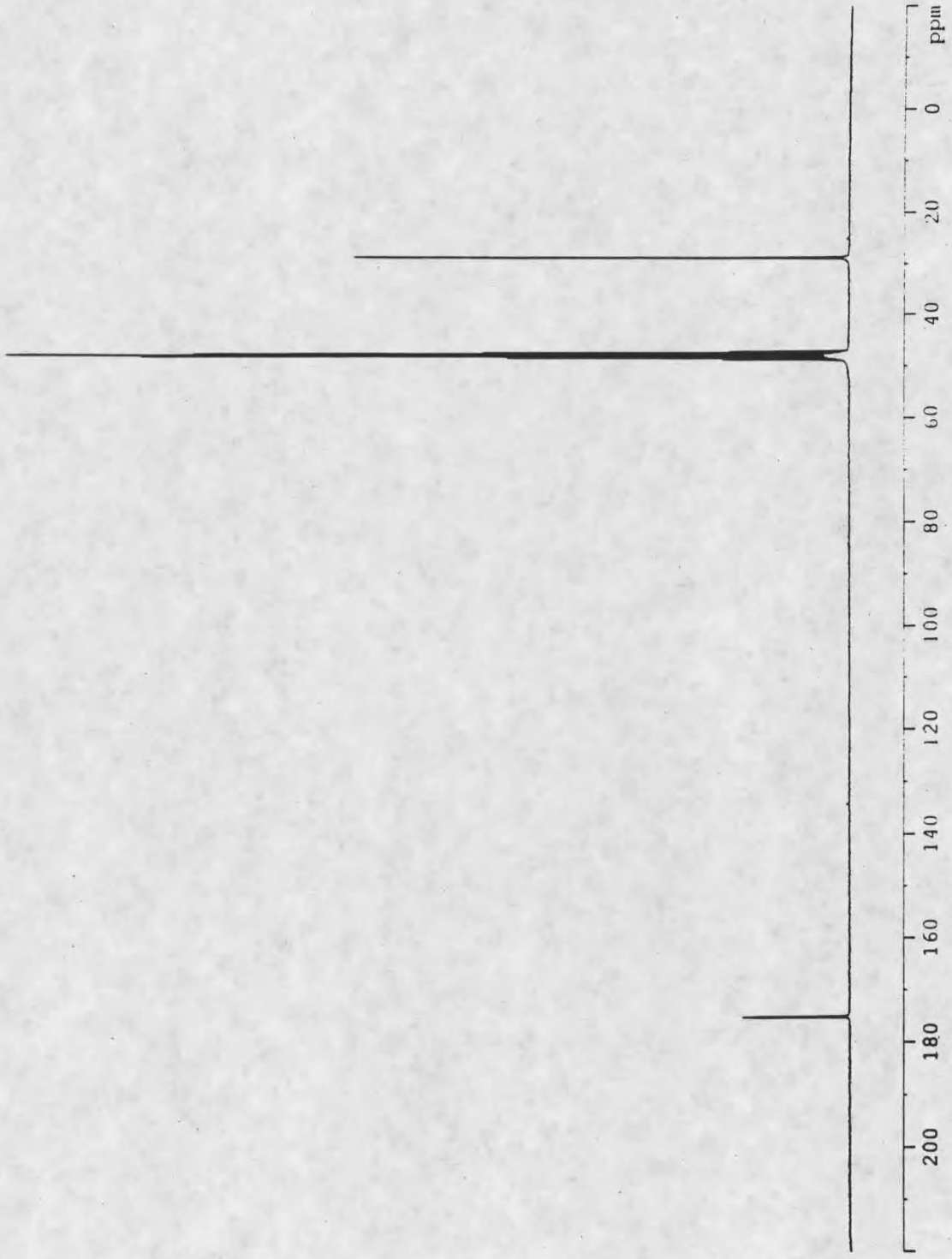


Figure 5.8 ^{13}C NMR of Succinic Acid

Chapter 6

EPICORAZINE A

The second interesting activity from the preliminary bioassays of the crude extracts was the strong anti-fungal activity of the ME19 F/O fraction (Table 1.1). Activity-guided fractionation of this extract led to the isolation of the previously known compound, epicorazine A (Figure 6.1).

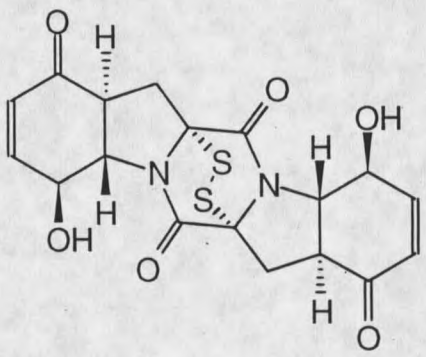


Figure 6.1 Epicorazine A

Epicorazine A is in the class of epipolythiopiperazine-3,6-diones, a potent class of fungal metabolites containing the basic ring structure shown in Figure 6.2. Compounds within this class show a wide variety of biological activity from antiviral and antifungal activities to high mammalian toxicities.[73] The simplest member of this family, 1,4-dimethyl-2,5-epidithiopiperazine-3,6-dione has many

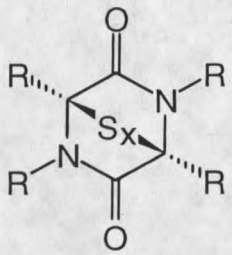
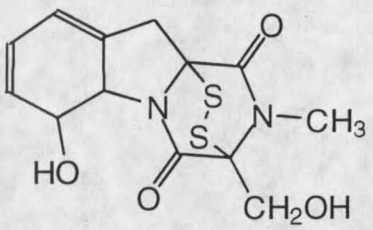


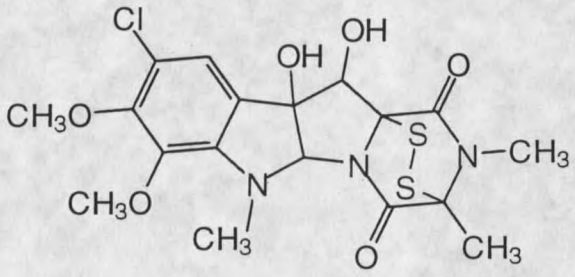
Figure 6.2 General epipolythiopiperazine-3,6-dione

of the biological properties of the more complex, naturally occuring, compounds, showing the importance of the basic ring structure in the biological activity.[74]

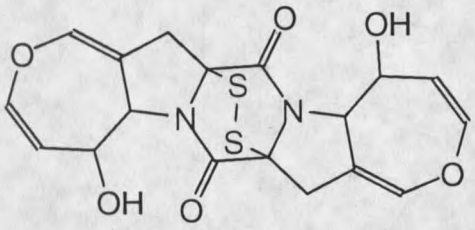
The first molecule in this class, gliotoxin (Figure 6.3), was originally isolated in 1932, but it's structure wasn't elucidated until 1958. Gliotoxin, originally isolated from *Gliocladium* spp., is a common antifungal compound and many related compounds have been discovered.[75] Another example discovered at about the same time is Sporidesmin (Figure 6.3), produced by *Pithomyces chartarum*. It was isolated as a toxin inhibiting the growth of sheep and cattle eating moldy fodder.[76] With the developement of antiviral screens, several more classes of epipolythio piperazine-3,6-diones were discovered, including aranotin and hyalodendrin[77] (Figure 6.3). Beyond the central ring structure, the structures in these compounds vary widely, including spirocyclic structures such as sirodesmin A[78] (Figure 6.3). There are even some variations in the attachment of the polysulfide bridge, such as in aspirochlorine[79] (Figure 6.3), as well as in the number of sulfur atoms bridging the central ring structure such as sirodesmin C[80] and Sporidesmin G[81] (Figure 6.3).



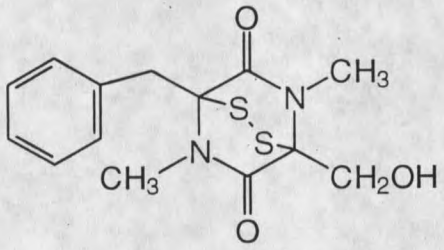
Gliotoxin



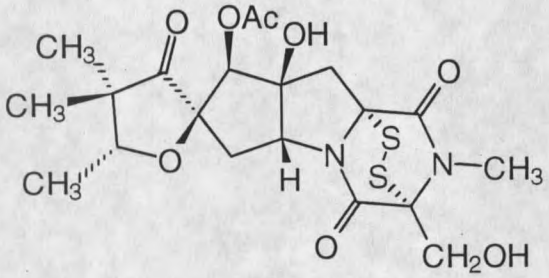
Sporidesmin



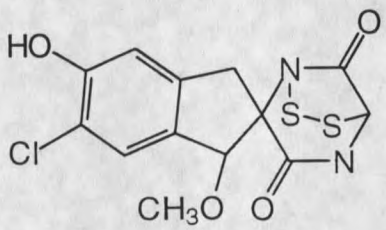
Aranotin



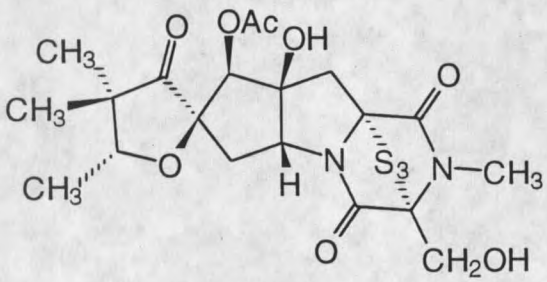
Hyalodendrin



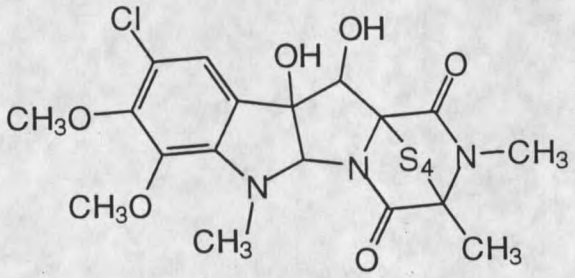
Sirodesmin A



Aspirochlorine



Sirodesmin C



Sporidesmin G

Figure 6.3 Epipolythiopiperazine-3,6-diones

Isolation and Structure Elucidation of Epicorazine A

The *Epicoccum* sp., labled as ME-19, isolated from the inner bark of the mexican yew tree, *Taxus globosa*, was grown in still culture in R-1 media for 21 days. The mycelia were removed by filtration through eight layers of cheesecloth and the filtrate was extracted with methylene chloride. The extract was chromatographed on Sephadex LH-20 and eluted with 1:1 $CHCl_3$:MeOH. The active fractions were triturated with methanol and the insoluble fraction contained Epicorazine A which crystallized from 2:1 $CHCl_3$:MeOH.

The EIMS showed an intense peak at 356 which corresponded to the $M^+ - S_2$ mass having the formula $C_{18}H_{16}N_2O_6$. The molecular formula of $C_{18}H_{16}N_2O_6S_2$ allowd for twevle sites of unsaturation. Since the number of carbon and proton signals was exactly half the number in the molecular formula the molecule must be a dimer. The carbon NMR signals at 194, 151, and 130 ppm suggested the presence of an unsaturated ketone, and the signal at 165 ppm suggested the presence of an acid, ester, or amide functional group. The IR signal at 1674 cm^{-1} was also idicative of an unsaturated ketone and the peak at 1700 cm^{-1} confirms that the other carbonyl signal was an amide. The IR signal at 3360 cm^{-1} denoted the presence of an alcohol. This accounted for six of the sites of unsaturation (three sites doubled for the dimerization), so the remaining six sites must be rings.

Starting with the unsaturated ketone, and using the COSY spectrum (Figure 6.4) and the information from the coupling constants (Table 6.1) the structural

C	Chemical Shift[a]	H	Chemical Shift (300 MHz, J Hz)
A	31.8 t	a	2.57 (1H, dd, 14.8, 5.5)
B	49.7 d	b	3.02 (1H, dd, 14.8, 12.6)
C	70.2 d	c	3.25 (1H, dt, 13.0, 12.6, 5.5)
D	71.8 d	d	3.80 (1H, dd, 13.0, 8.1)
E	76.5 s	e	4.78 (1H, dd, 8.1, 1.6)
F	129.9 d	f	5.83 (1H, OH)
G	151.4 d	g	6.13 (1H, dd, 10.3, 1.6)
H	165.0 s	h	6.92 (1H, d, 10.3)
I	194.2 s		

Table 6.1 NMR Data of Epicorazine A
[a] multiplicities based on DEPT spectra

backbone of Figure 6.5 was put together. The assignments of the alkene protons g and h were typical of an unsaturated ketone with the alkene proton opposite the carbonyl being downfield of the alkene proton adjacent to the carbonyl. The coupling constant between protons g and e, 1.6 Hz, was in the range of 1-2 Hz of a typical allylic relationship.[82] The carbon signal at 31.8 ppm was determined to be a methylene carbon by the DEPT spectra, requiring two of the remaining protons to be geminal protons. Of the remaining protons, the largest coupling constant of 14.8 Hz between protons a and b was typical of geminal protons (12-15 Hz for typical geminal coupling[83]). The vicinal connections of protons e to d, d to c, and c to a and b were made from the coupling constants given in Table 6.1.

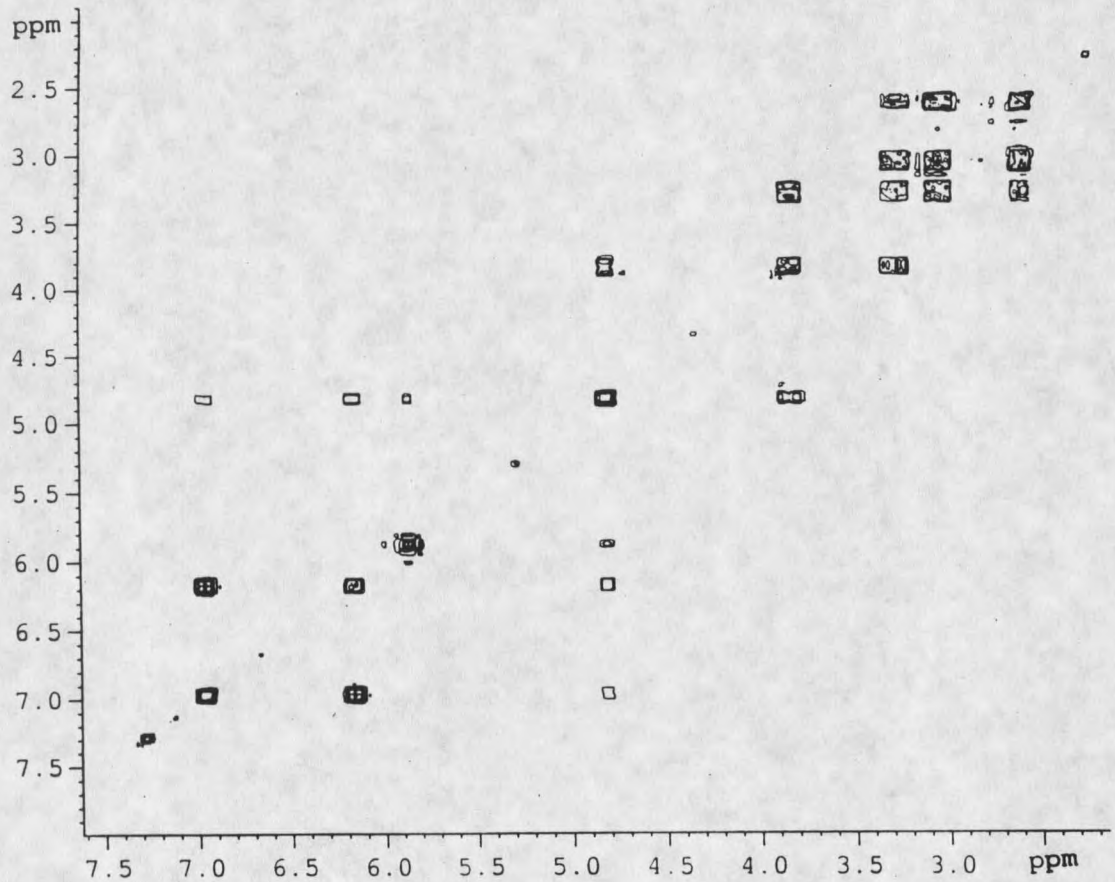


Figure 6.4 COSY of Epicorazine A

The assignments of the carbon backbone of Figure 6.6 were made by using the HETCOR spectrum and typical carbon signals. The alkene carbons G and F were assigned in a typical manner with the carbon opposite the carbonyl being downfield of the carbon adjacent to the carbonyl. The remaining carbons were assigned by their correlations to the protons found in the HETCOR

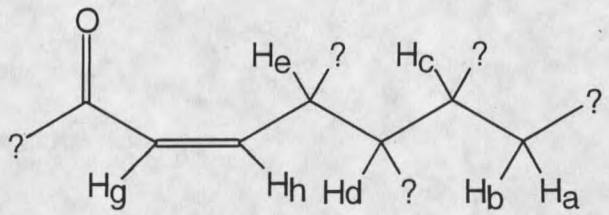


Figure 6.5 ^{1}H Backbone of Epicorazine A

spectrum (Figure 6.7). Additionally, carbons C and D with chemical shifts of 70.2 and 71.8 ppm respectively, were typical of methine carbons adjacent to a heteroatom, namely oxygen or nitrogen but probably not sulfur which typically come upfield of 70 ppm.[84] The chemical shift of carbon A and protons a and b

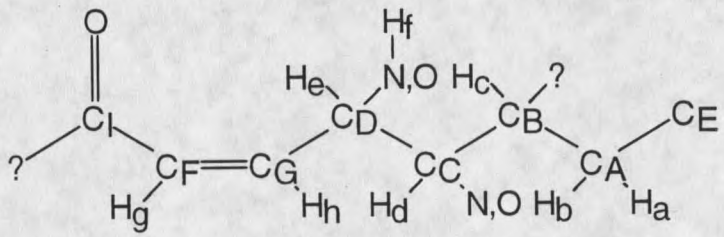


Figure 6.6 ^{13}C Backbone of Epicorazine A

were typical of methylene signals attached only to other carbons. Carbon E, the quaternary carbon was chosen over the ketone carbon I or the amide carbonyl H because of the relatively upfield chemical shift of carbon A (31.8 vs 35.4 for 3-pentanone). The slight correlation between protons e and f shown on the COSY spectrum suggested that proton f was attached to the heteroatom on carbon D.

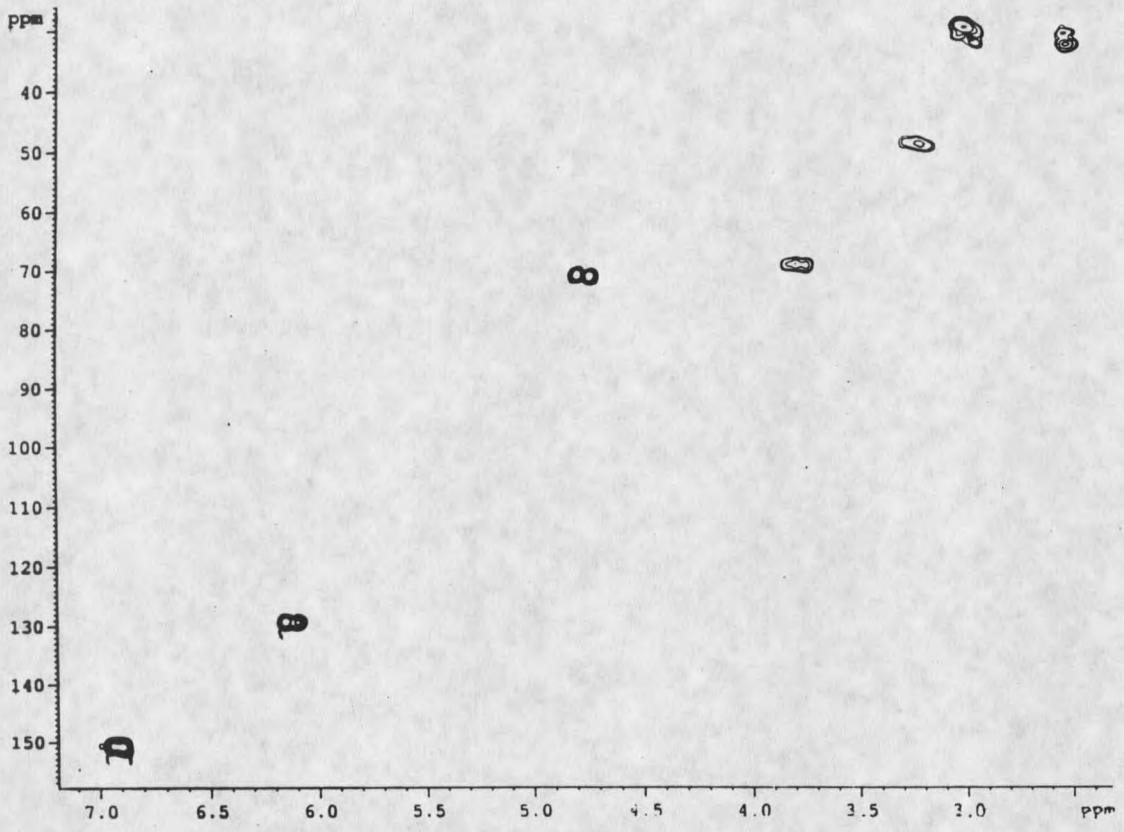
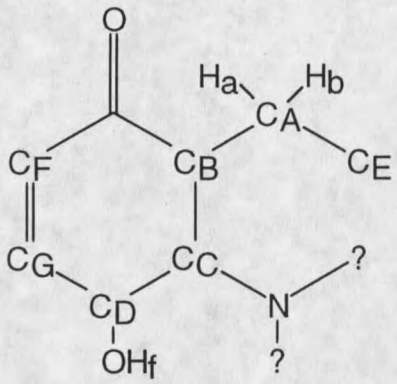
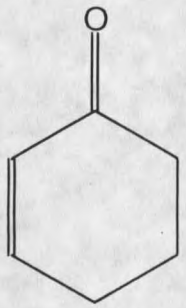


Figure 6.7 HETCOR of Epicorazine A

The need for six rings in the final structure suggested the need to include the carbon skeleton in a ring. The ring given in Figure 6.8 was based on the chemical shifts of carbons F and G. These carbons had shifts of 129.9 and 151.4 respectively, which compared very well to those of 2-cylcohexenone (129.3 and 150.7 respectively[85]). The molecular formula allowed for six oxygens (three each half), and since two of the three oxygens were accounted for with the ketone and amide functional groups, the remaining oxygen must be





2-cyclohexenone

Figure 6.8 Hexenone ring of Epicorazine A

the alcohol suggested by the IR spectrum. Since carbon D was downfield of carbon C, the more electron withdrawing oxygen was attached to carbon D.

Since the molecular formula allowed for only one nitrogen per half of the molecule, the nitrogen on carbon C must be the amide nitrogen. Since there were no remaining protons on heteroatoms, it must be a secondary amide. The only site remaining to attach to the nitrogen was carbon E. One of the two remaing sites on carbon E must then be attached to the sulfur atom giving the completed half-structure shown in Figure 6.9.

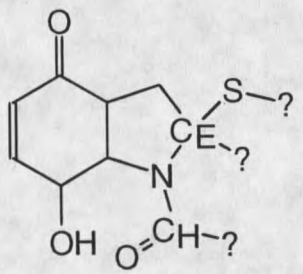


Figure 6.9 Half-structure of Epicorazine A

The two halves of the molecule were joined in a diketopiperazine fashion with a disulfide bridge. Diketopiperazines are a common class of structures and this molecule seemed to be a prime candidate for that arrangement. This gave the complete structure found in Figure 6.1 with the relative stereochemistry assigned through the 1D NOE difference data shown in Figure 6.10. Since the optical rotation data (-348°) and the published data for Epicorazine A were both levorotatory, the absolute configuration was taken from the published data.[86]

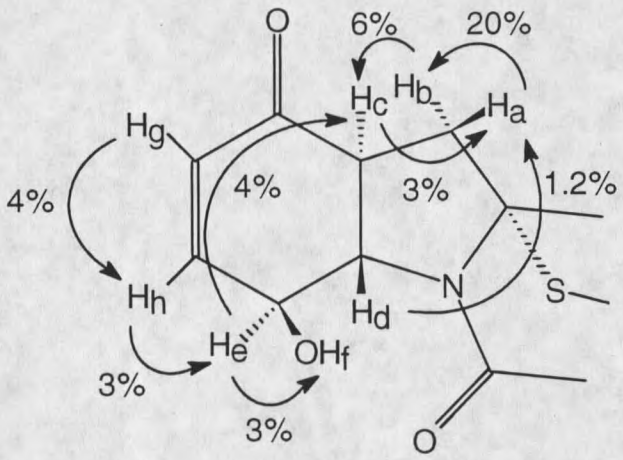


Figure 6.10 NOE data for Epicorazine A

Experimental

Culture Conditions

ME-19 was sustained on M-1-D agar containing 1% yew broth. ME-19 was grown in still culture in 2 liter erlenmeyer flasks and 1000 ml Roux flasks in R-1 media containing 1% yew broth for 21 days. Since ME-19 grew on the surface

of the culture liquid, the amount of liquid used in each flask was chosen to give the largest surface area for growth.

Extraction and Isolation

The fungal mycelia were removed by filtration through eight layers of cheesecloth, and the filtrate was extracted three times with 400 mL portions of methylene chloride. The extracts were combined and evaporated to dryness on a rotoevaporator (30 mg/L). The extracts were chromatographed on a Sephadex LH-20 column (2.8 X 110 cm), and eluted with 1:1 $CHCl_3$:MeOH. Seven fractions were collected and isolation of Epicorazine A was carried out by activity-guided fractionation using *G. cand.*, *C. alb.*, *B. sub.*, and *E. coli* overspray bioassays. Fractions 3 and 4 (18 mg/L) were dissolved in methanol and the methanol insoluble compounds were removed by filtration through J.T. Baker SPE disposable filtration columns with 20 µm frits. The methanol insoluble fraction (3mg/L) contained Epicorazine A which crystalized from 2:1 $CHCl_3$:CH_3OH (3 mg/L).

Physical/Chemical Data

White crystals; mp 230 d; $[\alpha]^{25}D$ -348; UV (CH_3CN) $\lambda_{max}(\varepsilon)$ 213 nm (22,050); IR ν_{max} (cm^{-1}, KBr) 3360 br, 1700, 1674; EIMS m/z 356 (66), 175 (14), 64 (100); ^1H NMR δ ($CDCl_3$) data in Table I; ^{13}C NMR δ ($CDCl_3$) data in Table I.

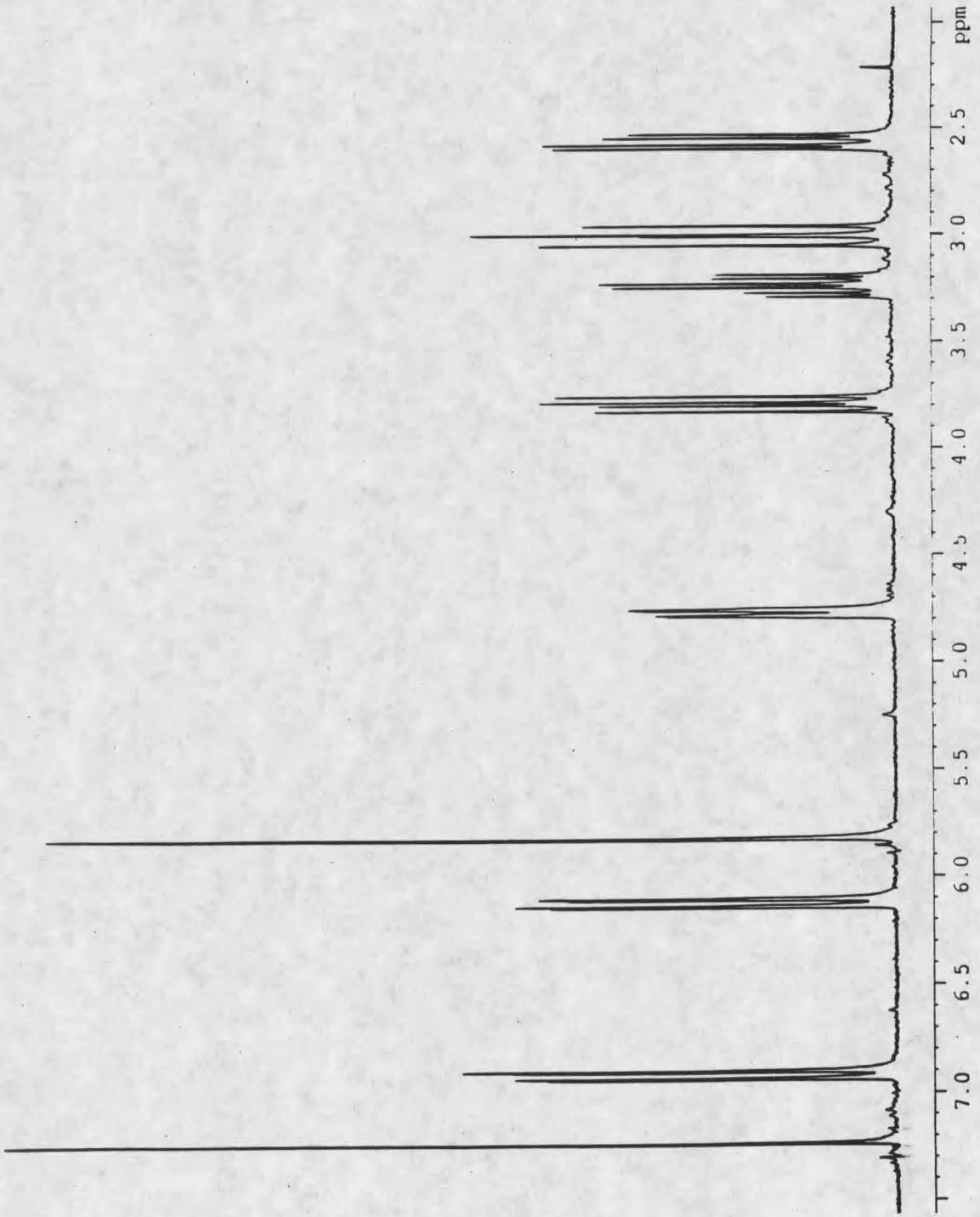


Figure 6.11 ^{1}H NMR spectrum of Epicorazine A

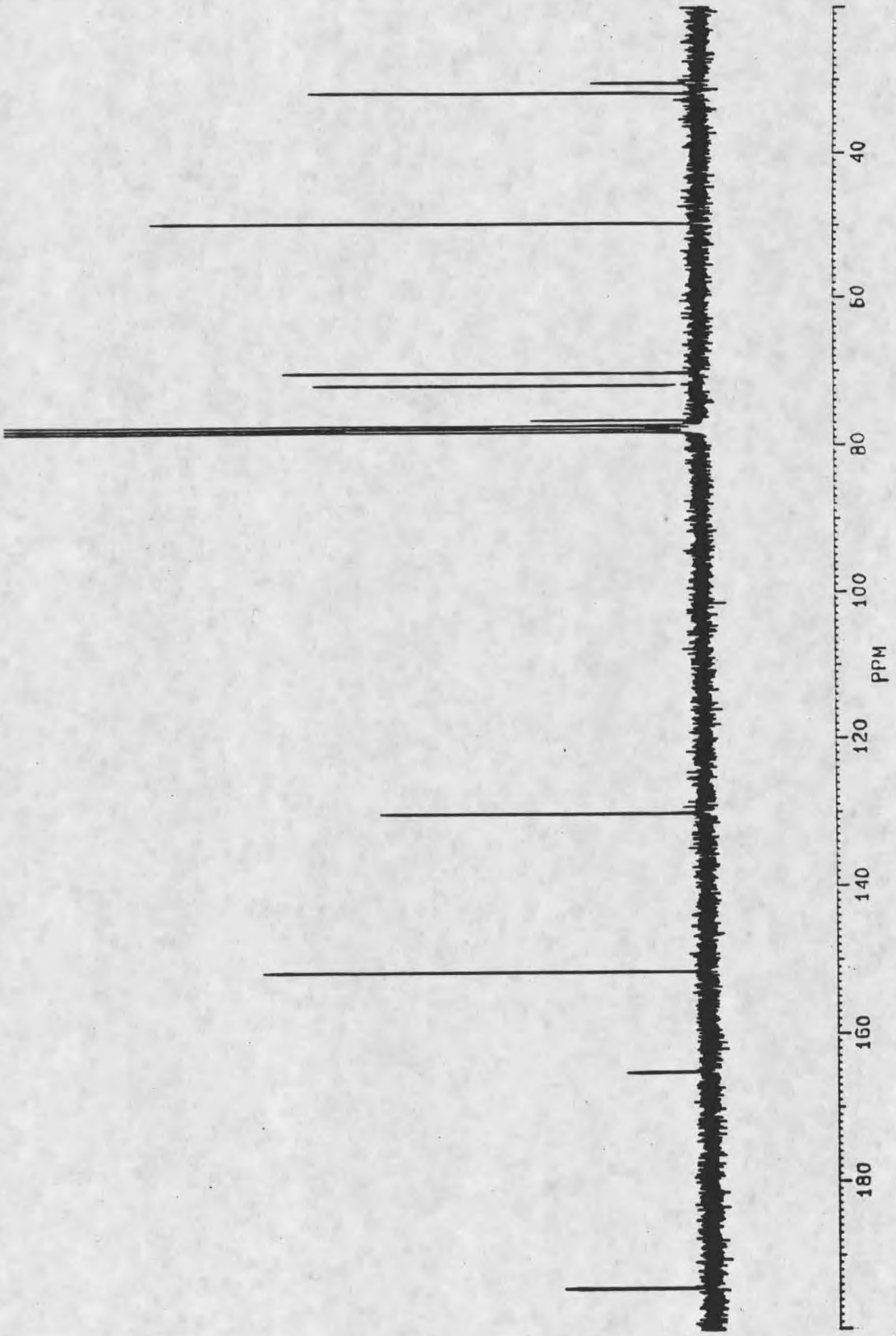


Figure 6.12 ^{13}C NMR specrum of Epicorazine A

Chapter 7

CONCLUSION

Due to the development of microbial resistance to current clinical antibiotics, the increasing number of immunocompromised hosts (AIDS patients, patients on chemotherapy, etc.), and the side effects of current antitumor drugs, there is a need for novel pharmaceuticals. Endophytic microbes are one possible source of novel bioactive compounds which are mostly unexplored. It is possible to isolate novel compounds from these organisms which could be developed as new pharmaceuticals, or could be used as templates for chemical synthesis of even more effective drugs.

In this study, a novel compound, sphaeric acid, was isolated from a *Sphaeriopsis* sp. isolated from a mexican yew tree. Chemical derivatization of this compound resulted in a variation in the activities observed in several bioassays. Although few consistent trends were observed throughout the entire range of bioassays, some clues might be observed that could direct future derivatizations attempting to increase specific activities.

It was also observed that changing the growth conditions of two fungi resulted in variations in the activities observed in the screening bioassays of the crude extracts of these fungi. This study used high and low nutrient media, an

ammonium ion trapping medium, a phosphate trapping medium, and a magnesium spiked medium to explore the effect of culture medium changes on bioactive metabolite production. The partial success of this can be seen in the appearance of activity in certain fractions for some media that were not observed in the others. Considering all the other variables in culture conditions (length of culture, temperature, pH, agitation, small molecule precursors, etc.) there exists a large source of novel bioactive compounds that has barely been tapped.

It only seems logical that the need for new pharmaceuticals, matched with the largely unstudied pool of endophytic fungi, is a catalyst for the continuation of this sort of research. All that is necessary is an investment of time, money, and a deal of luck in searches such as this that might lead to the next penicillin or taxol.

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