# Isolation, structure elucidation, and biological activities of toxic secondary metabolites from *Helminthosporium maydis*

by

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## ABBREVIATIONS

| | |
|---|---|
| BSA | Bovine serium albumin |
| DCIP | 2,6-Dichlorophenolindophenol |
| $Et_2O$ | Diethyl ether |
| EtOH | Ethanol |
| EtOAc | Ethyl acetate |
| ft-NMR | Fourier-transform nuclear magnetic resonance |
| GC/MS | Gas chromatography-mass spectrometry |
| GLC | Gas-liquid chromatography |
| Hz | Hertz |
| HMT toxin | Host specific toxin mixture from H. maydis Race T |
| HPLC | High performance liquid chromatography |
| IR | Infrared |
| MeOH | Methanol |
| NMR | Nuclear magnetic resonance |
| TLC | Thin layer chromatography |

# INTRODUCTION

One can think of perhaps no members of the Plant Kingdom more uniquely significant in human affairs than the fungi. In recent times, fungal species have yielded a vast treasure of biodynamic organic compounds including antibiotics, antineoplastic agents, psychoactive compounds, and various other pharmaceuticals. On the other hand, the role of the sclerotium of the fungus Claviceps purpurea as a poisonous contaminant of edible grain can be traced as far back as a 600 B.C. Assyrian tablet describing a "noxious pustule in the ear of grain" (1). A similar modern day problem has been the fungal production of aflatoxin and other food toxins.

Some of the most important fungal species in modern agriculture are the plant pathogens which infest a wide variety of food crop plants. The concept that some of these pathogens produce toxins affecting disease development in the host plant originated almost a century ago, but it has only been recently that research efforts have substantiated this claim (2). In plant pathology, a nonenzymatic substance that injures or disrupts the metabolism of a cell is considered a toxin. A host-specific plant toxin is a metabolic product of a pathogen which is toxic only to the host of that pathogen, essentially without toxicity for other living things (3). The known host-specific toxins produce all the symptoms of the disease caused by the respective pathogens. An example of even greater host specificity is the selective toxin of Helminthosporium victoriae.

Only cultivars derived from the once widely used oat cultivar Victoria are susceptible (4).

Helminthosporium maydis

Race T of this fungus causes a severe leaf blight of corn cultivars containing the Texas male-sterile cytoplasm (Tcms) but only minor leaf spot on cultivars that do not contain this cytoplasm. The disease, called Southern Corn Leaf Blight, is essentially world-wide in distribution, but has been observed mainly in tropical or subtropical areas. Great interest has developed in this disease which reached nearly epidemic proportions in the U.S. in 1970 causing serious losses for corn producers.

In 1925, the ascigerous (sexual) stage of a Helminthosporium species was described and named Ophiobolus heterostrophus Drechsler (5). The asexual stage was identified in 1926 in Japan and named Helminthosporium maydis Nisikado and Miyake (6). In 1934, Drechsler revised the Ophiobolus genus and a new genus, Cochliobolus, was coined. The sexual stage of H. maydis was assigned the new name Cochliobolus heterostrophus (7). More recently, three species of Helminthosporium attacking corn, including H. maydis, have been classified in the genus Bipolaris. This is due to the fact that germinating spores put out germ tubes only from each end of the conidium (8). In this writing, the name H. maydis will be retained.

Although minor leaf spotting of maize had been noted from H. maydis, the first observation of severe hyper-susceptibility of corn possessing Tcms cytoplasm was in the Philippines in 1961 (9). Tcms

cytoplasm was a favorite cytoplasm used in the commercial production of hybrid seed corn. The disease symptoms observed were small, water-soaked, light green spots followed a few days later by large yellow blotches up to 4 centimeters long appearing first on the lower leaves. As the disease progressed to the upper leaves, lesions were formed on the husks and the mycelium penetrated to the ear. There was an unofficial report of the fungus in Mexico in 1968 and in 1969 it was detected as far north as Minnesota (10). It was soon established that the disease was produced by a new biotype (race) of H. maydis. This race has been named Race T and the older, slightly pathogenic type was named Race O. Because of widespread use of Tcms cytoplasm in commercial corn hybrids, the stage for a catastrophe was set in 1970. In late February and early March, the disease was seen in Florida. By August all of the eastern U.S. had been infected by wind blown spores. Two excellent reviews cover additional historical information (11,12).

## Effects of H. maydis Race T toxin (HMT toxin) on Tcms cytoplasm

Early work by Nelson and Hooker demonstrated the production of a host specific pathotoxin by H. maydis both in vitro and in vivo (13,14). When Tcms cytoplasm is treated with toxin-containing filtrates from Race T cultures or partially purified toxin preparations, morphological, physiological, biochemical, and structural changes occur. Most reported responses are host specific although a few are not. Table 1 lists these responses. It appears that mitochondrial effects

are the most easily reproduced. Chlorotic streaks and inhibition of root elongation are also widely reported. Host specific stimulation of electrolyte leakage has been difficult to repeat (16,17) as is the inhibition of microsomal potassium-stimulated ATPase (17,18). Mitochondria as well as the plasma membrane have been suggested as the primary site of early toxin action. Much experimental data and speculation exists on the mechanism of action (15). It has been hard to draw a sound comparison of various data because of differences in fungal isolates used, fungal growth conditions, methods of toxin isolation, quantitation of toxin used as well as differences in biological and biochemical handling of the host tissue.

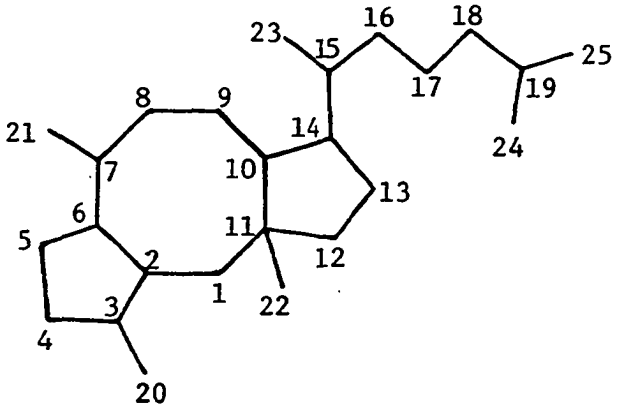
The HMT toxin

Early work with the toxin presented very little chemical data. Nelson described the toxin as dialyzable and heat stable (14). Since culture filtrates from a 10 day old, toxin producing fungal culture reacted readily with ninhydrin, the toxin was speculated to be a low molecular weight polypeptide. At least 14 separate isolation procedures have been reported (15). Most were initiated in an attempt to produce pure materials for physiological studies of host-pathogen interaction. A more complete chemical characterization of the host specific toxins was attempted by Karr et al. (19). By using a very tedious and lengthy separation method, microgram amounts of 4 toxic substances were isolated. It was further suggested that each was chemically pure and equal in biological activity with the single

bioassay used. Evidence was presented for toxins I and II being tetracyclic triterpenoids and toxin III a glycoside, the aglycone being very similar to toxins I and II. A fifth toxin and a chemical assay was later reported by this group (20). The involvement of these materials in toxin specificity has been questioned (21). A comparison of Race T and Race 0 using the described assay revealed that both fungal races produced components similar in chemical reactivity. Another problem of the work of Karr et al. is the use of a very insensitive leaf puncture bioassay (22). Several reported bioassays are much more sensitive and it is possible that the assay reported was insensitive to certain toxic components. There have been at least two attempts to reproduce the entire isolation procedure using more sensitive bioassay methods (21,23). In both cases, all detectable biological activity was lost during the isolation.

## Other secondary metabolites from H. maydis

Ophiobolin A 1 was the first reported compound of a series of terpenoidal compounds containing the ophiobolane ring system 2. These compounds have been shown to arise from the head to tail condensation of 5 isoprene units followed by cyclization to give a molecular skeleton containing 25 carbon atoms, thus they fall into the larger class of terpenes known as sesterterpenes. Originally, they were isolated from H. oryzae (24) and later from Ophiobolus heterostrophus and other Helminthosporium species known to be pathogenic to plants (25,26). The absolute structure of ophiobolin A was

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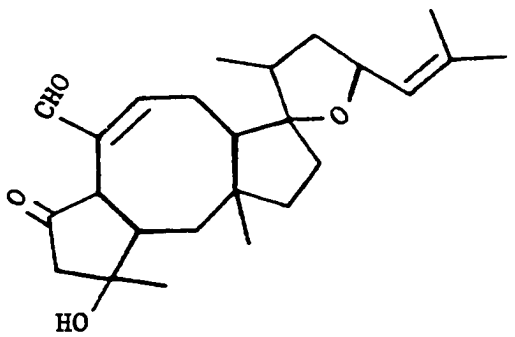
determined by Nozoe using X-ray crystallographic analysis of a bromomethoxy derivative (27). Canonica used chemical and spectral techniques to arrive at the same structure for cochliobolin (28). Confusion in nomenclature of these compounds was resolved in 1967 with the trivial name ophiobolin assigned to the basic 5-8-5 ring system (29). Ensuing research resulted in the isolation and characterization of additional ophiobolins anhydroophiobolin A 3, ophiobolin B 4, ophiobolin C 5, ophiobolin D 6, and ophiobolin F 7 (30,31,32). Ophiobolins A, B, C, and F have been isolated from Cochliobolus heterostrophus (H. maydis) along with the sesterterpene geranylnerolidol 8 and ergosterol, kaurene 9, isokaurene 10, and squalene have been tentatively identified (32). Three reviews have appeared on the chemistry of these compounds (33,34,35).

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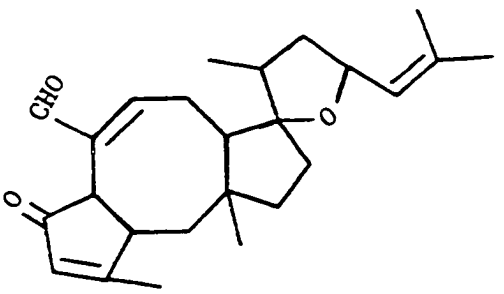


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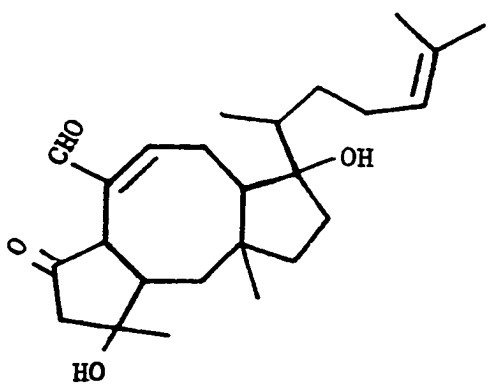


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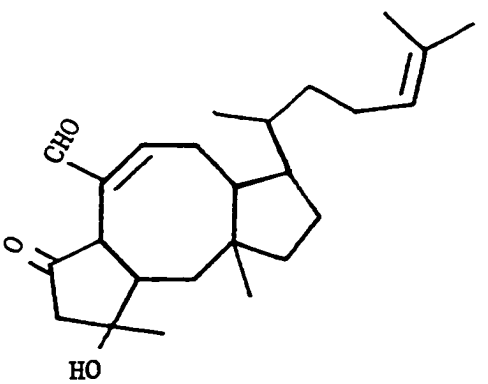


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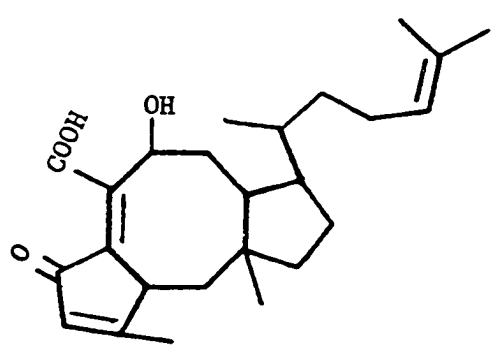


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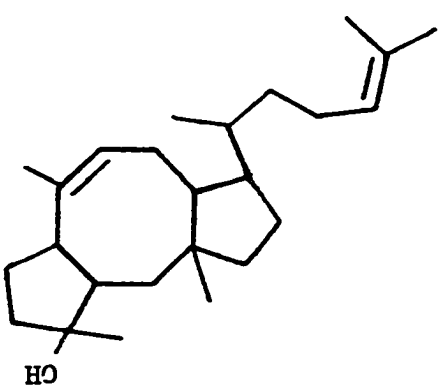


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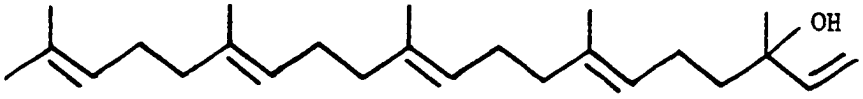


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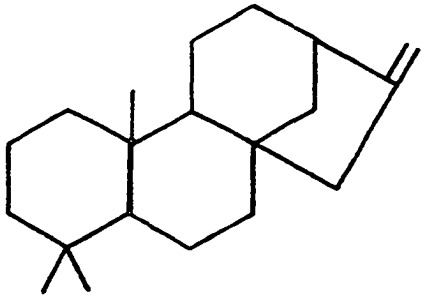

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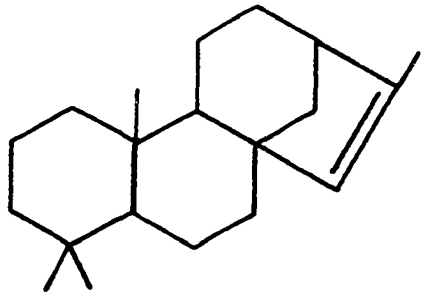

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Phyllosticta maydis

In 1968, a severe epidemic of an unusual seedling blight of corn occurred in central Pennsylvania. Field observations and laboratory testing indicated a wide range of susceptibility to the disease among inbred seedling plants. Resistance of several inbreds was lost upon their conversion to the male sterile inbred (36,37). The causal agent of the disease was tentatively identified as Phyllosticta zeae Stout. The disease had also been reported in Ohio as early as 1965 (38) and by 1969 was widespread in Wisconsin (39). Because of confusion in proper identification, the designation Phyllosticta sp. was used until 1971 when Arny and Nelson showed the fungus to be different from

Phyllosticta zeae Stout, was new to science, and formally named it Phyllosticta maydis (40).

Host susceptibility and response was similar to that observed with H. maydis Race T. Involvement of a new host-specific toxin from P. maydis was concurrently shown by Comstock et al. (41) and Yoder (42). Culture filtrates from isolates of P. maydis pathogenic to corn inhibited root growth, shoot development, induced leaf chlorosis, and caused increased leakage of electrolytes from corn containing Texas male sterile cytoplasm. Toxin-treated mitochondria isolated from Tcms maize exhibited an immediate irreversible swelling, uncoupled oxidative phosphorylation and had altered $O_2$ uptake. The toxin did not affect maize without Tcms cytoplasm except at high concentrations.

The similar host-specific activities of toxins from P. maydis and H. maydis suggest that they are the same or closely related chemically. There have been no reports concerning the isolation or chemical characterization of the P. maydis toxin(s).

The purpose of the study initiated by this author was to examine the natural products produced by H. maydis. Emphasis was placed on the isolation of materials exhibiting host specific responses on Tcms maize cultivars with the goal of chemical characterization of the toxic component. Toxic secondary metabolites from P. maydis were also examined to determine if any chemical similarities existed in toxic materials produced by these two unrelated fungi.

Table 1. Effects of H. maydis Race T toxin on T cytoplasm material (15)

| Toxin application (if not in ambient solution) | Effect |
|---|---|
| | **Plants** |
| Sprayed | lesion formation |
| Injected into stem | chlorosis of leaves |
| Pipetted onto leaf whorl | |
| Punctured with needle | chlorotic streaks |
| | **Seedlings** |
| | inhibition of root growth |
| | inhibition of uranyl uptake into vascuoles of root cells |
| | stimulation of electrolyte leakage |
| | ultrastructural damage to mitochondria in root cells |
| | failure of root mitochondria to respond to deoxyglucose treatment |
| | depolarization of membrane potential difference of root cells |
| | **Detached Leaves - Leaf Discs** |
| Leaf floated on toxin | water soaking |
| Injected into leaf | lesion formation |
| Leaf discs | inhibition of dark and light $CO_2$ fixation |
| Cut end in toxin | stimulation of electrolyte leakage |
| Epidermal peels floated on toxin | inhibition of light-stimulated $K^+$ uptake by guard cells |

Table 1. (cont.)

| | |
|---|---|
| | Isolated Roots |
| | stimulation of electrolyte leakage |
| | inhibition of $^{86}Rb$ uptake |
| | Callus |
| In agar medium | inhibition of growth |
| | Pollen (from Tcms cytoplasm plants with TRf genes) |
| In agar medium | inhibition of germination and pollen tube growth |
| | Protoplasts |
| | inhibition of volume increase |
| | collapse |
| | ultrastructural damage to mitrochondria |
| | Isolated Mitochondria |
| | uncoupling of oxidative phosphorylation |
| | stimulation of NADH oxidation |
| | inhibition of malate-pyruvate oxidation |
| | inhibition of α-ketoglutarate oxidation |
| | partial inhibition of succinate oxidation |
| | stimulation of succinate oxidation |
| | stimulation of ATPase activity |

Table 1. (cont.)

| | |
|---|---|
| | activation of cytochrome oxidase |
| | inhibition of $^{32}P$ accumulation |
| | increase in light transmittance (swelling) |
| | ultrastructural changes |
| | Microsomes |
| | inhibition of $K^+$-stimulated ATPase |

# MATERIALS AND METHODS

## Chemicals and supplies

Sephadex LH-20 was obtained from Pharmacia Fine Chemicals. Silica gel 60 GF-254 and silica gel 60 F-254 pre-coated TLC plates were from E. Merck. Deuterated NMR solvents were from Aldrich. A μ Bondapak $C_{18}$ HPLC column was obtained from Waters Associates. Potato dextrose agar and yeast extract were obtained from Difco. $^{14}C$ sodium acetate was purchased from New England Nuclear. Arasan A was from Du Pont. Distilled-in-glass solvents were obtained from Burdick and Jackson Laboratories. All routine solvents and chemicals were of reagent grade or better. Corn seed (Zea mays L., var. W64A) with T and N cytoplasm was obtained from Clyde Black and Son Seed Farms, Ames, Iowa. Routinely-used isolates of H. maydis race T and O and Phyllosticta maydis were a gift from Dr. C. A. Martinson, Department of Botany and Plant Pathology, Iowa State University, Ames, Iowa. Authentic ophiobolin samples were the generous gift of Dr. S. Nozoe, Institute of Applied Microbiology, The University of Tokyo, Japan. A gas filter obtained from Koby Incorporated was utilized when $N_2$ was used to remove solvent from small samples.

## Instrumental analysis

$^{1}H$ Nuclear magnetic resonance spectra were recorded on Varian Associates EM-360 or HA-100 instruments. $^{1}H$ and $^{13}C$ Fourier transform

NMR spectra were obtained using a Bruker HX-90 instrument equipped with a Nicolet 1089 data system. In some cases, a Nicolet 535 Signal Averager was used with the HA-100 instrument. In all cases, chemical shifts are reported as parts-per-million (ppm, $\delta$ scale) from tetramethylsilane (TMS, internal standard). Ultraviolet and visible spectra were obtained on Cary 15 or 118 instruments. Infrared spectra were recorded on Beckman IR 4250 or IR-18A instruments. Low resolution mass spectra were obtained on a Finnigan 4023 instrument. High resolution mass spectra were obtained on an AEI MS902 mass spectrometer with a direct solid inlet system. Gas chromatography was done on a Packard 409 instrument. High performance liquid chromatography was conducted with a Waters Associates Model 202 chromatograph equipped with a Model U6K injector and ultraviolet detector. Radioactivity measurements were done using a Beckman LS-3150T liquid scintillation spectrometer. Elemental micro-analyses were conducted by Galbraith Laboratories, Inc., Knoxville, Tennessee. Melting points were taken on a Kofler hot stage and are corrected.

Chromatography

Thin layer chromatography To prepare analytical thin layer plates a slurry of 25 gm silica gel GF-254 in 50 ml water was prepared by shaking for 60 seconds and spread onto hexane-washed plates to a thickness of 0.25 mm. The plates were allowed to air dry and placed in a drying oven at 110° C overnight. After activation the plates were allowed to cool and used immediately or stored in a desiccator until used. Precoated silica gel 60F-254 plates 0.25 mm and 0.50 mm

thick were used without activation. Solvents were allowed to equilibrate in the developing tanks before the plates were developed. Table 2 lists analytical TLC data. Preparative plates for ophiobolin isolations were made 0.75 mm thick. Preparative HMT toxin isolations were always done using 0.50 mm thick pre-coated plates. In all cases, from 10 to 30 mg of material was applied as a band with the aid of a commercial applicator. HMT toxins were visualized by spraying the plate with water while ophiobolins were observed by UV quenching at 254 nm.

High performance liquid chromatography HPLC was conducted using a stainless steel analytical column 4 mm ID x 30 cm commercially packed with μ Bondapak $C_{18}$ Porasil. The flow rate of the mobile phase; MEOH-$H_2O$ 35:65 v/v was 0.7 ml/min. An ultraviolet detector operating at 254 nm with a sensitivity of 0.02 absorbance unit was used for detection of various compounds.

Gel filtration Gel filtration chromatography was conducted in open glass columns using Sephadex LH-20. Two columns were used: A: 1.3 cm x 43 cm, and B: 2.5 cm x 99 cm. To prepare the columns a quantity of LH-20 was allowed to swell in methanol overnight and poured into the column as a slurry. Materials were carefully banded to the column top using methanol. Elution was always done with methanol using a flow rate of 0.5 ml/min. To determine the void volume of column A, 8 mg of soluble Polyvinylpyrrolidone (PVP, mw - 40,000) was dissolved in 0.5 and MeOH and applied to the column. Fractions were assayed by spotting aliquots on silica gel TLC plates

Table 2. TLC data of various compounds studied

| Compound | Rf values in solvent systems[a] A | B | Visualization color[b] C | D | E |
|---|---|---|---|---|---|
| Ophiobolin A | .46 | | Blue | Brown | |
| Anhydroophiobolin A | .53 | | Blue | Brown | |
| Ophiobolin C | .59 | | Blue | | |
| Ophiobolin B | .22 | | Blue | | |
| Unknown Ophiobolin | .60 | | Blue | | |
| P1 | | .47 | | Pink | Yellow |
| B1 | | .50 | | Brown | Yellow |
| P2 | | .52 | | Pink | Yellow |
| B2 | | .53 | | Brown | Yellow |
| L1 | | .33 | | Pink | Yellow |
| L2 | | .38 | | Violet | Yellow |
| L3 | | .43 | | Pink | Yellow |
| 2 Deoxy-D-ribose | | .20 | | Black | |

[a]A(Chloroform:Methanol; 50:1 (v/v)), B(Chloroform:Methanol; 50:5 (v/v)).

[b]C(1% Phosphomolybdic acid in methanol followed by heating 2 min. at 100°C), D(Sulfuric acid/methanol, 1:1 (v/v)), E(2,4-Dinitrophenylhydrazine spray) (43).

and visualizing with $H_2SO_4$ without plate development. PVP was located in milliters 12-13.

Gas chromatography Gas chromatography was done on a stainless steel column 1/8 in. x 6 ft. which had been packed with 3% SE-30(w/w) on 100/120 mesh Gas Chrom Q and was operated isothermally at 240°C. A flame ionization detector was used. The flow rate of nitrogen was 40 ml per minute with injector temperature at 300° C and detector temperature at 235° C. Flow rates of air and hydrogen were 250 and 30 ml per minute, respectively.

Fungal methods

H. maydis Race T and O were maintained on potato-dextrose agar slants and stored under refrigeration at 4° C prior to use. Various isolates were sometimes received as lesions on dried corn leaves. These were placed onto sterile slants for germination and subcultured to new slants for growth and storage. Pieces of mycelium were used to inoculate primary culture flasks; 250 ml Erlenmeyer flasks containing 50 ml sterile potato-dextrose agar sealed with a cotton plug. After 8 days growth, inoculum was prepared by adding 100 ml sterile water and a quantity of sterile glass beads. Vigorous shaking produced spore and mycelium suspensions. Ten ml of this suspension was used as inoculum to each of the sterile 2800 ml Fernbach secondary flasks. Table 3 lists various growth media used. Initially, 200 ml and later 400 ml medium was contained in each culture flask. In some cases, for ophiobolin production, 500 ml medium was used.

Table 3. Media used

Medium A; Defined medium of Karr et al. (19)

| | | | |
|---|---|---|---|
| $Ca(NO_3)_2 \cdot 4 H_2O$ | 405 mg/l | $MnSO_4 \cdot H_2O$ | 5.8 mg/l |
| $KNO_3$ | 80 mg/l | $ZnSO_4 \cdot 7 H_2O$ | 4 mg/l |
| KCl | 65 mg/l | $H_3BO_3$ | 1.3 mg/l |
| $MgSO_4$ | 360 mg/l | KI | 0.7 mg/l |
| $NaH_2PO_4 \cdot H_2O$ | 22 mg/l | Glucose | 30 g/l |
| $Na_2SO_4$ | 200 mg/l | pH adjusted to | 5.5 |
| $FeCl_3 \cdot 6 H_2O$ | 3.4 mg/l | | |

Medium B; Modified Fries Medium (44)

| | | | |
|---|---|---|---|
| Ammonium tartrate | 5 g/l | $CaCl_2 \cdot 2 H_2O$ | 0.13 g/l |
| $NH_4PO_4$ | 1 g/l | Sucrose | 30 g/l |
| $KH_2PO_4$ | 1 g/l | Yeast extract | 1 g/l |
| $MgSO_4 \cdot 7 H_2O$ | 0.5 g/l | Iron solution | 1 ml |
| NaCl | 0.1 g/l | ($FeSO_4 \cdot 7 H_2O$ in 100 ml $H_2O$) | 2 gm |

Medium C; Defined medium of Watrud et al. (45)

| | | | |
|---|---|---|---|
| $NH_4NO_3$ | 1 g/l | $CaCl_2$ | 100 mg/l |
| $KNO_3$ | 1 g/l | $FeCl_3$ | 1 mg/l |
| $KH_2PO_4$ | 1 g/l | $ZnSO_4$ | 1.6 mg/l |
| $MgSO_4 \cdot 7 H_2O$ | 480 mg/l | $MnSO_4$ | 0.3 mg/l |
| NaCl | 100 mg/l | $CaSO_4$ | 0.2 mg/l |
| Glucose | 2 g/l | | |

Medium D; PDA

| | |
|---|---|
| Potato Dextrose Agar | 3.9 gm |
| $H_2O$ | 100 ml |

The flasks were usually incubated at approximately 26° C in a growth chamber with 1200 ft-C continuous light supplied by incandescent and fluorescent lamps.

## Bioassays

Leaf streak bioassay Assays for host-specific toxin activity were carried out on 7-day-old plants from one susceptible line of corn, W64A T cytoplasm and one resistant line of corn, W-64A N cytoplasm using the plant whorl assay of Turner and Martinson (46). Corn seeds were slurried in Arasan 50, 0.25% w/w in a small amount of water, and spread on paper towels to dry. The treated seeds were planted in vermiculite and kept under continuous light at 26° C until the plants were used. Fifty µl of test solution was injected into the whorl of each plant using a microliter syringe. Care was taken to insure that most of the solution remained in the whorl. Experience showed that this was more difficult with plants younger or older than 7 days. Normally, from 3 to 5 replicates were conducted for each assay. The treated seedlings were returned to their growing conditions and the assay scored after 24 hours. The dilution end point was determined as the highest dilution of culture medium or purified toxin that would produce a yellow lesion 0.1 cm wide around the hole left in the leaf from injection. At lower dilutions this lesion must enlarge into a streak toward the leaf tip before a test was scored as positive.

Mitochondrial assay A second assay used was the measurement of toxin inhibition of mitochondrial malate oxidation (47). Non-treated W-64A T or N corn seeds were germinated in trays containing vermiculite. After 3 or 4 days in the dark, 5 g of shoots were excised. All subsequent steps were carried out at 4-5° C. The shoot tissue was ground using a mortar containing 15 ml of extraction buffer containing 0.5 M mannitol, 1 mM EDTA, 10 mM TRIS, 10 mM $KH_2PO_4$, and 0.2% BSA, pH taken to 7.2 with HCL. The solution was filtered through 4 layers of cheesecloth and centrifuged to 12,000 and stopped. The supernatant from the 12,000 g run was then centrifuged for 1 min at 30,000 g. The supernatant was discarded and the pellet was homogenized in 15 ml of the extraction buffer minus BSA. The suspension was centrifuged at 17,000 g for 1 min. The supernatant was again discarded and the pellet was homogenized in 5 ml assay buffer containing 0.3 M mannitol, 4 mM EDTA, 10 mM $KH_2PO_4$, 10 mM Tris, 5 mM $MgCl_2$, 10 mM KCL, and 0.2% BSA, pH taken to 7 with KOH. Stock solutions of 0.21 mM DCIP, .805 M malic acid taken to pH 7 with NaOH, and 192 mM $NaN_3$ were prepared. The reaction mixture routinely contained 0.2 ml malic acid, 0.1 ml DCIP, 0.3 ml toxin solution or if running a control, 0.3 ml $H_2O$ and 2.8 ml assay buffer containing 0.1 ml $NaN_3$ per 10 ml buffer. The reaction was initiated by addition of 0.2 ml T or N mitochondria. The mitochondria were kept at 4° C while all other solutions were at room temperature. DCIP reduction was measured spectrophotometrically at 600 nm using a plastic cuvette. After addition of the solutions, the cuvette was covered with a small

piece of parafilm, inverted twice, and placed into the spectrophotometer. Measurement of time needed for complete inhibition of DCIP reduction was begun 30 seconds after addition of the mitochondria.

# RESULTS

## Ophiobolin characterization

Isolation of Ophiobolin A  H. maydis Race T had been grown for 12 days using modified Fries media. The mycelial mats were removed and expressed through 4 layers of cheesecloth. The resulting solution was added to the growth medium to give 12 liters of solution. This material was filtered through Whatman No. 1 filter paper and placed in a large liquid-liquid extractor. Two liters of $Et_2O$ was added and the material extracted under reflux for 7 hours. At this time, 1 liter fresh $Et_2O$ was added and the extraction continued for 7 additional hours. The two ether fractions were pooled, dried with sodium sulfate, and taken to an orange, nondrying oil by evaporation under vacuum at 45° C. The oil was redissolved in 20 ml $Et_2O$. On standing this solution deposited large quantities of crystals. The crystals were filtered through sintered glass and washed 3 times with 10 ml portions of cold $Et_2O$ to give a total of 558 mg crude Ophiobolin A. Recrystallization from methylene chloride/heptane several times yielded white, needle-shaped crystals. Purification was monitored by solvent system A using UV quenching and phosphomolybdic acid for visualization.

Ophiobolin A 1  m.p. 178-181° (lit (28) m.p. 181°). nmr ($CDCl_3$) 100 MHz $\delta$ .84 (s, 3, C11-$CH_3$), 1.10 (d, J=7, 3, C15-$CH_3$), 1.37 (s, 3, C3-$CH_3$), 1.71 (d, J=1, 3, C19-$CH_3$), 1.73 (d, J=1, 3, C19-$CH_3$), 2.66 (q, J=19, 2, C4-$H_2$), 3.28 (d, J=10, 2, C6-H, C3-OH),

4.44 (q, J=7, 1, C17-H), 5.17 (d, J=7, 1, C18-H), 7.20 (t, J=8, C8-H, 9.24 (s, 1, C21-H) (See Fig. 1). Mol. wt. required for $C_{25}H_{36}O_4$, 400.2614; found 400.2584 (high resolution mass spectrometry). (See Fig. 2 for low resolution mass spectral results).

Isolation of other ophiobolins The mother liquor from the ophiobolin A crystallization was taken to dryness under vacuum. This brownish residue was dissolved in 4 ml $CH_2Cl_2$. Analytical TLC examination using solvent system A revealed the presence of ophiobolin A, two other major metabolites with Rf values higher than ophiobolin A, and several minor components with lower RF values. This material was applied to 7 preparative GF 254 TLC plates 75 mm thick and developed with solvent system A. After development, the plates were removed from the tanks, visualized with UV light, and the two major bands removed from the plates. The silica gel was placed on MeOH-washed Whatman #1 filter paper and eluted with three 10 ml portions of methanol.

Upper band This material was concentrated to 1 ml under vacuum and placed on the small Sephadex LH-20 column. The column was developed with MeOH at a flow rate of .32 ml/min. and 1.4 ml solvent was collected per fraction. Ten ml solvent was collected before tube #1 was collected using a fraction collector. After 30 tubes were collected, analytical TLC using solvent system A revealed tubes 13 to 15 contained very pure material. Tubes 13 and 14 were combined and taken to dryness under vacuum to give 6.3 mg yellow, nondrying oil.

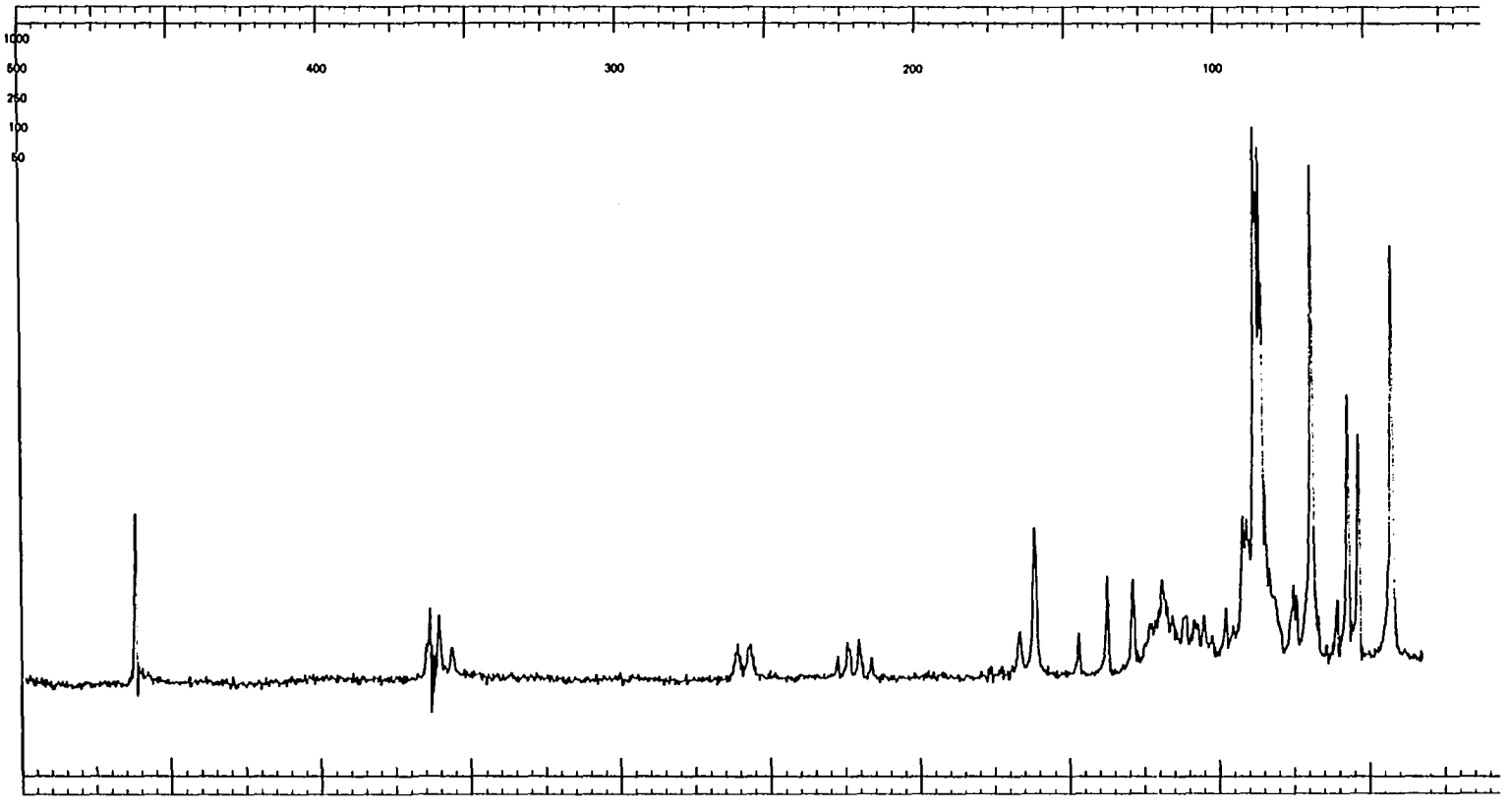
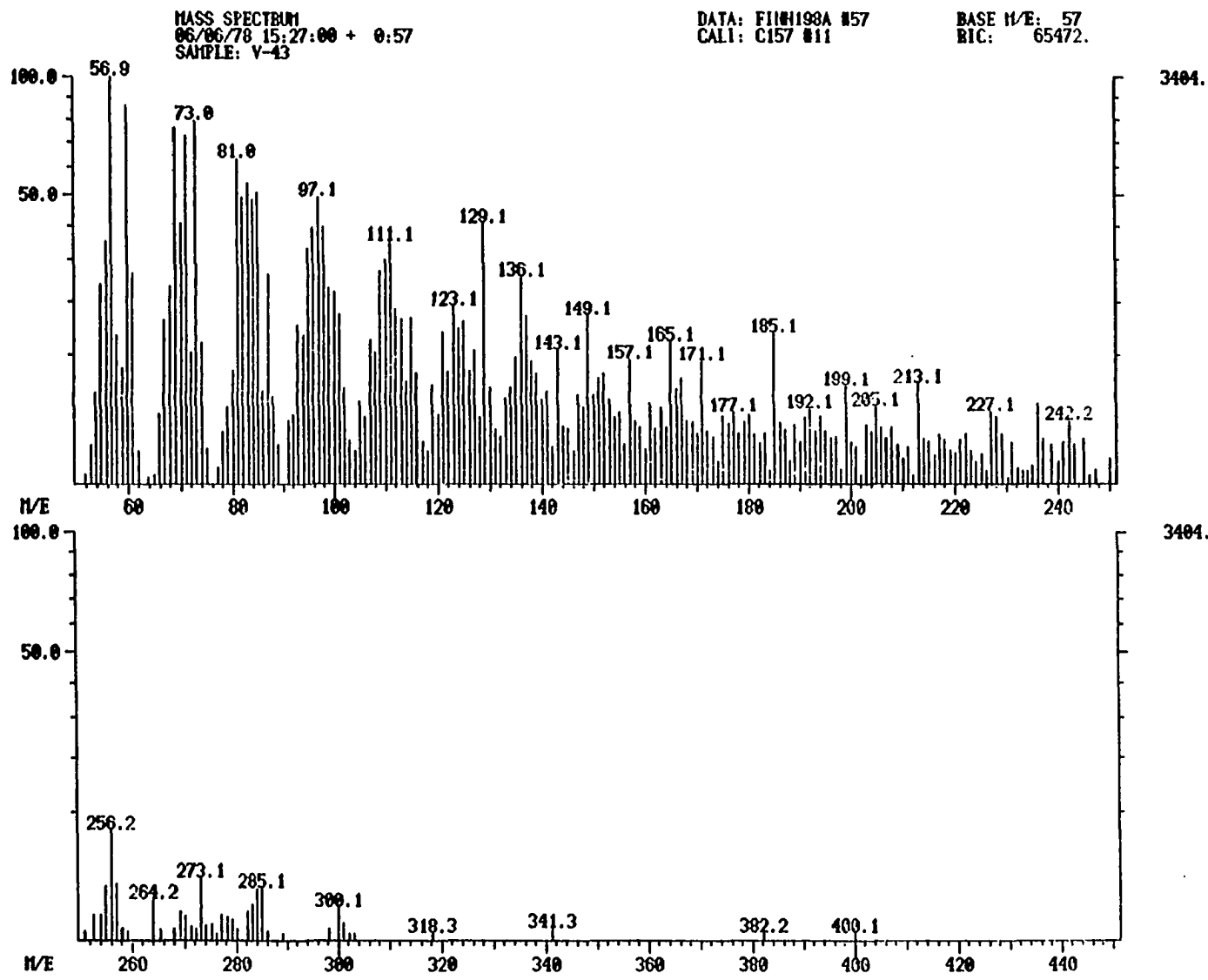



Figure 1. 100 MHz proton NMR spectrum of ophiobolin A. The scan width was 1000 Hz

Figure 2. Low resolution mass spectrum of ophiobolin A


MASS SPECTRUM
06/06/78 15:27:00 + 0:57
SAMPLE: V-43
DATA: FIIH198A #57
CALI: C157 #11
BASE M/E: 57
RIC: 65472.
100.0
50.0
3404.
M/E
56.9
73.0
81.0
97.1
111.1
123.1
129.1
136.1
143.1
149.1
157.1
165.1
171.1
177.1
185.1
192.1
199.1
213.1
227.1
242.2
60
80
100
120
140
160
180
200
220
240
256.2
264.2
273.1
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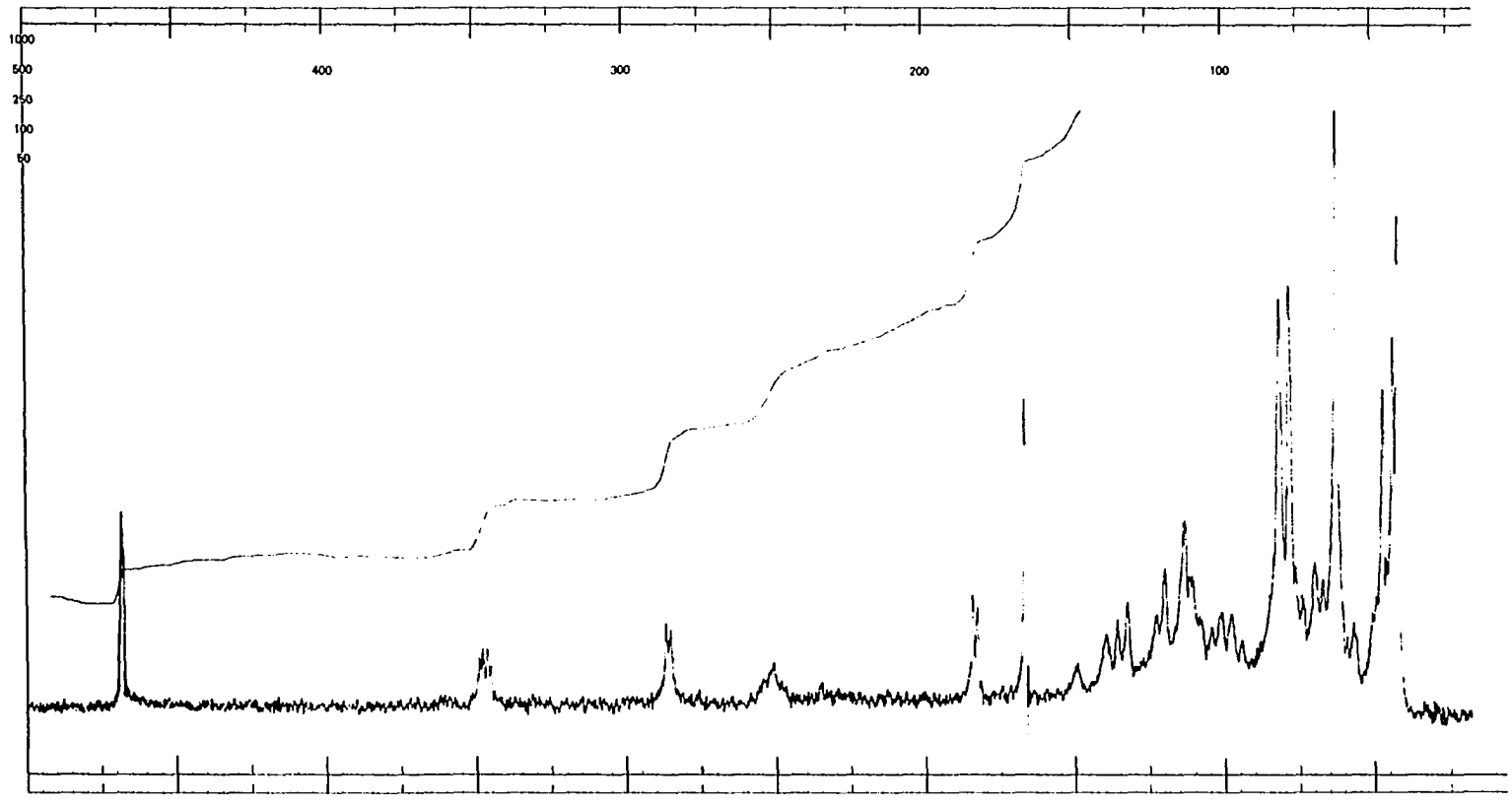



Figure 3. 100 MHz proton NMR of unknown ophiobolin. The scan width was 1000 Hz, signal at δ 3.34 is residual methanol

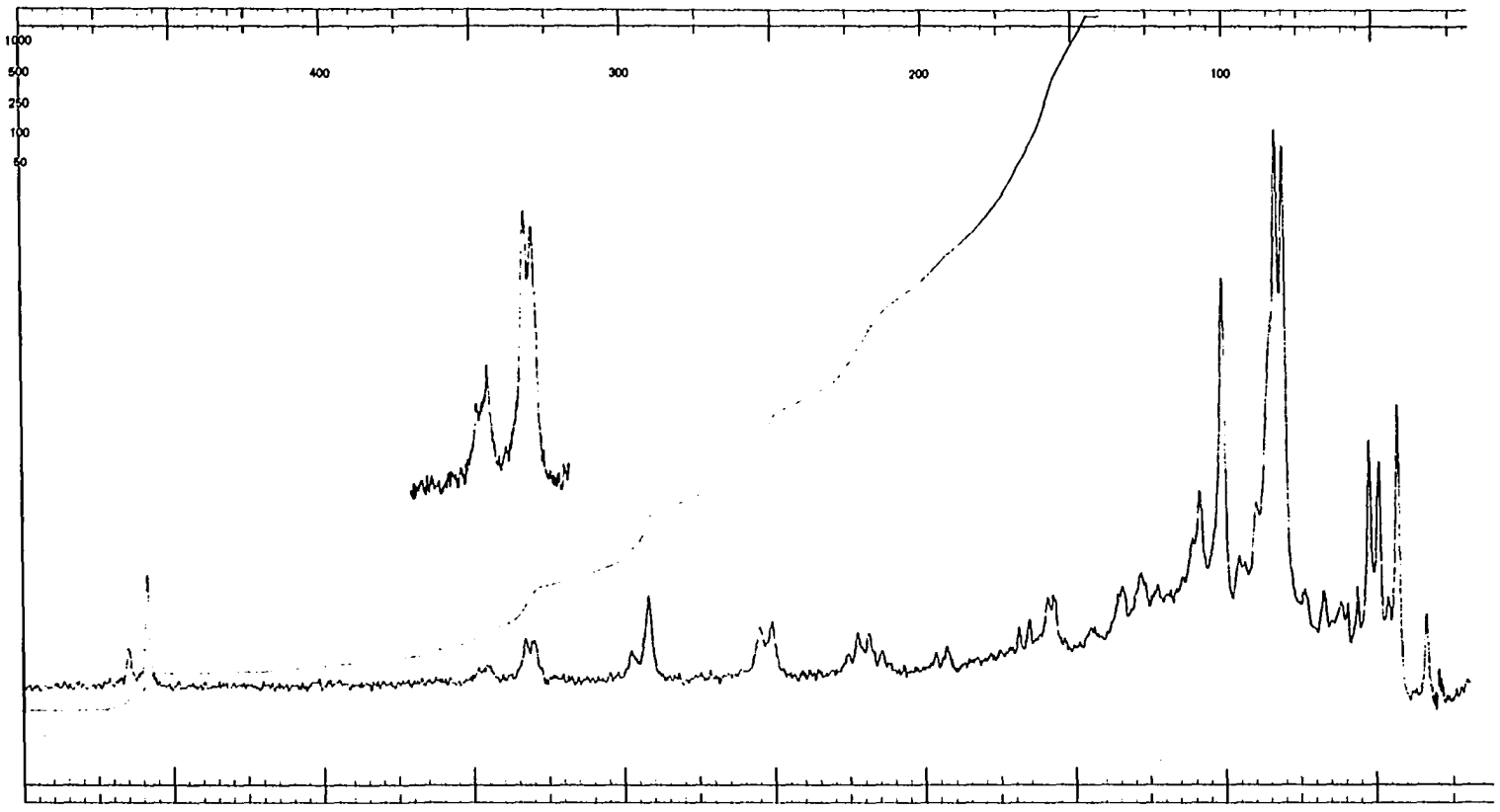



Figure 4. 100 MHz proton NMR spectrum of isolated anhydroophiobolin A. The scan width was 1000 Hz

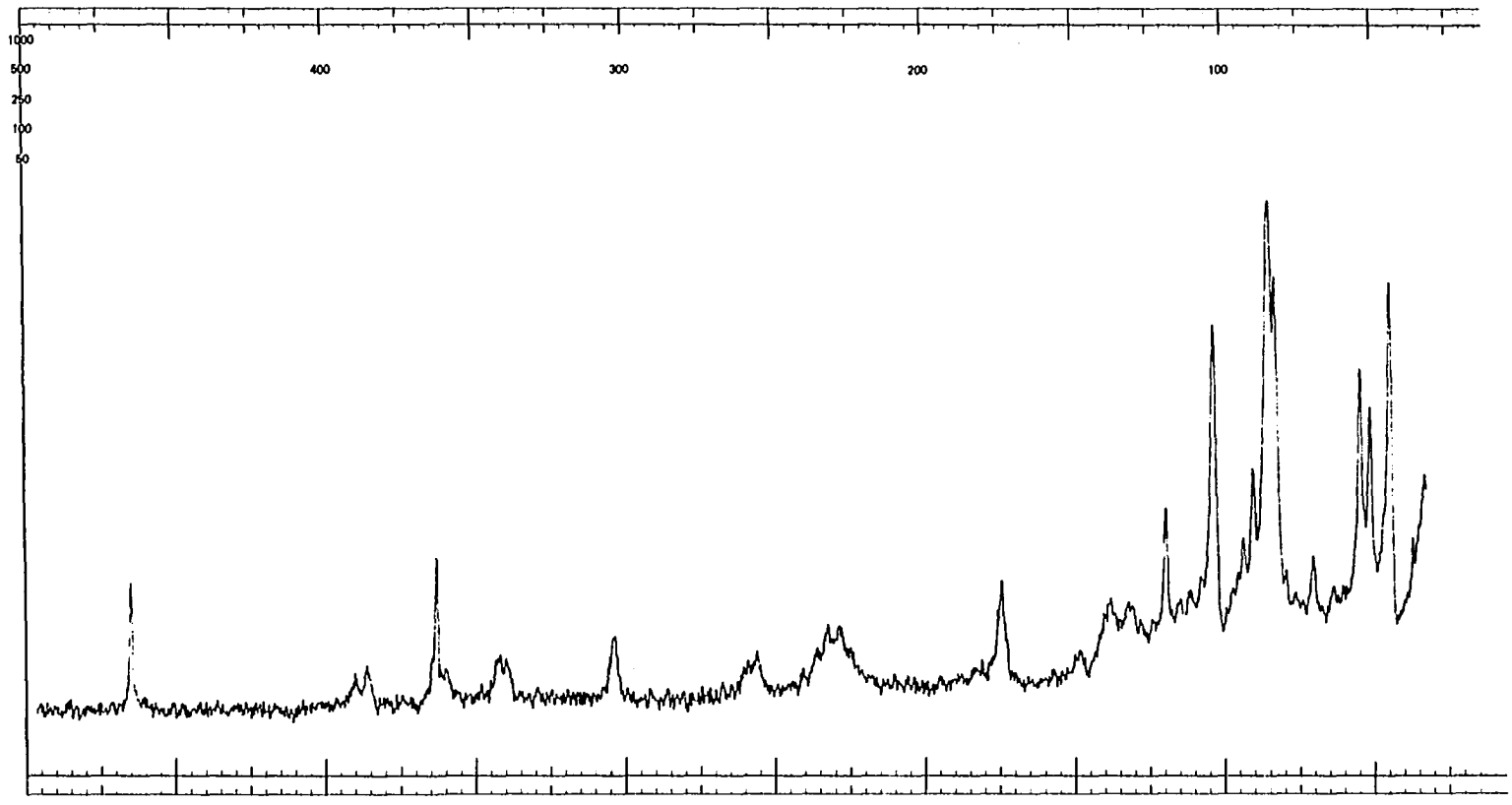



Figure 5. 100 MHz proton NMR spectrum of semi-synthetic anhydroophiobolin A. The scan width was 1000 Hz, signals at δ 2.35 and 7.75 are due to p-toluenesulfonic acid

Unknown ophiobolin nmr($CCL_4$) 100 MHz δ .84 (s, 3), .90 (d, J=7, 3), 1.24 (s, 3), 1.56 (s, 3), 1.63 (s, 3), 9.36 (s, 1). (See Fig. 3. Mol. wt. required for $C_{25}H_{32}O_3$, 380.2352; found 380.2344 (high resolution mass spectrometry).

Lower band This material was handled in a similar fashion to the upper band except it was rechromatographed by preparative TLC before placing the sample on the same Sephadex column. After 20 tubes were collected, TLC revealed that tubes 10 to 12 contained the second compound in a very pure state. Tubes 10 and 11 were pooled and taken to dryness under vacuum to give 23.3 mg material.

Anhydroophiobolin A 3. nmr($CCL_4$) 100 MHz δ .85 (s, 3, C11-$CH_3$), 1.01 (d, J=7, 3, C15-$CH_3$), 1.60 (s, 3, C19-$CH_3$), 1.65 (s, 3, C19-$CH_3$), 2.02 (s, 3, C3-$CH_3$), 5.84 (s, 1, C4-H), 9.17 (s, 1, C21-H) (See Fig. 4).

Semi-synthetic anhydroophiobolin A Ophiobolin A (13.5 mg) was dissolved in 0.8 ml tetrahydrofuran. p-Toluenesulfonic acid (7.2 mg) was added and dissolved by swirling the vial. The solution was allowed to stand at room temperature for 16 hours. TLC examination revealed the quantitative conversion of ophiobolin A (Rf=.46) to a material with a higher Rf value (.53). This material was isolated by gel filtration using the small Sephadex LH-20 column or in later experiments, $H_2O$ was added to the completed reaction and the product extracted with $Et_2O$. nmr($CDCL_3$) 100 MHz δ .88 (s, 3, C11-$CH_3$), 1.04 (d, J=6, 3, C15-$CH_3$), 1.66 (s, 3, C19-$CH_3$), 1.70 (s, 3, C19-$CH_3$), 2.06 (s, 3, C3-$CH_3$), 6.07 (s, 1, C4-H), 9.30 (s, 1, C21-H) (See Fig. 5).

Phytochemical interrelationships of Ophiobolin and HMT toxin

Ophiobolin biosynthesis To determine possible nutritional factors affecting ophiobolin biosynthesis, several macro and micronutrients were examined. Table 4 lists the media tested. Two Fernbach flasks each containing 200 ml were prepared for each different medium. The flasks were autoclaved and inoculated with H. maydis Race T. The cultures were allowed to grow for 11 days in the usual manner. At the end of this time, the mycelium from each flask was removed and each pair of replicate cultures were pooled. 30 ml portions were removed from each medium and extracted with three, 30 ml portions of ethyl acetate. The combined solvent from each experiment was taken to dryness under vacuum, quantitatively reconstituted with 0.5 ml MeOH, and stored overnight at $0^{\circ}$ C. The samples were chromatographed using a $\mu$ $C_{18}$ Bondapak HPLC column using a solvent system composed of MeOH/water 65/35 (v/v). Compounds were detected using a UV detector operating at 254 nm. (See Fig. 6).

Table 4. Media variations

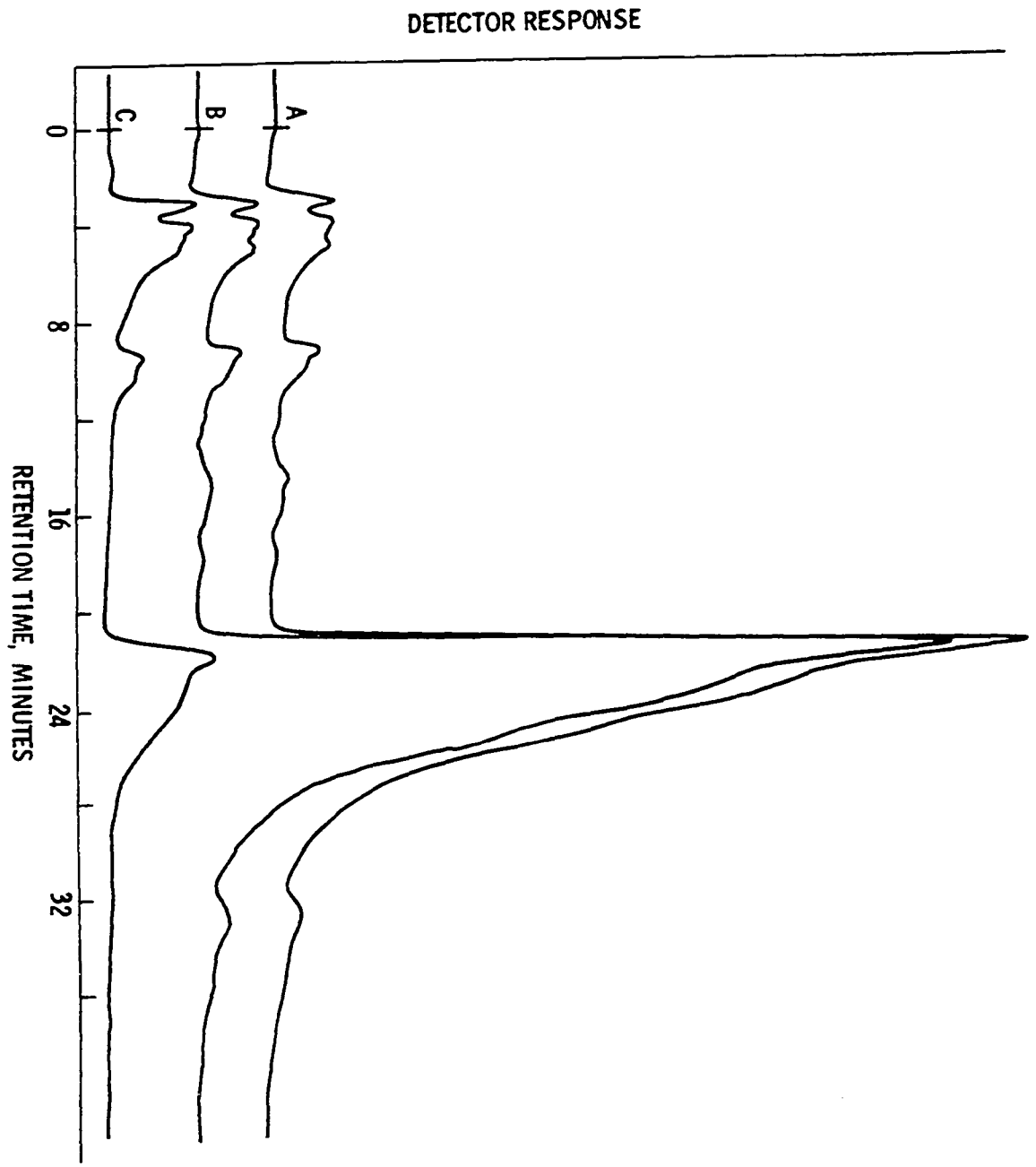
| | |
|---|---|
| 1. | Modified Fries with yeast extract |
| 2. | Same as #1 but glucose substituted for sucrose |
| 3. | Same as #1 but without tartaric acid |
| 4. | Same as #1 but with 4 mg/l $ZnSO_4 \cdot 7 H_2O$ |
| 5. | Same as #1 but with 6 mg/l $ZnSO_4 \cdot 7 H_2O$ |
| 6. | Same as #1 but with both 4 mg/l $ZnSO_4 \cdot 7 H_2O$ and 6 mg/l $MnSO_4 \cdot H_2O$ |
| 7. | Same as #1 but no yeast extract |
| 8. | Same as #7 but culture allowed to grow 18 days |
| 9. | Defined medium of Karr et al. (19). |
| 10. | Defined medium of Watrud et al. (45). |

Figure 6. High performance liquid chromatograms of media extracts

A = Medium B

B = Medium B plus 1.95 ppm manganese

C = Medium B plus 0.90 ppm zinc


DETECTOR RESPONSE
A
B
C
0
8
16
24
32
RETENTION TIME, MINUTES

Bioassay of materials separated by HPLC Thirty ml of Fries culture filtrate was worked up as usual and extracted twice with 30 ml portions of ethyl acetate. The combined extracts were pooled and taken to dryness under vacuum. The residue was taken up in 0.5 ml MeOH and 7 µl were injected into the HPLC. Operating conditions were described earlier. Separations were monitored by UV at 254 nm while HMT toxin activity was determined by the collection of 0.1ml solvent from exit port of the chromatograph every 2 min. Each fraction was diluted to 1/100 the original culture concentration with $H_2O$ and the leaf streak bioassay performed. Ophiobolin A co-comatographed with an authentic analytical standard. The retention times of toxin(s) activity and ophiobolin A were 3-13 and 18 min., respectively, at 1300 PSI. Figure 7 shows the results.

Separation of HMT toxin activity from Ophiobolins by gel filtration 100 ml of Fries culture filtrate was worked up as usual and extracted twice with 100 ml of ethyl acetate. The ethyl acetate was dried with sodium sulfate and evaporated to dryness. The resulting brown residue was reconstituted with 1 ml methanol and applied to the small Sephadex LH-20 column. Methanol, with a flow rate of 0.47 ml/min., was used to develop the column. A total of 10 ml solvent was collected in a beaker before the column was attached to the fraction collector. A total of 20, one milliliter fractions were collected. 25 µl of solvent was removed from each tube and spotted on a 20 x 20 cm analytical silica gel TLC plate. The plate was developed with solvent system A and visualized with phosphomolybdic acid. For bioassay,

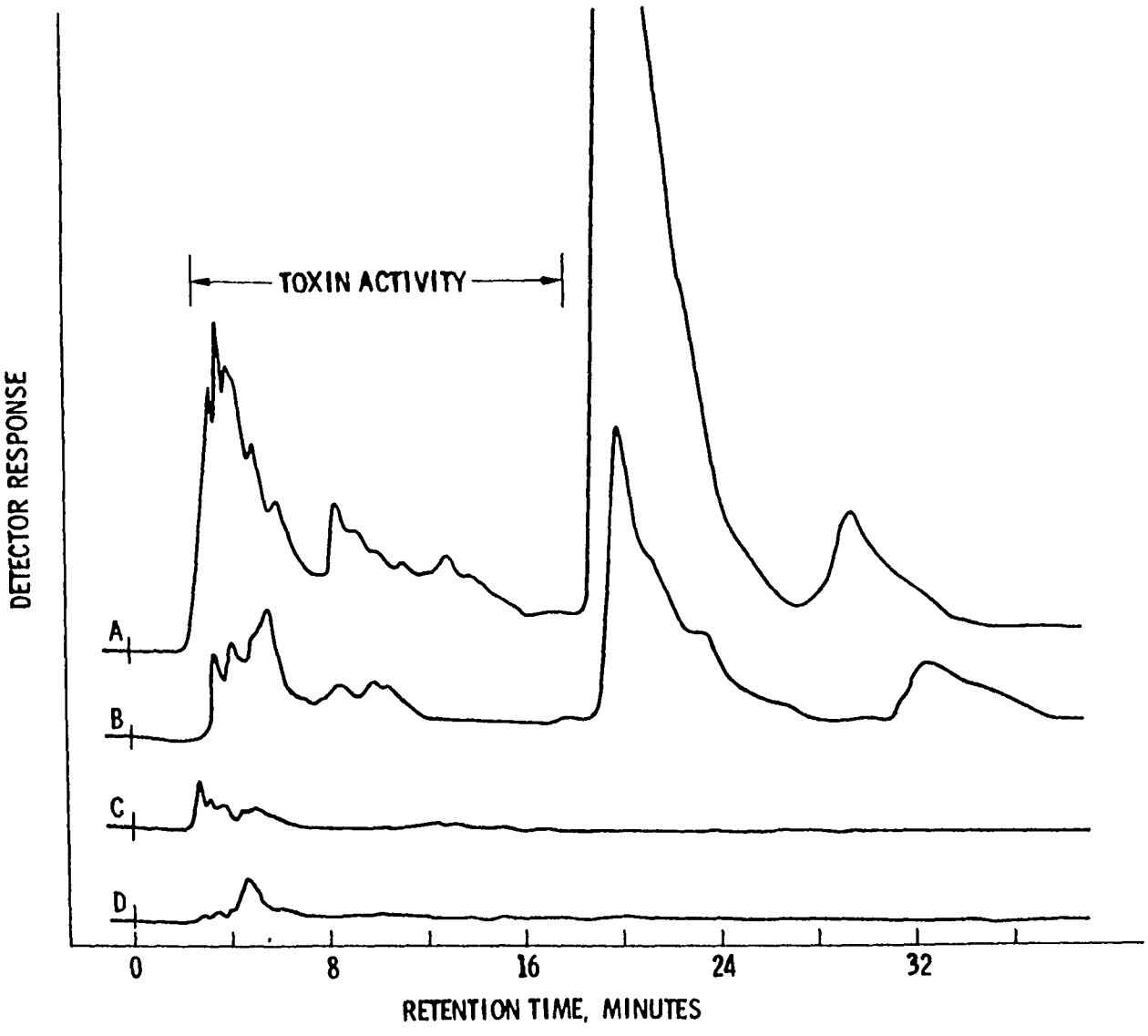
Figure 7. High performance liquid chromatograms of media extracts

A = Medium B

B = Medium D

C = Medium A

D = Medium C


TOXIN ACTIVITY
DETECTOR RESPONSE
A
B
C
D
0
8
16
24
32
RETENTION TIME, MINUTES

0.05 ml solvent was removed from each fraction and 5 ml $H_2O$ added. The leaf streak bioassay was done as usual. A control consisting of 0.5 ml MeOH and 5 ml of $H_2O$ was also tested. Tubes 14-18 contained material which co-comatographed with authentic ophiobolin A along with several unknown metabolites in minor amounts. Tubes 9-13 contained material at the plate origin but none of the less polar metabolites found in later fractions. Bioassay indicated that only tubes 9-13 contained material responsible for leaf streak production. The control had no effect. When the material in tubes 9-13 was pooled and examined by TLC using solvent system B and visualized with $H_2SO_4$/MeOH, only a large, brown spot at the plate origin was observed.

Comparison of H. maydis races T and O Six Fernbach flasks each containing 400 ml of the defined medium A were inoculated with H. maydis Race T while 5 similar flasks were inoculated with Race O. At the end of 18 days the cultures were harvested, filtered and the culture filtrate bioassayed by checking for leaf streak activity. Race T was active at 1/1000 dilution of the original culture concentration while Race O was not active undiluted. Culture filtrates from both races were extracted with ethyl acetate. In the case of Race T, evaporation of the solvent gave a white precipitate while only a nondrying oil was observed for Race O. The Race O extract was dissolved with 0.5 ml MeOH. Thirty µl of the clear, yellow solution was removed for TLC and the remainder was fractionated using the small Sephadex LH-20 column. 10.3 mg of the white precipitate from the Race T extract was also dissolved in 0.5 ml MeOH with gentle steam heating.

A small amount was removed for TLC and the rest fractionated in the same manner as the Race 0 extract.

The fractions collected from the Sephadex column were allowed to evaporate to dryness over a period of 3 days. With Race T, tubes 9-12 contained a white material while the corresponding tubes from Race 0 contained nothing. The contents of each tube collected were dissolved in 0.2 ml MeOH aided by gentle heating. Examination of each fraction by TLC revealed that the white material collected from the column was identical to the precipitate before the gel filtration. Corresponding fractions from the Race 0 extract contained none of these materials. Finally, dilutions were made from each fraction such that Race 0 fractions were equal to the original culture concentration while Race T were 1/10 original culture concentration. Leaf streaking activity was observed with Race T only in the tubes that contained the white precipitate. No leaf streaking ability was noted with any of the Race 0 fractions.

Isolation of HMT toxin

Gel filtration For preparative isolation of toxin the defined medium of Karr et al. (20) was used. After 12 days of fungal growth a total of 5400 ml culture medium was collected and filtered. One liter fractions were each extracted twice with 750 ml ethyl acetate. The pooled extracts were taken to dryness under vacuum. Four hundred ml toluene was added, the mixture taken to a clear, tan oil under vacuum at 40° C, 4 ml of MeOH was added and the oil brought into

solution by gentle heating. This solution was filtered through MeOH-washed cotton twice, placed on the large Sephadex LH-20 column and developed with MeOH. A total of 21 ml of solvent was collected in a beaker before the column was attached to the fraction collector. A flow rate of 0.66 ml/min. was used to collect 90, 3 ml fractions. For leaf streak bioassay, 0.1 ml was removed from every other tube and diluted to 1/100 the concentration of the culture filtrate. Tubes 24-30 were the only ones to contain active material. All tubes were allowed to evaporate to near dryness over a three day period. Only tubes 24-30 contained a white, waxy like substance. Hot MeOH was used to dissolve this material and the fractions were pooled in a tared flask. Evaporation of the solvent under vaccum gave 260 mg of the creamy yellow material. HPLC analysis was conducted by injecting 10 µl of a MeOH solution (10 µg/µl) of this material onto the reversed-phase system. With a sensitivity setting of 0.02 absorbance unit on the UV detector, only 3 extremely minor peaks were observed. For further purification, the sample was dissolved in approximately 2 ml hot MeOH and allowed to stand until a white precipitate formed. The yellow mother liquor was decanted and the precipitate washed with cold ethyl acetate to yield 35 mg white material. The mother liquor precipitated large quantities of additional material which TLC analysis in solvent system B revealed to be of lesser purity.

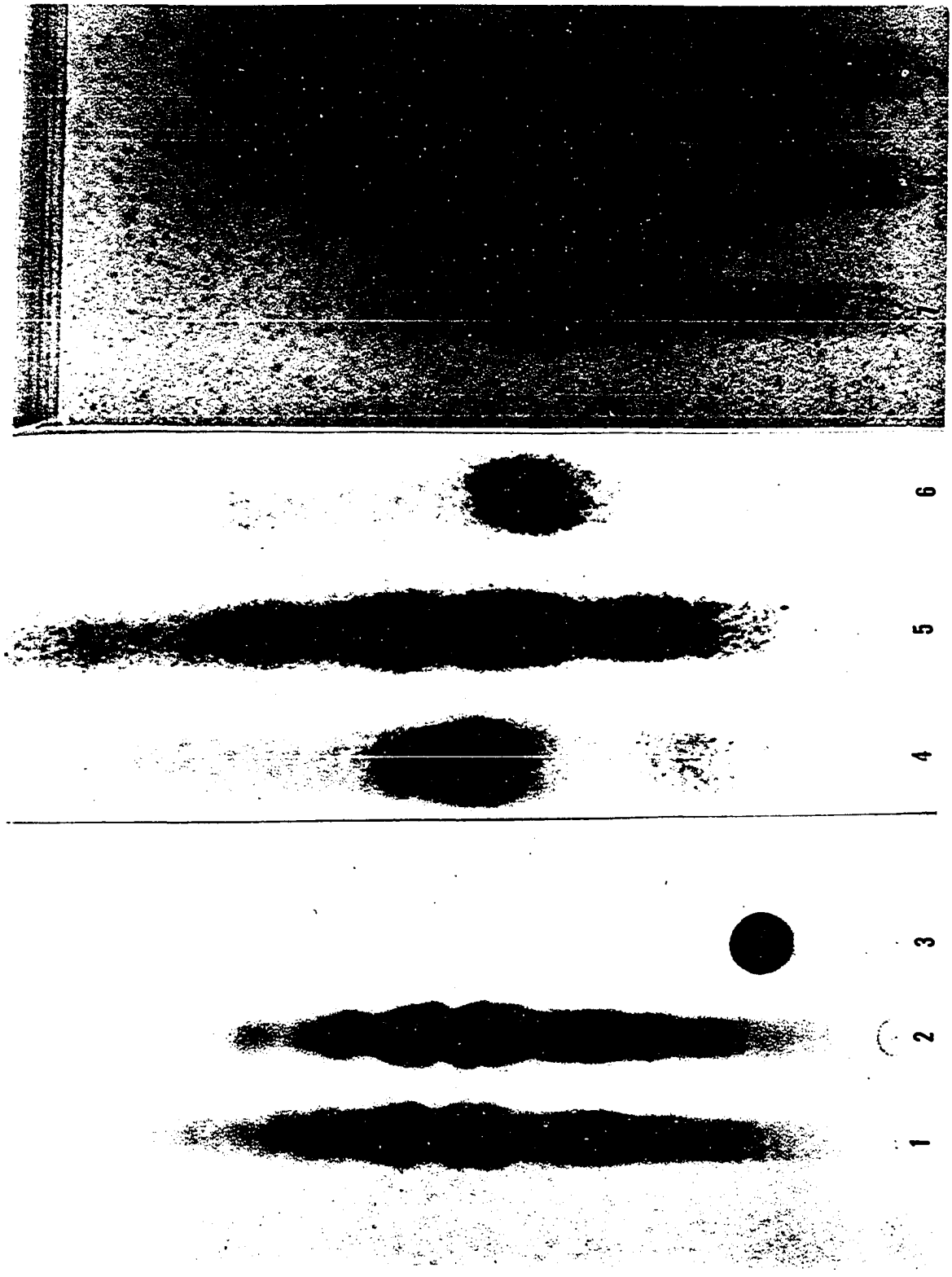
Solvent precipitation After 16 days of fungal growth, a total of 6550 ml culture media was collected and filtered. One liter

fractions were each extracted 4 times with 500 ml ethyl acetate. The pooled ethyl acetate extracts were evaporated under vacuum at 40° C to approximately 75 ml. At this point, the solvent appeared cloudy and was transferred to a 125 ml Erlenmeyer flask. After two hours, a quantity of chalky white precipitate formed from the light yellow mother liquor. The solvent was decanted and the precipitate washed with cold ethyl acetate. Fresh ethyl acetate was added and the material transferred to a tared flask. Evaporation of the solvent under vacuum yielded 129.8 mg white material which was dried 5 days over $CaSO_4$ to give a total of 129.2 mg material (19.7 mg/l culture media). TLC in solvent B showed this material to be nearly identical to that purified by gel filtration. A small amount of glucose (Rf = 0.0) was found in the precipitated toxins. (See Fig. 8).

Paper chromatography Whatman #1 chromatographic paper was used for analytical descending paper chromatography. The paper (19 x 50 cm) was irrigated for 18 hours with a solvent system composed of EtOAc/pyridine/water, 8:3:1 (v/v) at room temperature. To detect reducing sugars the dried chromatogram was first dipped into a silver nitrate solution (2.5 ml saturated $AgNO_3$ in 1 l acetone) and allowed to dry. The chromatogram was then visualized by spraying with base (10 ml of 40% NaOH in 1 l MeOH). After dark spots appeared, the chromatogram was dipped into the fixer (240 g $Na_2S_2O_3 \cdot 5H_2O$, 25 g $Na_2S_2O_5$, and 10 g $Na_2SO_3$ in 1 l $H_2O$) until the background was white. The excess reagents were removed with running water. In a typical chromatogram, 100 to 200 µg of the HMT toxin mixture dissolved in

Figure 8. Thin layer chromatograms of HMT toxins. Solvent system B was used

1 = HMT toxin complex purified by gel filtration
2 = HMT toxin complex purified by solvent precipitation
3 = External standard 2-deoxy-D-ribose
4 = Purified B1
5 = HMT toxin standard
6 = Purified P1
7 = Purified P2
8 = HMT toxin standard
9 = Purified B2


6
5
4
3
2
1

MeOH was applied to the paper. A standard composed of glucose, arabinose, xylose, and rhamnose dissolved in water was also supplied. Glucose was the only sugar obtained from EtOAc precipitated toxin samples. Toxin samples passed through Sephadex LH-20 contained no glucose. HMT toxins did not react with the visualizing reagents.

HMT toxin stability

Chemical A 20 x 20 cm silica gel TLC plate 0.25 mm thick was spotted in the lower left corner with approximately 200 µgm of the toxin mixture dissolved in MeOH. The plate was developed in solvent system B, removed and dried, rotated 90 degrees and placed in fresh solvent for development in the second dimension. After the second development and visualization by spraying with $H_2SO_4$/MeOH all the components were found on a straight line 45 degrees from horizontal. This indicates that no decomposition took place during chromatography.

Any possible effects of $P_2O_5$ and $CaSO_4$ on the toxin mixture was tested. EtOAc precipitated toxin (1 mg) was placed in each of two vials. One was held under vacuum over $P_2O_5$ for one week. The other was held over $CaSO_4$ without vacuum for one month. At the end of the respective times TLC examinations indicated no decomposition had taken place. Leaf streak bioassays indicated full biological activity from each sample.

One half milligram of the toxin mixture was placed in each of 3 vials. MeOH (0.3 ml) was added and the mixture warmed until dissolved. Reagent grade $CHCl_3$ (0.7 ml) was added to one vial, reagent grade $CHCl_3$ passed through neutral alumina (0.7 ml) was added to the second and

MeOH (0.7 ml) added to the third. The vials were allowed to stand at 27° C for 12 hours. The solvents were removed under a stream of nitrogen, 0.1 ml MeOH added with gentle warming to redissolve the samples which were then examined by TLC. A control, which was the toxin mixture dissolved in warm MeOH immediately before TLC, had no component with an Rf value greater than .67. The sample which stood in MeOH had one faint pink and one faint brown spot with Rf values greater than .67 as did the sample in alumina treated $CHCl_3$. The sample in untreated $CHCl_3$ had several pink or brown spots with Rf values larger than .67. A new toxin sample was dissolved in reagent grade $CHCl_3$ as before except it was stirred at 30° C for 26 hours. TLC examination indicated almost complete conversion (approximately 90% by comparison of spot sizes and color intensities) to materials with larger Rf values. In another experiment, one sample of the toxin mixture was stored under EtOAc for a period of 3 years and a second sample was held in an open flask for 2 years. TLC examination of these samples indicated no decomposition had occurred. Leaf streak bioassays showed full biological activity had been retained. Five milligrams of the toxin mixture was dissolved in 0.5 ml pyridine-$d_5$ by gentle heating over steam. NMR analysis indicated extensive decomposition of the sample.

Biochemical A 20 x 5 cm silica gel TLC plate was spotted and developed in solvent system B. All the silica gel was scraped from the plate and immediately eluted with MeOH. The solution was concentrated in vacuum, respotted on a new TLC plate and developed.

This procedure could be followed for three times without altering the appearance of the final chromatogram when visualized. To test for possible loss of biological activity during TLC, 2.15 mg of the toxin mixture was dissolved in 1.075 ml MeOH with gentle steam heating. Dilutions were made with MeOH to give a final concentration of 20 µg/ml. Two milliliters were removed and 1 ml was placed in each of two test tubes. The MeOH from each was evaporated with a stream of nitrogen. A total of 75 µl MeOH was used to apply the toxin from tube 1 to a 20 x 5 cm silica gel TLC plate that had been predeveloped in a solvent system of $CHCl_3$:MeOH; 50:6 (v/v) and allowed to dry. The plate was subjected to multiple development in a solvent system of $CHCl_3$:MeOH; 50:3 (v/v) three times. Fresh solvent was used for each development. After chromatography all the silica gel was removed from the plate and eluted with 6 ml MeOH 4 times and filtered through Whatman #2 filter paper. The MeOH was removed under vacuum at $40^{\circ}$ C. To serve as a control, sample number 2 was taken up in a total of 75 µl MeOH and placed in a flask. The MeOH was removed under vacuum using the same conditions as sample 1. MeOH (0.2 ml) was added to each sample which dissolved with gentle heating. Water was added to each flask to give a concentration of 0.5 µg/ml. Dilutions were made from these stock solutions to .05 µg/ml and bioassays were run. Data from the leaf streak bioassay was complicated but it appeared that a small amount of activity may have been lost (See Table 5). The mitochondrial assay indicated that there has been no loss of activity.

Table 5. Leaf streak bioassay of HMT toxins after TLC

| Sample | Concentration (mg/ml) | Response | Lesion size (cm) | | |
|---|---|---|---|---|---|
| $H_2O$ only #1 | 0 | 000 | | | |
| | .050 | +++ | .8 | .5 | .2 |
| | .056 | +++ | .8 | .5 | 1.0 |
| | .063 | +++ | .3 | .4 | .9 |
| | .071 | +++ | .9 | .4 | .6 |
| | .083 | +++ | .3 | 1.0 | 1.0 |
| | .100 | +++ | .6 | .7 | .7 |
| | .125 | +++ | .7 | .6 | .9 |
| | .167 | +++ | 1.0 | 1.0 | 1.0 |
| | .250 | +++ | 1.0 | 4.0 | .6 |
| | .333 | ++ | 2.0 | .6 | |
| | .500 | ++ | 3.0 | 2.5 | |
| | | Sample #2 Control | | | |
| $H_2O$ only #2 | 0 | 000 | | | |
| | .05 | +++ | 1.5 | .6 | 2.0 |
| | .056 | +++ | .8 | 1.6 | .5 |
| | .063 | +++ | 1.0 | 1.5 | 1.3 |
| | .071 | ++ | .6 | .6 | |
| | .083 | ++ | .6 | 2.0 | |
| | .100 | ++ | 1.5 | 2.0 | |
| | .125 | +++ | 3.0 | 2.0 | .9 |
| | .167 | +++ | 1.0 | 1.6 | 2.0 |
| | .250 | +++ | 1.0 | 2.0 | 3.0 |
| | .333 | +++ | 1.0 | 2.5 | 1.5 |

Chemical tests for toxin functionality

Ferric chloride test (48) Two to five mg of the compounds tested were placed in a test tube and dissolved in 0.1 ml MeOH. One milliliter of $H_2O$ was added and then 5 drops 1% aqueous ferric chloride were quickly added. Any instantaneous color change was noted. Ferric chloride solution was added to 0.1 ml MeOH in 1 ml $H_2O$ as a control (See Table 6).

Table 6. Ferric chloride test results

| Sample | Color formed |
|---|---|
| Control | Colorless |
| Glucose | Colorless |
| Acetylacetone | Red-brown |
| 1,2-Cyclohexanedione | Violet-brown |
| Phloroglucinol | Violet |
| HMT toxin | Yellow |

There was no change in coloration of the toxin sample after standing at room temperature 2 hours; however, the control and glucose samples had started to turn yellow.

Sodium metaperiodate-benzidine (43) The HMT toxin mixture was resolved by TLC using solvent system B. A 0.1% aqueous solution of sodium metaperiodate was sprayed on the TLC plate and allowed to dry. A second spray reagent was made of 0.9 g benzidine dissolved in 25 ml EtOH and 25 ml $H_2O$. Acetone (10 ml) and 5 ml 0.2 N HCl was added to this solution which was sprayed on the periodate treated TLC plate. White spots on a blue background are observed with 1,2-diol groups or functionality which under the oxidizing conditions of the spray reagent can give rise to 1,2-diols. In the case of the toxins, all components yielded a bluish-white spot against a dark blue background. A control (glucose) produced a clean, white spot.

Feeding experiments

Incorporation of 1,2-$^{14}$C sodium acetate into HMT toxin Two Fernbach flasks each containing 400 ml medium A were inoculated with H. maydis Race T and placed in a hood supplied with continuous fluorescent lighting. After 3 days, 25 µCi $^{14}$C sodium acetate were added (38 µg sodium acetate, 54 mCi/mmol, in 0.25 ml EtOH). Eight days after inoculation an additional 25 µCi sodium acetate were added taking care not to disturb the mycelium. After 12 days of growth the cultures were pooled and the HMT toxins isolated as usual to give 15 mg white material. Approximately 250 µg were dissolved in hot MeOH, spotted on a 5 x 20 cm precoated silica gel TLC plate and developed in solvent system B. After visualization with $H_2SO_4$, Kodak no-screen medical X-ray film was placed over the TLC plate and kept in contact with the chromatogram for 2 weeks in a light-tight cassette. Development of the radioautogram showed a chromatographic pattern quantitatively and qualitatively similar to the pattern obtained from $H_2SO_4$ visualization.

Radioassay Liquid scintillation counting was done in a Bray's solution of the following composition: 60 g napthalene, 4 g 2,5-diphenyloxazole (PPO), 100 mg 1,4-bis-2(5-phenyloxazoyl) benzene (POPOP), 20 ml ethylene glycol, 100 ml methanol, and dry, peroxide-free dioxane to make one liter. The sample of HMT toxins (1.32 mg) to be counted was weighed on a microanalytical balance. The sample was dissolved in enough MeOH to make 1 ml of solution, 0.05 ml removed (66 µg toxin), and added to 15 ml of the Bray's solution. Counting

efficiencies were determined by the internal standard method using calibrated $^{14}C$ labeled toluene. For this experiment, efficiency was determined to be 65%. Percent incorporation was calculated using the following formula:

$$\% \text{ incorporation} = \frac{\text{total activity of HMT toxins}}{\text{total activity fed}} \times 100$$

With this experiment, percent incorporation was determined to be 2.55%.

Isolation of individual HMT toxin components

129 mg of ethyl acetate-precipitated toxin was dissolved in 3.25 ml MeOH with gentle, steam heating. A commercial preparative TLC applicator was used to apply a total of 0.5 ml of the solution to each of 5 precoated silica gel plates 0.5 mm thick. On a sixth plate, a total of 29 mg was applied. A $CHCl_3$:MeOH; 50:3 (v/v) system was used to develop the plates. After the first development the plates were removed, dried and quickly placed in fresh solvent for the second development. Upon completion the plates were removed, dried, and visualized by spraying them with distilled $H_2O$. The resolved bands appeared opaque against the translucent silica gel background. Bands corresponding to the 5 major components were scraped from each plate with the silica gel from similar bands being pooled. Each fraction was placed on a filter of MeOH-washed filter paper and eluted with MeOH. The MeOH was taken to dryness under vacuum and the residue dissolved in 1 ml MeOH for spotting onto analytical TLC plates. Analytical examination revealed that compounds B2 and P2 were not satisfactorily separated so the above procedure was repeated

on samples containing these compounds (See Fig. 8). To get an accurate weight of each component for bioassay purposes, dissolved silica gel and other contaminants had to be removed. For each compound, the sample was dissolved in 0.5 ml hot MeOH, placed on the small Sephadex LH-20 column and developed with MeOH. Twelve ml of solvent was always collected before the fraction collector was started. A flow rate of 0.46 ml/min. was used to collect from 15 to 20 tubes, each containing 1.4 ml solvent. 10 µl from each fraction were spotted onto a single TLC plate which was visualized by spraying with $H_2SO_4$/MeOH without plate development. In the case of P1 and P2, tubes 7, 8, and 9 were found to contain organic material giving a pink coloration. With B1 and B2, tubes 7, 8, and 9 were found to contain organic material giving a brown coloration. This procedure gave the same results with sample L, which is actually at least 3 minor compounds with Rf values lower than the values observed with major toxins. In each case, the positive tubes were pooled, subjected to analytical TLC, and placed in tared flasks. The flasks were allowed to evaporate to dryness under $N_2$ and stored over $CaSO_4$ for several days before weighing. The total amount of individual compounds isolated were: P1, 1.94 mg; B1, 2.08 mg; P2, 3.23 mg; B2, .29 mg; L, 2.46 mg for a total recovery of approximately 8%. It was later found that recovery of unaltered individual toxin components was much higher if MeOH solutions of the compounds were never taken to complete dryness by rotary evaporation. In one typical isolation, preparative TLC of 11.55 mg of HMT

toxin followed by gel filtration of isolated components resulted in the recovery of 2.2 mg P2 and 2.8 mg of a mixture of P1 and B1. In several cases, rotary evaporation from MeOH produced materials having much higher Rf values on analytical TLC examination. Slow evaporation over a period of hours or days never resulted in the production of these materials.

Bioassay of individual compounds

Leaf streak assay Known weights of the pure compounds were dissolved in 1 ml MeOH. Ten µl aliquots were removed from each and diluted with $H_2O$ to give solutions of 1 µg/ml. Similar conditions were made using the HMT toxin mixture to serve as a control. Results of the assay are shown in Table 7.

Mitochondrial assay Individual compounds at 0.2 µg/ml were used as stock solutions in this assay. A standard curve was prepared from the HMT toxin mixture at 1.0, 0.2, 0.1, and 0.02 µg/ml. Host specificity was checked on mitochondria from corn containing N cytoplasm at toxin concentrations of 1 µg/ml. Figure 9 shows the results.

Bioassay of "decomposed toxin" On one occasion, 27 mg of the toxin mixture dissolved in MeOH was taken to dryness under vacuum at 40° C. TLC examination of the material before and after evaporation indicated that the entire amount had been converted to materials with mugh higher Rf values but retained the characteristic pink and brown coloration when visualized by spraying with sulfuric acid. The material was tested for leaf streaking activity at 7.6, 1.0, 0.1, and 0.02 µg/ml. Table 8 lists the results.

Table 7. Leaf streak assay of purified HMT toxin components

| Sample | Concentration (µg/ml) | Response | Lesion size (cm) |
|---|---|---|---|
| $H_2O$ | 0 | 00 | .2, .4, .3, .4, .3 |
| P1 | 0.02 | +++++ | .2, .4, .3, .4, .3 |
| | 0.2 | +++++ | .4, .2, .5, .4, .5 |
| B1 | 0.02 | ++++0 | .3, .2, .2, |
| | 0.2 | ++++0 | .6, .4, .4, .7 |
| P2 | 0.02 | +++00 | .1, .1, .1 |
| | 0.2 | +++++ | .1, .3, .1, .4, .3 |
| B2 | 0.02 | +0000 | .1 |
| | 0.2 | +++++ | .5, .3, .3, .7, .5 |
| L | 0.02 | ++++0 | .2, .1, .2, .1 |
| | 0.2 | +++++ | .6, .1, .2, .3, .8 |
| Control (HMT toxin mixture) | 0.02 | ++++0 | .1, .3, .3, .2 |
| | 0.2 | +++++ | .4, .5, .8, .5, .5 |

Table 8. Leaf streak assay of "decomposed toxin"

| Sample | Concentration (µg/ml) | Response | Lesion size (cm) |
|---|---|---|---|
| $H_2O$ | | 000 | |
| Decomposition product | 0.02 | +000 | .2 |
| | 0.1 | ++++ | .4, .3, .7, .3 |
| | 1.0 | ++++ | 5.5,4.0, .3,3.0 |

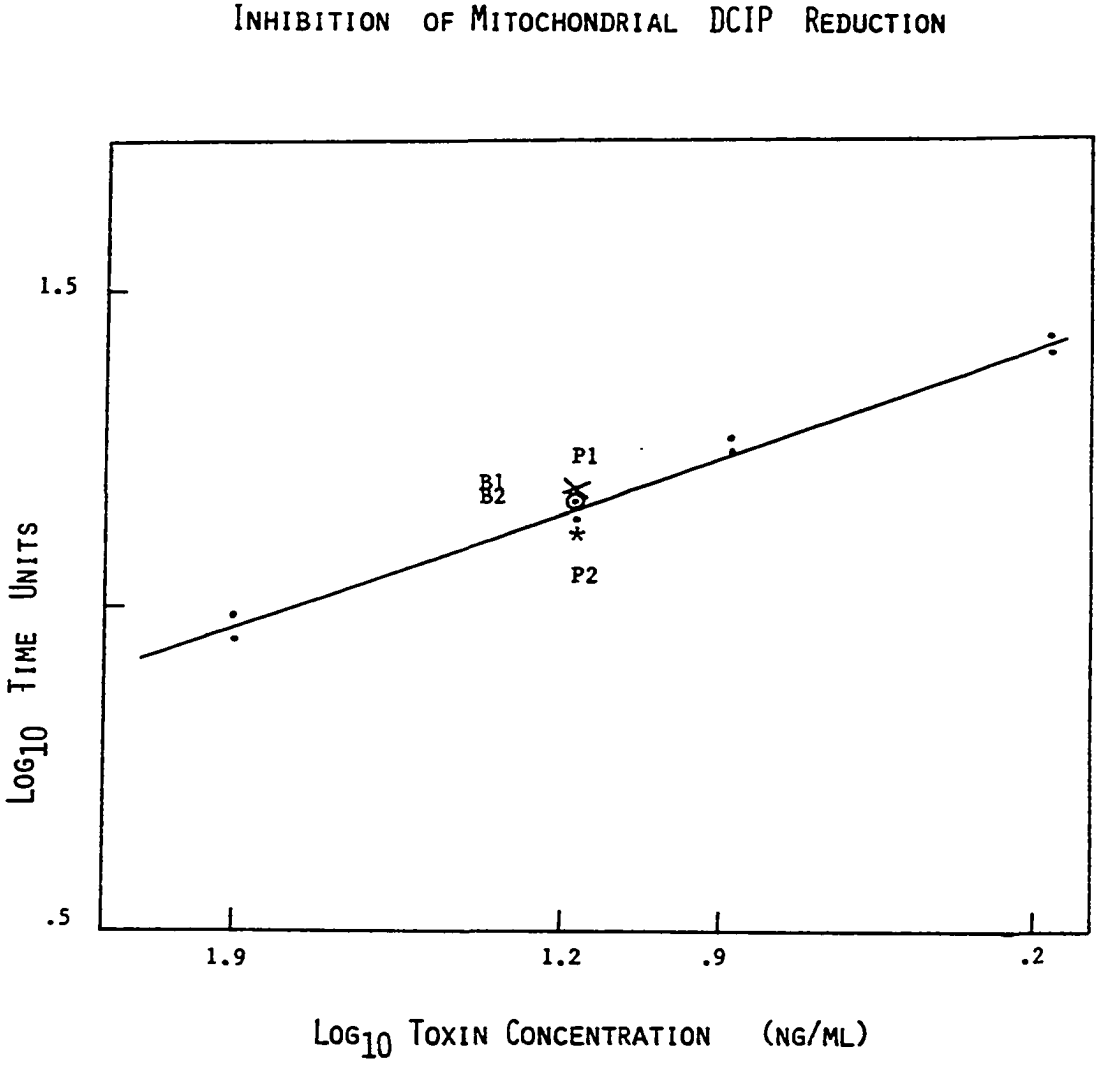



Figure 9. Inhibition of Tcms maize mitochondrial DCIP reduction by purified HMT toxins

X = P1
O = B1 and B2
* = P2

Chemical characterization of toxin

100 MHz $^{1}H$ NMR data on toxin mixture HMT toxin (6.4 mg) precipitated from EtOAc was dissolved in 0.25 ml MeOH-$d_4$ with gentle warming of solvent. δ 0.90 (t, $-CH_2CH_3$ methyl), 1.40, 1.57, 1.63, 1.78, 1.85 (broad multiplets, aliphatic regions), 2.11, 2.15 (s, $-CH_2COCH_3$ methyls), 2.43, 2.50, 2.55, 2.61 (broad multiplets, $-CH_2COCH_2-$), 3.74, 4.29, 4.47 (q, $-CH_2CHOHCH_2-$ methine). The singlet at 2.11 was more intense than the singlet at 2.15 (see Fig. 10).

Mass spectral data on toxin mixture Several milligrams of HMT toxin precipitated from EtOAc was dried under vacuum for several days. A direct solid inlet system was used. For typical data see Figs. 11, 12, and 13. No molecular ions were observed.

22.6 MHz $^{13}C$ Ft-NMR data on toxin mixture Sixty mg HMT toxin precipitated from EtOAc was dissolved in 2.5 ml MeOH-$d_4$ with warming of solvent (See Fig. 14). A similar toxin sample (70 mg) was passed through the large Sephadex LH-20 column. Fractions containing the toxin mixture were pooled and the solvent removed under vacuum. The sample dissolved in approximately 1 ml warm MeOH-$d_4$/MeOH (1:1). Figure 15 shows the spectrum obtained. TLC examination of the sample after NMR analysis showed a pattern similar to the standard along with several components having Rf values greater than the standard.

Other data obtained on toxin mixture IR (KBr) 1100, 1260, 1375, 1405, 1720, 2830, 2870, 2880, and 3420 $cm^{-1}$ (See Fig. 16). λ max (MeOH) 269, m.p. 108°. Elemental analysis of EtOAc precipitated toxin; % C, 59.90, H, 9.07, O, 31.28, N, 0.09. The sample contained

1.15% ash. Elemental analysis after removal of contaminating glucose by gel filtration; % C, 63.48, H, 9.32, O, 26.96.

90 MHz $^{1}$H Ft-NMR data on individual toxin components P1: 960 µg P1 was dissolved in 150 µl MeOH-$d_4$, 100.0 atom % D. A spectrum identical to the 100 MHz spectrum of the HMT toxin mixture was obtained except the signal at δ 2.12 was only a shoulder. The signal at 2.16 was retained (See Fig. 17). P2: 1.60 mg P2 was done as P1. Again the only differences were at δ 2.12 and 2.16. The singlet at 2.12 contained more area than the singlet 2.16 (See Fig. 18). B1: 1.03 mg B1 was done as P1. The only differences were at δ 2.12 and 2.16. The singlet at 2.16 was only a small shoulder while the signal at 2.12 was retained (See Fig. 19). B2: 144 µg B2 was done at P1. Resolution of this small sample was poor but sufficient information was obtained to indicate structural similarities to P1, P2, and B1 (See Fig. 20). In all these spectra, solvent peaks appear at δ 3.2, 3.3, and 4.9.

100 MHz $^{1}$H NMR data on two individual toxin components The isolation of B1 and P2 was done a second time from a different sample of the HMT toxin mixture. The larger samples (2 to 3 mg) were reexamined at 100 MHz using a signal averager (CAT). Although resolution was improved, the only detectable differences appear in the δ 2.11 to 2.16 region (See Fig. 21 and 22). After NMR analysis sample P2 was rechromatographed by preparative TLC and reexamined by 90 MHz $^{1}$H Ft-NMR. Figure 23 shows the spectrum which was obtained after the sample had been dissolved in MeOH for several days.

Figure 10. 100 MHz proton NMR spectrum of HMT toxin mixture. The scan width was 1000 Hz, 8 scans were accumulated and averaged

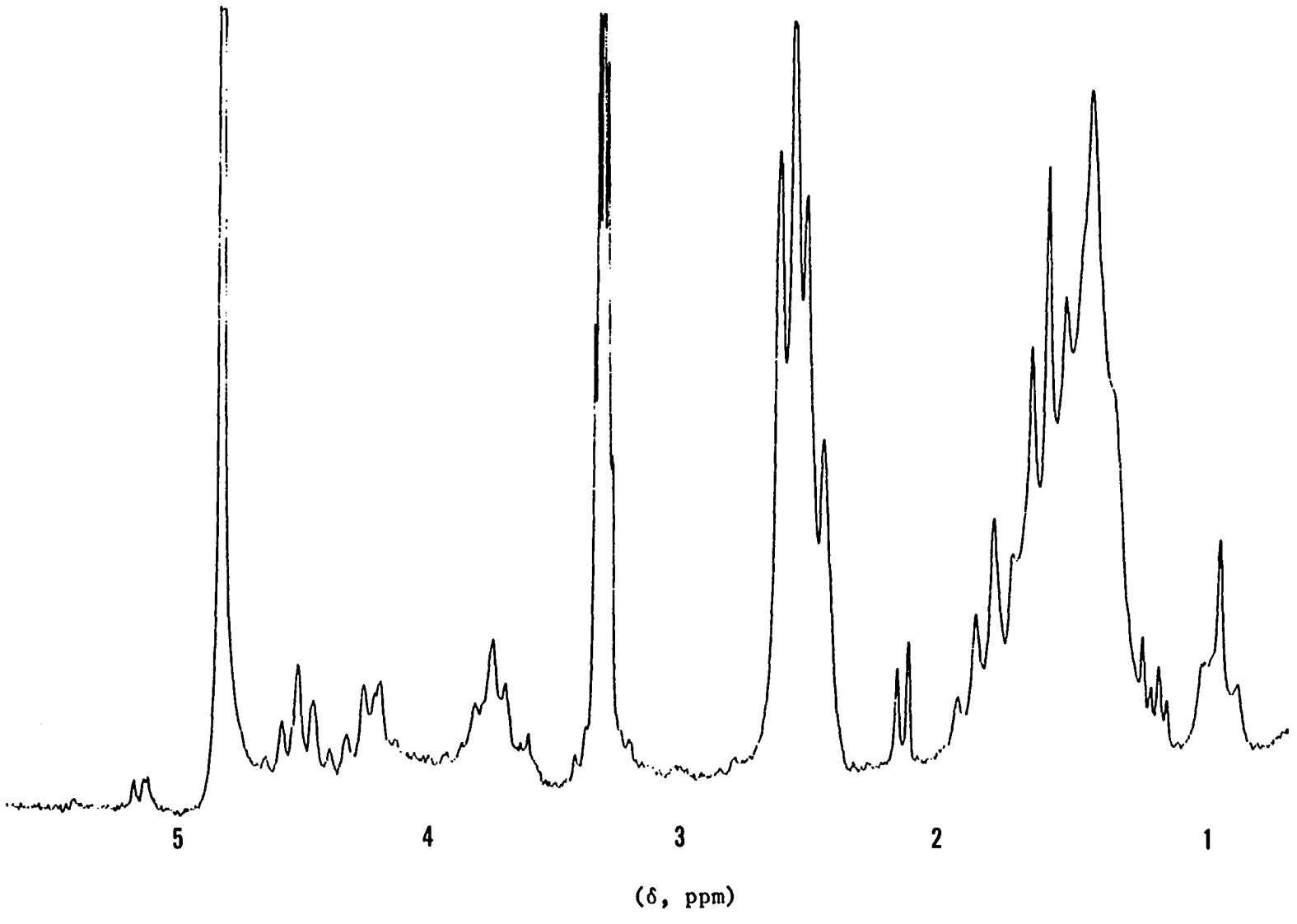

5
4
3
2
1
(δ, ppm)

Figure 11. Electron impact mass spectrum of HMT toxin mixture. This was an early scan

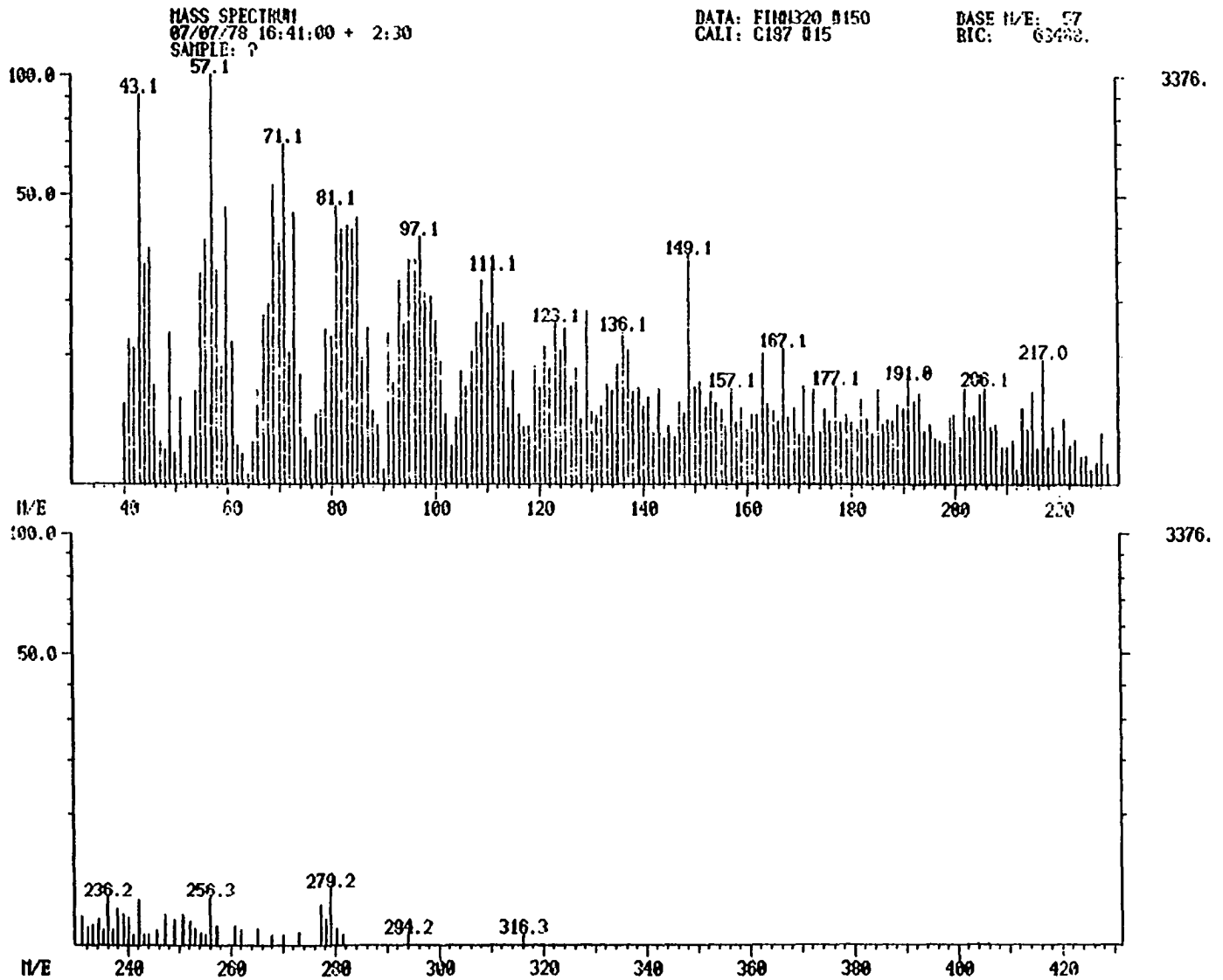

MASS SPECTRUM
07/07/78 16:41:00 + 2:30
SAMPLE: ?
DATA: FIN0320 0150
CALI: C197 015
BASE M/E: 57
RIC: 63488.
100.0
50.0
3376.
43.1
57.1
71.1
81.1
97.1
111.1
123.1
136.1
149.1
157.1
167.1
177.1
191.0
206.1
217.0
M/E
40
60
80
100
120
140
160
180
200
220
100.0
50.0
3376.
236.2
256.3
279.2
294.2
316.3
M/E
240
260
280
300
320
340
360
380
400
420

Figure 12. Electron impact mass spectrum of HMT toxin mixture. This was a scan at the ionization midpoint

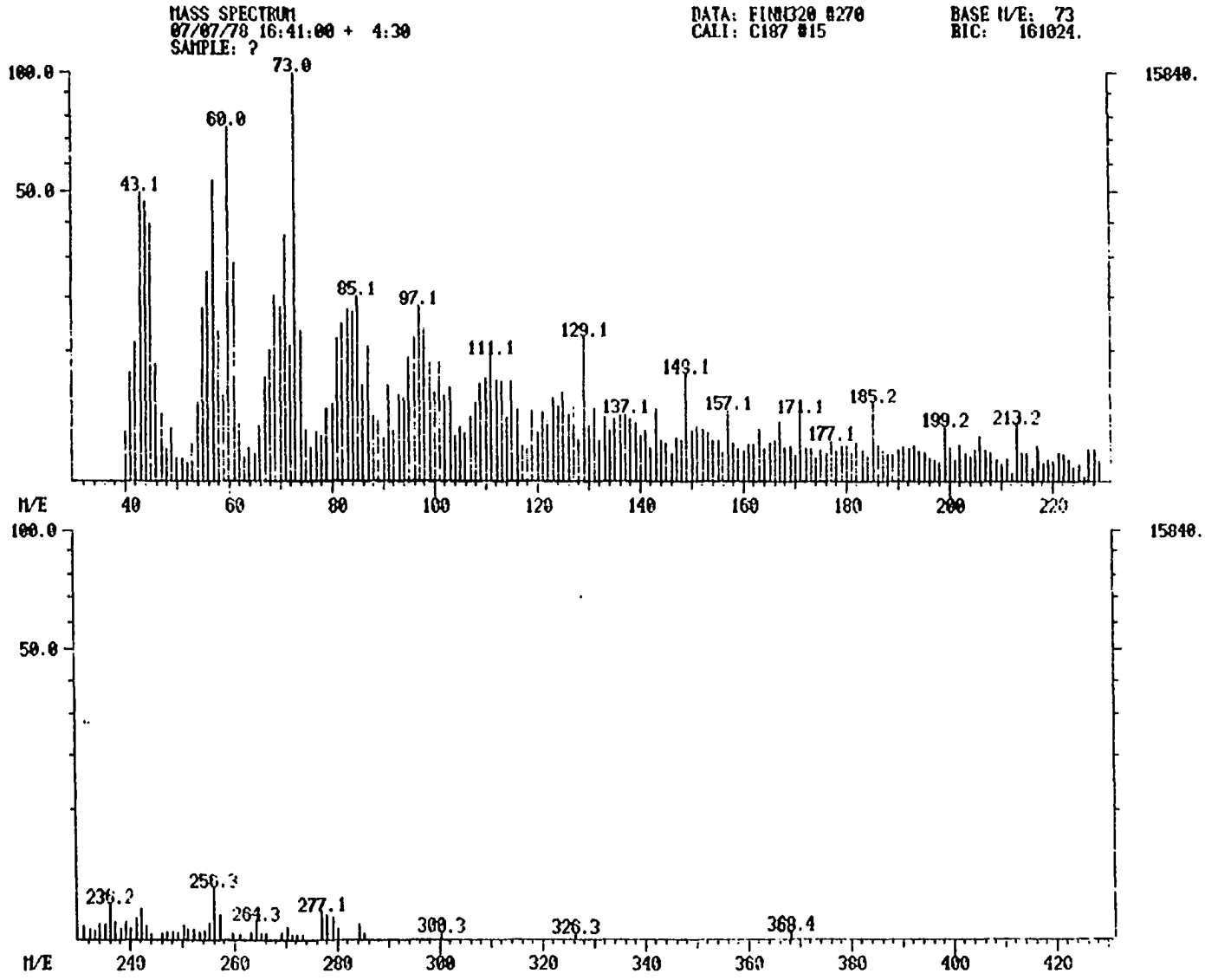

MASS SPECTRUM
07/07/78 16:41:00 + 4:30
SAMPLE: ?
DATA: FIN0320 0270
CALI: C187 015
BASE M/E: 73
RIC: 161024.
100.0
50.0
15840.
73.0
60.0
43.1
85.1
97.1
111.1
129.1
149.1
137.1
157.1
171.1
177.1
185.2
199.2
213.2
M/E
40
60
80
100
120
140
160
180
200
220
100.0
50.0
15840.
236.2
256.3
264.3
277.1
300.3
326.3
368.4
M/E
240
260
280
300
320
340
360
380
400
420

Figure 13. 70eV electron impact mass spectrum of HMT toxin mixture. This was a late scan

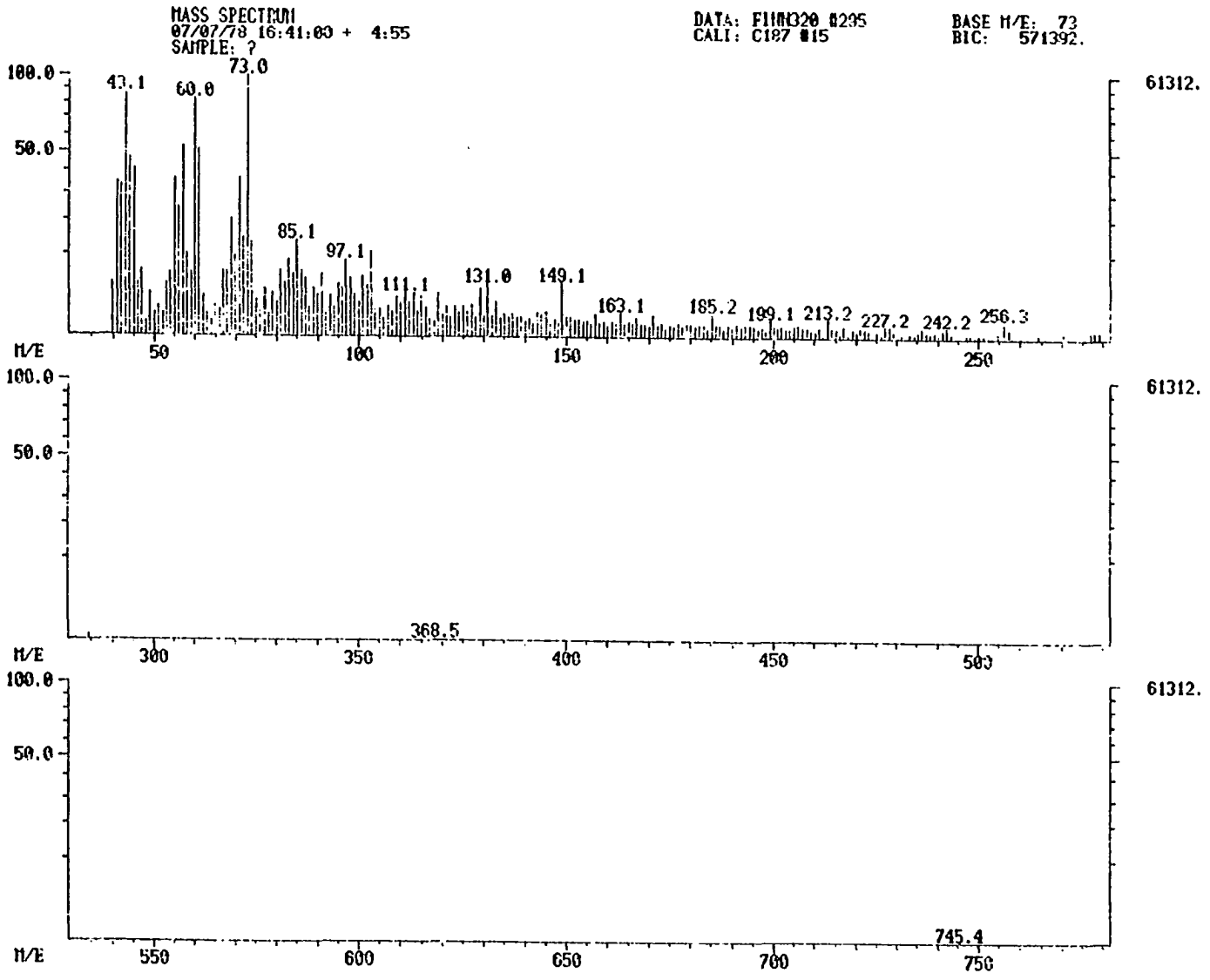

MASS SPECTRUM
07/07/78 16:41:00 + 4:55
SAMPLE: ?
DATA: F1MM320 0295
CALI: C187 815
BASE M/E: 73
BIC: 571392.
100.0
50.0
43.1
60.0
73.0
85.1
97.1
111.1
131.0
149.1
163.1
185.2
199.1
213.2
227.2
242.2
256.3
61312.
M/E
50
100
150
200
250
100.0
50.0
368.5
61312.
M/E
300
350
400
450
500
100.0
50.0
745.4
61312.
M/E
550
600
650
700
750

Figure 14. Broadband decoupled $^{13}$C-NMR spectrum of HMT toxin mixture before gel filtration

%XP
HMT TOXIN IN CD3OD 2JUN78 P86701

| NO. | CURSOR | FREQ. | PPM | INTENS. | AREA |
|---|---|---|---|---|---|
| 1 | 872 | 4795.304 | 211.844 | 38912 | 240896 |
| 2 | 889 | 4782.803 | 211.291 | 23040 | 83200 |
| 3 | 2023 | 3948.901 | 174.452 | 17024 | 86336 |
| 4 | 4371 | 2222.267 | 98.174 | 16832 | 80448 |
| 5 | 4501 | 2126.670 | 93.950 | 31360 | 96064 |
| 6 | 4991 | 1766.342 | 78.032 | 21504 | 104512 |
| 7 | 5088 | 1695.012 | 74.881 | 34496 | 95296 |
| 8 | 5119 | 1672.216 | 73.874 | 32704 | 117632 |
| 9 | 5147 | 1651.626 | 72.964 | 34688 | 112832 |
| 10 | 5180 | 1627.359 | 71.892 | 41536 | 223616 |
| 11 | 5206 | 1608.239 | 71.047 | 19904 | 89280 |
| 12 | 5303 | 1536.909 | 67.896 | 18368 | 46720 |
| 13 | 5347 | 1504.553 | 66.467 | 19200 | 28672 |
| 14 | 5384 | 1477.344 | 65.265 | 44736 | 196800 |
| 15 | 5460 | 1421.457 | 62.796 | 32192 | 131072 |
| 16 | 5788 | 1180.258 | 52.140 | 29504 | 105856 |
| 17 | 5797 | 1173.639 | 51.848 | 155776 | 752256 |
| 18 | 5817 | 1158.932 | 51.198 | 69376 | 348288 |
| 19 | 5826 | 1152.314 | 50.906 | 537024 | 2567744 |
| 20 | 5840 | 1142.019 | 50.451 | 100288 | 475072 |
| 21 | 5846 | 1137.607 | 50.256 | 102912 | 447872 |
| 22 | 5855 | 1130.988 | 49.964 | 1051520 | 5185088 |
| 23 | 5875 | 1116.281 | 49.314 | 112256 | 576960 |
| 24 | 5884 | 1109.663 | 49.022 | 1231680 | 6169920 |
| 25 | 5913 | 1088.337 | 48.079 | 1060224 | 6017792 |
| 26 | 5942 | 1067.012 | 47.137 | 529344 | 2956416 |
| 27 | 5971 | 1045.686 | 46.195 | 167936 | 1015232 |
| 28 | 6012 | 1015.536 | 44.863 | 28928 | 241280 |
| 29 | 6031 | 1001.564 | 44.246 | 27136 | 121792 |
| 30 | 6062 | 978.768 | 43.239 | 46592 | 189056 |
| 31 | 6374 | 749.335 | 33.103 | 21952 | 39232 |
| 32 | 6585 | 594.173 | 26.249 | 30144 | 140864 |
| 33 | 6638 | 555.199 | 24.527 | 19008 | 72960 |
| 34 | 6662 | 537.550 | 23.747 | 23360 | 50240 |
| 35 | 6828 | 415.480 | 18.354 | 29952 | 110016 |
| 36 | 6948 | 327.236 | 14.456 | 17024 | 28416 |
| 37 | 7393 | 0.000 | 0.000 | 225728 | 896320 |

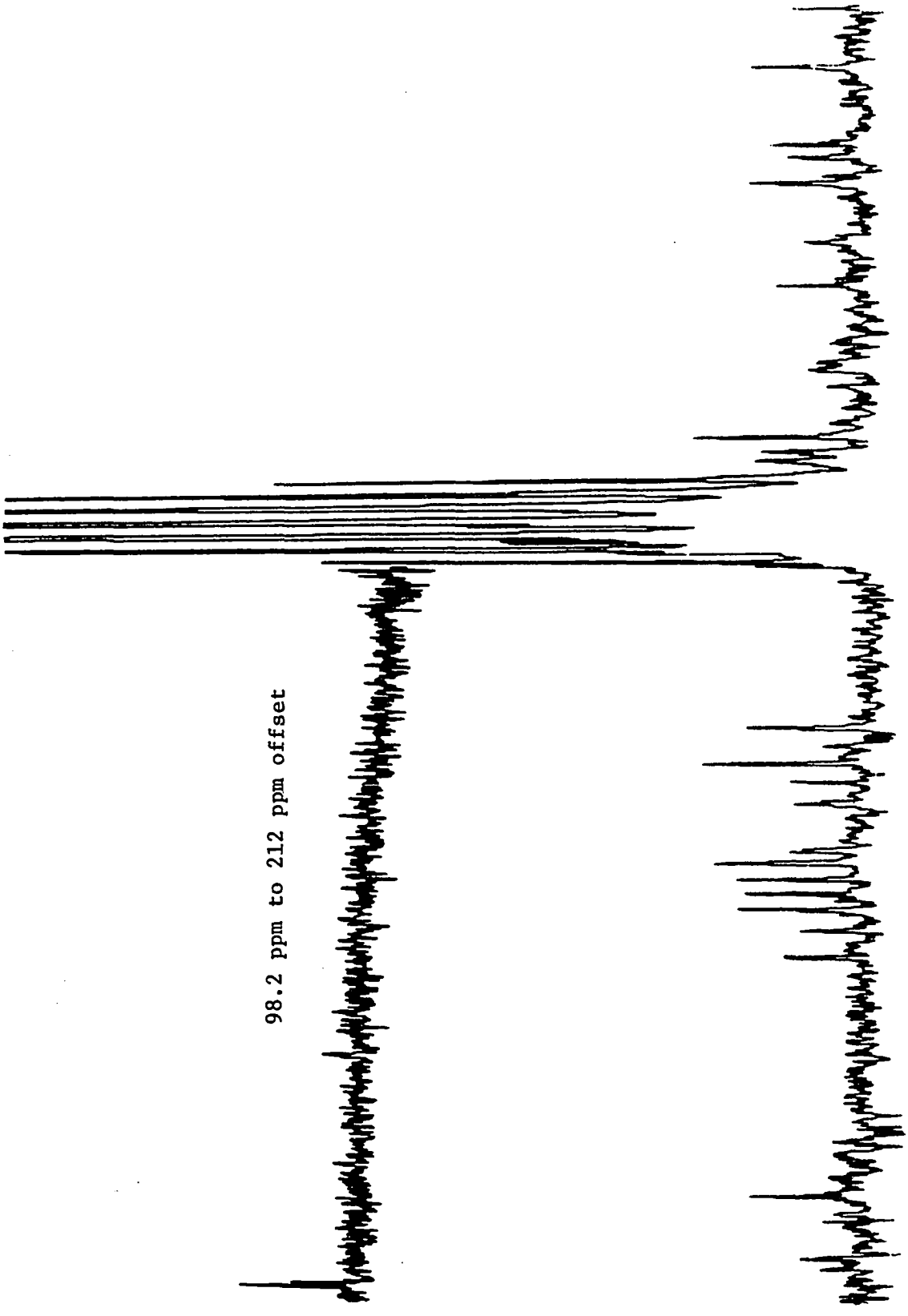
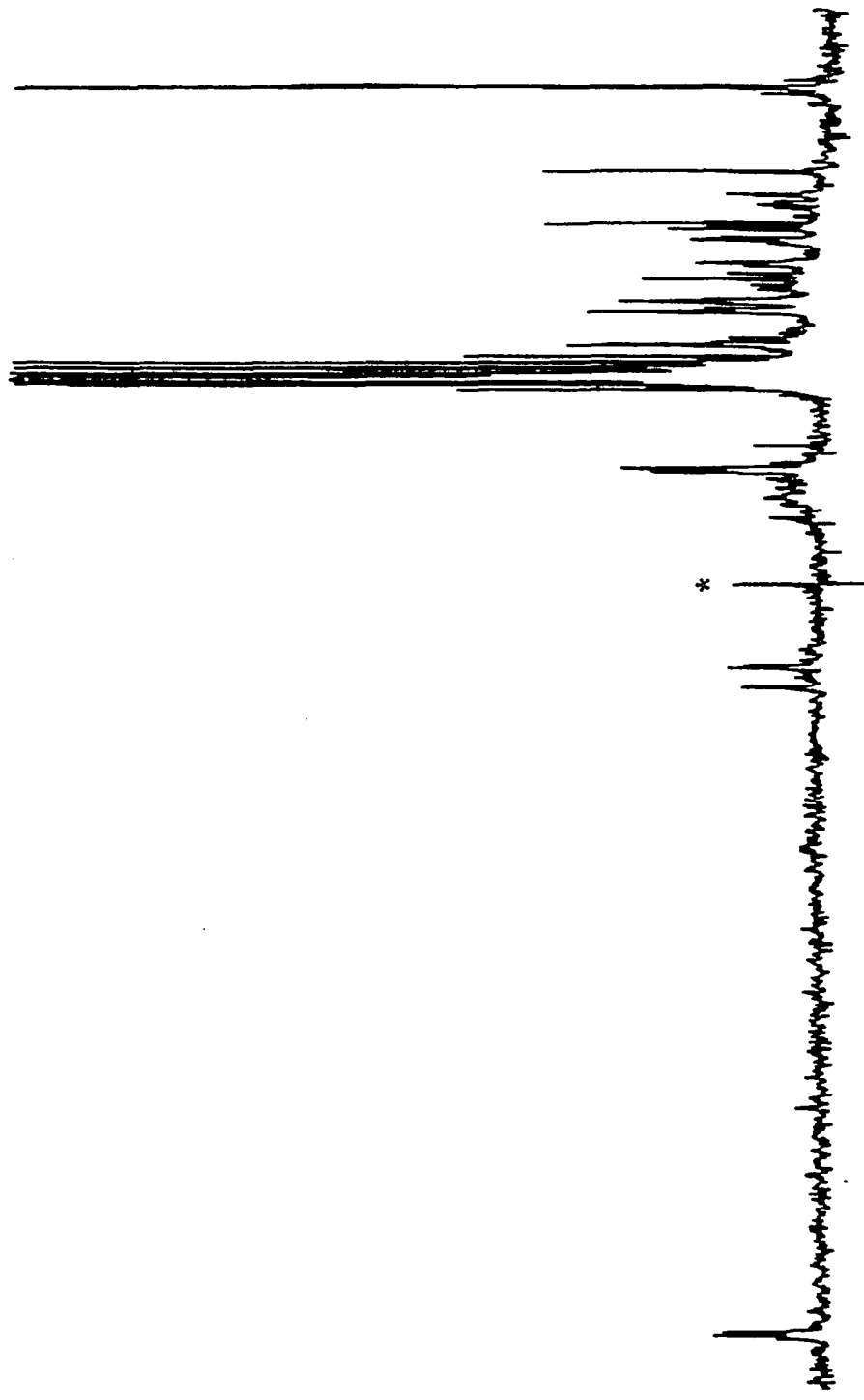

98.2 ppm to 212 ppm offset

Figure 15. Broadband Decoupled $^{13}C$ NMR spectrum of HMT toxin mixture after gel filtration

| NO. | FREQ. | PPM | INTENS. | AREA |
|---|---|---|---|---|
| 1 | 4803.073 | 212.187 | 314368 | 1073152 |
| 2 | 4791.259 | 211.665 | 621568 | 3781120 |
| 3 | 4780.430 | 211.187 | 565248 | 3375104 |
| 4 | 2314.413 | 102.244 | 496128 | 3267072 |
| 5 | 2244.518 | 99.157 | 549888 | 2251264 |
| 6 | 1925.560 | 85.066 | 517120 | 301568 |
| 7 | 1672.560 | 73.889 | 364544 | 1433600 |
| 8 | 1615.462 | 71.366 | 305152 | 2381824 |
| 9 | 1589.867 | 70.236 | 377856 | 3909120 |
| 10 | 1553.443 | 68.627 | 335872 | 1672704 |
| 11 | 1524.894 | 67.365 | 314368 | 1590784 |
| 12 | 1515.050 | 66.930 | 372224 | 608256 |
| 13 | 1484.532 | 65.582 | 972800 | 11159552 |
| 14 | 1476.657 | 65.234 | 1114112 | 5646336 |
| 15 | 1458.937 | 64.452 | 360960 | 2101760 |
| 16 | 1390.026 | 61.407 | 435200 | 922112 |
| 17 | 1197.076 | 52.883 | 309760 | 1311232 |
| 18 | 1173.450 | 51.839 | 1920000 | 9440768 |
| 19 | 1162.621 | 51.361 | 982528 | 3147776 |
| 20 | 1151.792 | 50.883 | 5432832 | 26532352 |
| 21 | 1128.165 | 49.839 | 82905600 | XXXXXXXX |
| 22 | 1109.461 | 49.013 | 11409920 | 60534272 |
| 23 | 1087.804 | 48.056 | 10266112 | 51880960 |
| 24 | 1066.146 | 47.099 | 5377024 | 28853248 |
| 25 | 1044.488 | 46.142 | 1902592 | 8115200 |
| 26 | 1024.799 | 45.273 | 366080 | 2129408 |
| 27 | 1001.173 | 44.229 | 1386496 | 18825728 |
| 28 | 975.578 | 43.098 | 570880 | 5128704 |
| 29 | 956.873 | 42.272 | 317952 | 1118208 |
| 30 | 879.103 | 38.836 | 1290240 | 15659008 |
| 31 | 835.787 | 36.922 | 1128448 | 19476480 |
| 32 | 788.534 | 34.835 | 429056 | 3248640 |
| 33 | 771.799 | 34.096 | 457728 | 3726336 |
| 34 | 748.172 | 33.052 | 1009664 | 6923264 |
| 35 | 725.530 | 32.052 | 577536 | 4892160 |
| 36 | 685.168 | 30.268 | 740352 | 9541120 |
| 37 | 607.398 | 26.833 | 374272 | 2570240 |
| 38 | 593.616 | 26.224 | 772096 | 9416192 |
| 39 | 553.254 | 24.441 | 892416 | 6115328 |
| 40 | 535.534 | 23.658 | 1481216 | 6904320 |
| 41 | 471.545 | 20.881 | 344576 | 859646 |
| 42 | 457.763 | 20.222 | 437248 | 2024960 |
| 43 | 452.841 | 20.005 | 310272 | 767488 |
| 44 | 442.997 | 19.570 | 349184 | 2910208 |
| 45 | 414.448 | 18.309 | 583168 | 3663360 |
| 46 | 326.833 | 14.438 | 1490944 | 6423040 |
| 47 | 24.610 | 1.087 | 412672 | 1124864 |
| 48 | 0.000 | 0.000 | 11851776 | 48793088 |




* 85.066 ppm; computer noise

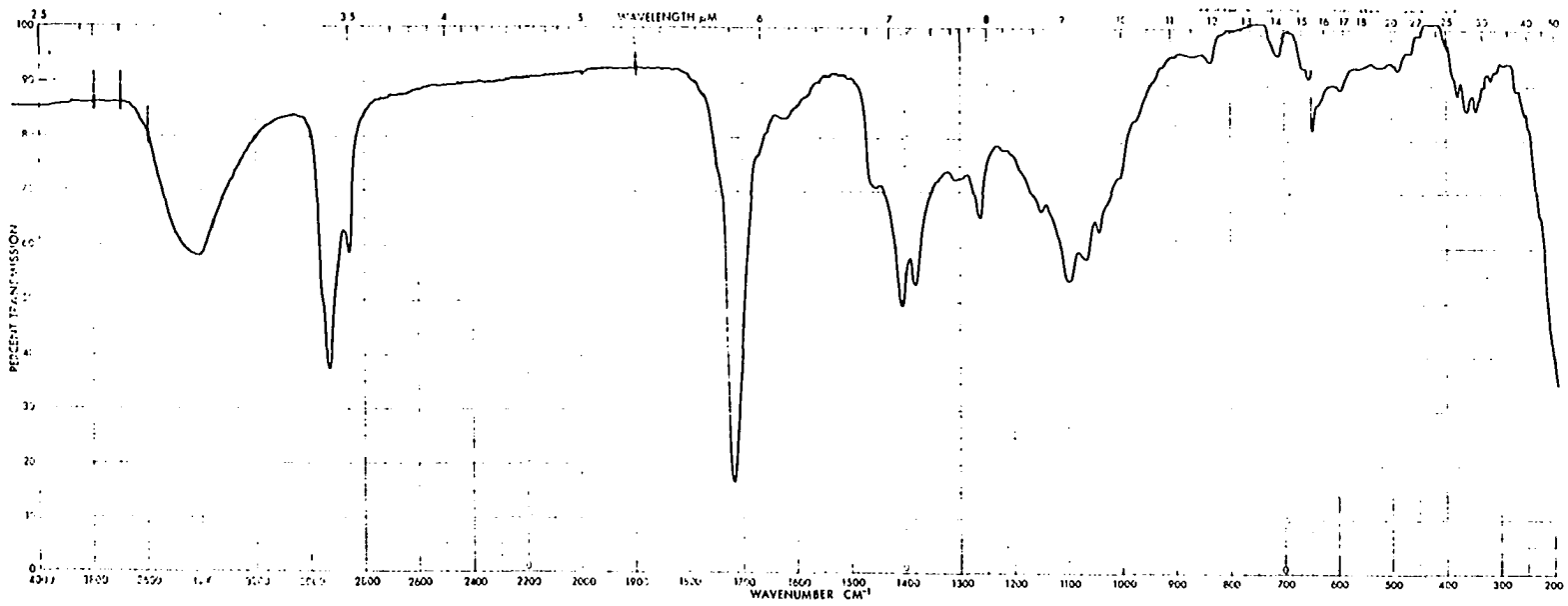



Figure 16. Infrared spectrum of HMT toxin mixture (KBr)

Figure 17. 90 MHz proton ft-MNR spectrum of P1

```
%XP
 RBP1 IN MEOH-D4(100%) 20DEC77 B60601
     NO.   CURSOR      FREQ.          PPM      INTENS.     AREA
      1     1499      471.555        5.239      25632    625760
      2     1615      437.516        4.861     758528  12359744
      3     1733      402.890        4.476      41280   1571520
      4     1810      380.295        4.225      27328   2209952
      5     1973      332.465        3.694      20512   1036736
      6     2092      297.546        3.306      41664   1092032
      7     2124      288.156        3.201      38048    471392
      8     2306      234.750        2.608     171296   4288032
      9     2327      228.588        2.539     191040   4839488
     10     2360      218.904        2.432      87904   1977312
     11     2444      194.256        2.158      60768   1311904
     12     2539      166.379        1.848      48768   1329216
     13     2561      159.923        1.776      62592   1038816
     14     2604      147.306        1.636      97344   2713440
     15     2626      140.850        1.565     132576   2319488
     16     2681      124.711        1.385     180480   8222560
     17     2711      115.908        1.287     157792   4562400
     18     2827       81.869        0.909      65696   2685184
     19     3106        0.000        0.000     150656   1714592
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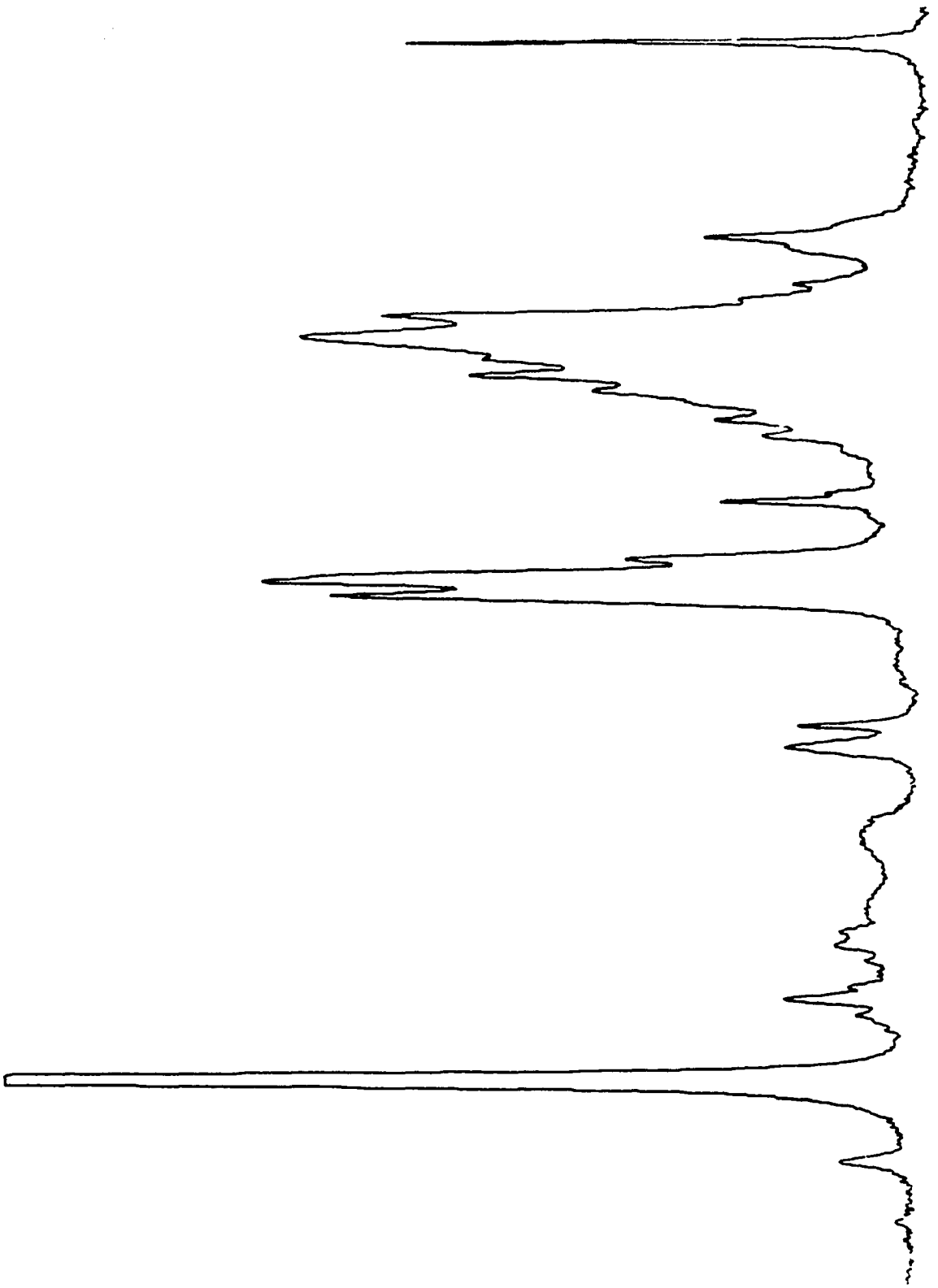


Figure 18. 90 MHz proton ft-NMR spectrum of P2

XP
PBP2 IN MEOH-D4(100%) 20DEC77 B60601

| NO. | CURSOR | FREQ. | PPM | INTENS. | AREA |
|---|---|---|---|---|---|
| 1 | 1616 | 437.516 | 4.861 | 1034368 | 11835136 |
| 2 | 1709 | 410.226 | 4.558 | 25023 | 249792 |
| 3 | 1730 | 404.064 | 4.489 | 35200 | 436416 |
| 4 | 1751 | 397.902 | 4.421 | 29760 | 316928 |
| 5 | 1771 | 392.033 | 4.355 | 29696 | 693248 |
| 6 | 1877 | 360.929 | 4.010 | 40832 | 3767488 |
| 7 | 2081 | 301.067 | 3.345 | 426944 | 4652672 |
| 8 | 2124 | 288.449 | 3.204 | 249984 | 3770496 |
| 9 | 2304 | 235.630 | 2.618 | 197248 | 4211968 |
| 10 | 2324 | 229.762 | 2.552 | 250112 | 10152448 |
| 11 | 2444 | 194.549 | 2.161 | 52160 | 401152 |
| 12 | 2457 | 190.734 | 2.119 | 49664 | 568832 |
| 13 | 2538 | 166.966 | 1.855 | 75328 | 3525696 |
| 14 | 2630 | 139.970 | 1.555 | 182400 | 9374208 |
| 15 | 2677 | 126.178 | 1.401 | 242624 | 12673024 |
| 16 | 2712 | 115.908 | 1.287 | 188224 | 3738496 |
| 17 | 2758 | 102.409 | 1.137 | 48960 | 908224 |
| 18 | 2828 | 81.869 | 0.909 | 117632 | 4072384 |
| 19 | 3107 | 0.000 | 0.000 | 290368 | 1869952 |

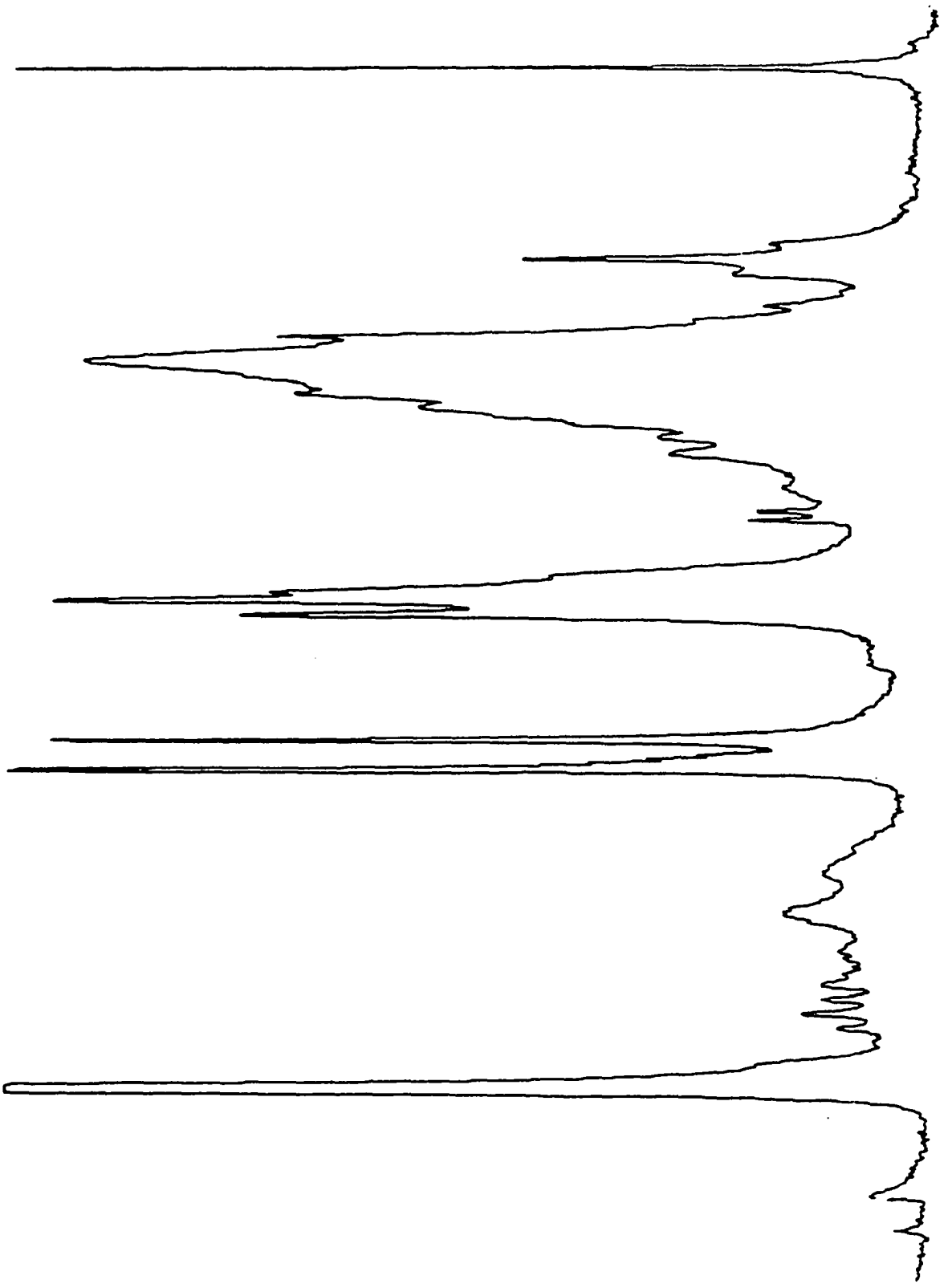


Figure 19. 90 MHz proton ft-NMR spectrum of B1

%XP

FBB1 IN MEOH-D4(100%) 20DEC77 B60601

| NO. | CURSOR | FREQ. | PPM | INTENS. | AREA |
|---|---|---|---|---|---|
| 1 | 1505 | 470.088 | 5.223 | 15936 | 178048 |
| 2 | 1616 | 437.516 | 4.861 | 852512 | 9450528 |
| 3 | 1709 | 410.226 | 4.558 | 17184 | 259104 |
| 4 | 1731 | 403.771 | 4.486 | 36384 | 771808 |
| 5 | 1812 | 380.002 | 4.222 | 23328 | 2055648 |
| 6 | 1965 | 335.106 | 3.723 | 17824 | 772960 |
| 7 | 2093 | 297.546 | 3.306 | 39808 | 800192 |
| 8 | 2124 | 288.449 | 3.204 | 42624 | 394048 |
| 9 | 2306 | 235.044 | 2.611 | 168064 | 2890208 |
| 10 | 2327 | 228.881 | 2.543 | 192832 | 2548096 |
| 11 | 2337 | 225.947 | 2.510 | 175040 | 1868672 |
| 12 | 2362 | 218.611 | 2.429 | 96224 | 2155744 |
| 13 | 2458 | 190.441 | 2.116 | 65696 | 1362624 |
| 14 | 2541 | 166.086 | 1.845 | 48704 | 1346848 |
| 15 | 2565 | 159.043 | 1.767 | 64512 | 1171328 |
| 16 | 2604 | 147.599 | 1.639 | 103360 | 2719872 |
| 17 | 2627 | 140.850 | 1.565 | 142496 | 2406944 |
| 18 | 2681 | 125.004 | 1.388 | 196480 | 8986368 |
| 19 | 2713 | 115.614 | 1.284 | 215712 | 4843456 |
| 20 | 2759 | 102.116 | 1.134 | 52512 | 1087072 |
| 21 | 2828 | 81.869 | 0.909 | 81568 | 3248544 |
| 22 | 3107 | 0.000 | 0.000 | 155584 | 1294176 |

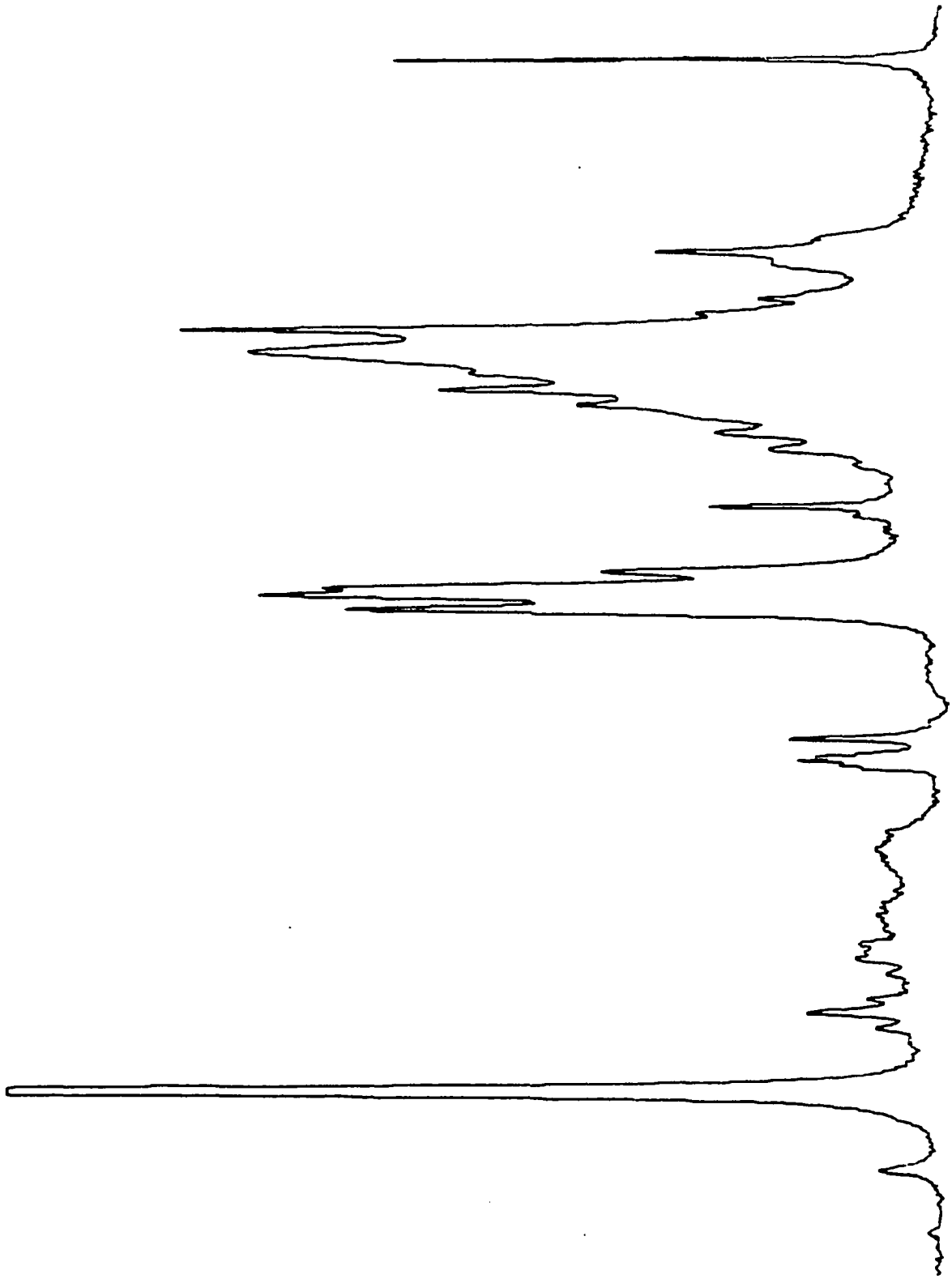


Figure 20. 90 MHz proton ft-NMR spectrum of B2

```
%XP
 FREQ IN MEOH-D4(100%) 20DEC77 B60601
     NO.   CURSOR      FREQ.          PPM      INTENS.      AREA
      1     1475      476.837        5.298      84432   4088592
      2     1613      436.342        4.848     461056  26570320
      3     1778      387.925        4.310      81584   5800704
      4     1882      357.407        3.971      48208   1628784
      5     1956      335.693        3.729      45296   2105808
      6     2059      305.469        3.394      33536     49160
      7     2095      294.905        3.276      86640   5790544
      8     2327      226.827        2.520     117056  10792768
      9     2707      115.321        1.281     276848  44093312
     10     2821       81.869        0.909     112304   9062206
     11     3100        0.000        0.000      33088    659872
```

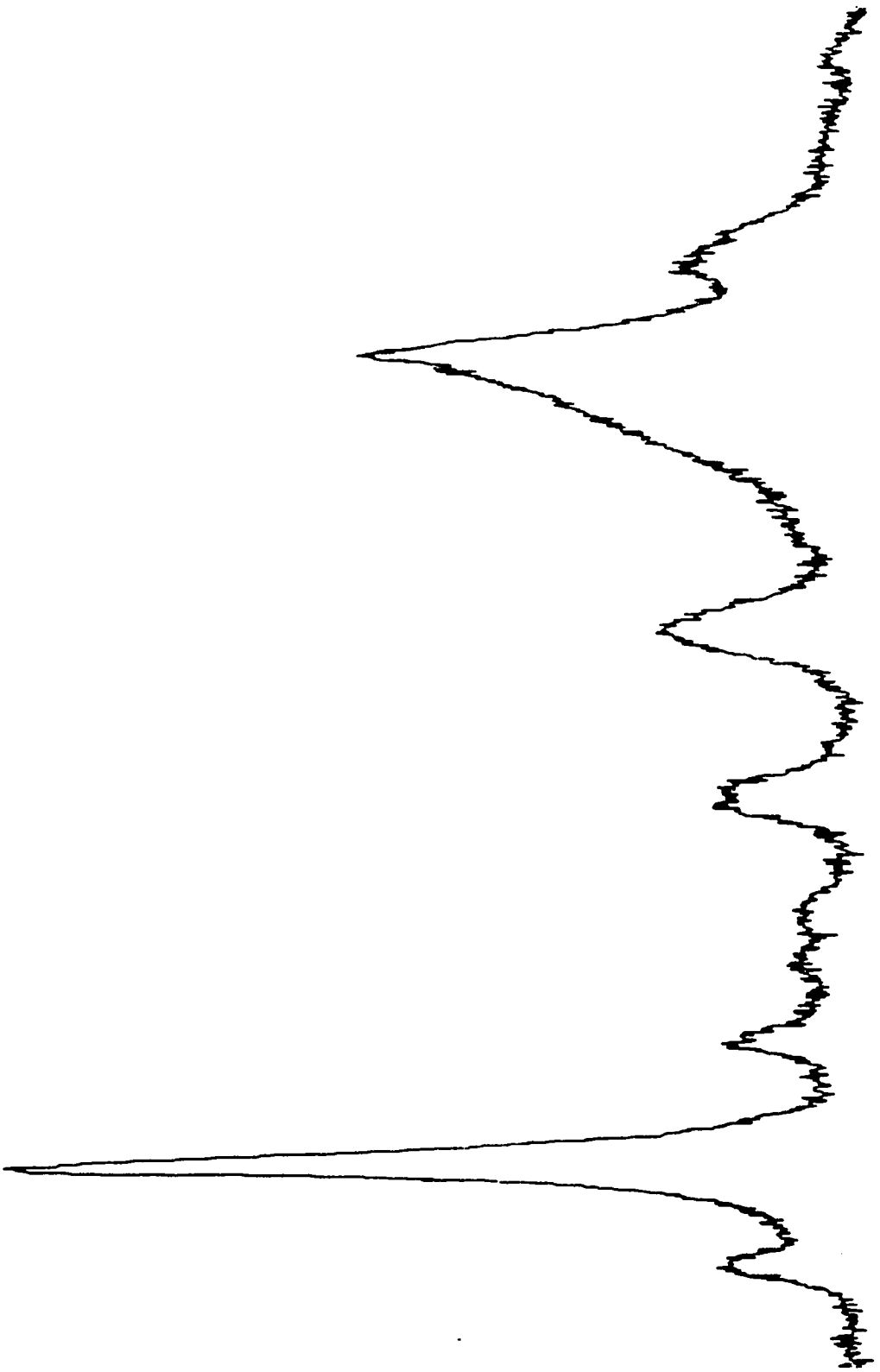


Figure 21. 100 MHz proton NMR spectrum of B1. The scan width was 1000 Hz, 27 scans were accumulated and averaged

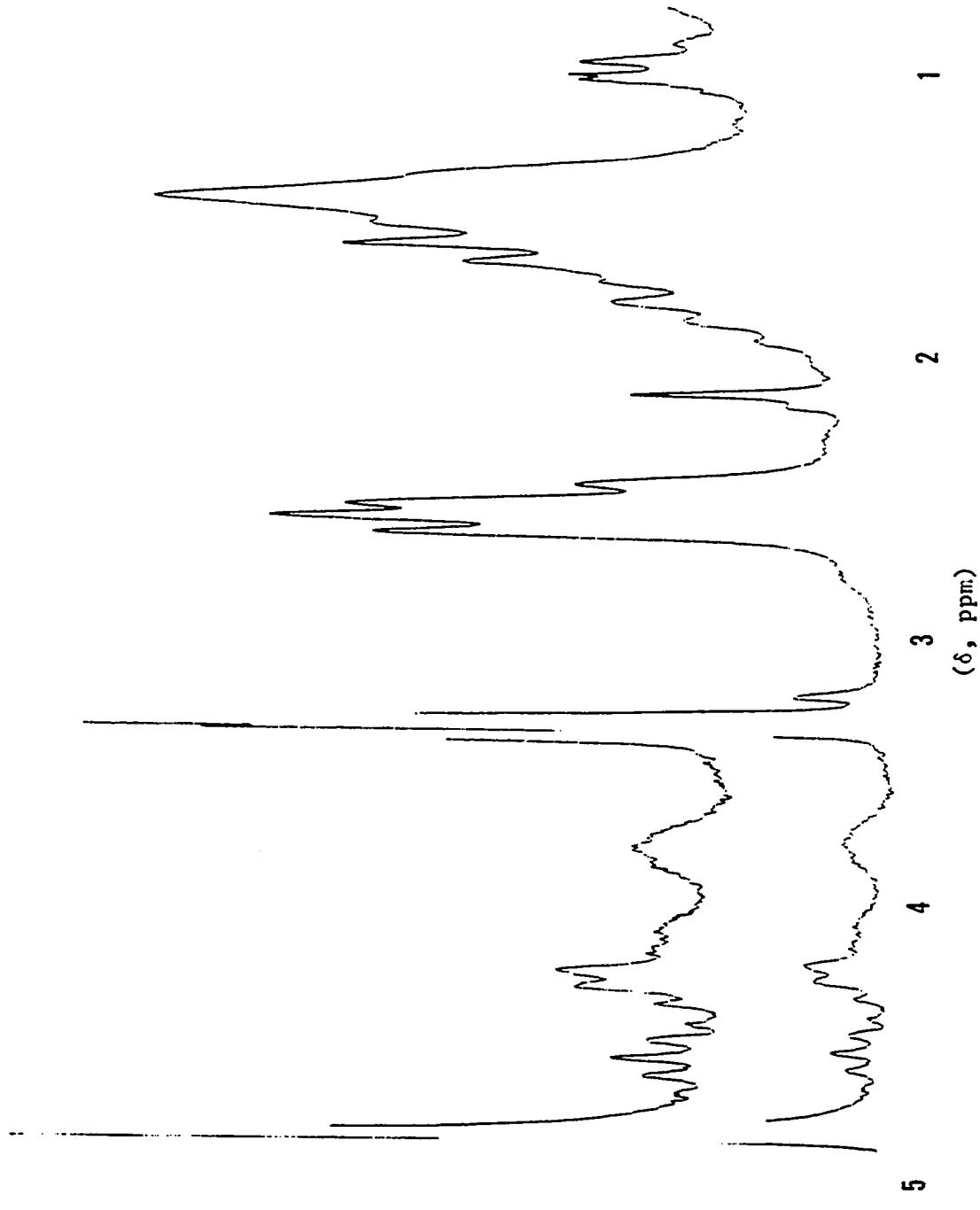

1
2
3
4
5
(δ, ppm)

Figure 22. 100 MHz proton NMR spectrum of P2. The scan width was 1000 Hz, 30 scans were accumulated and averaged

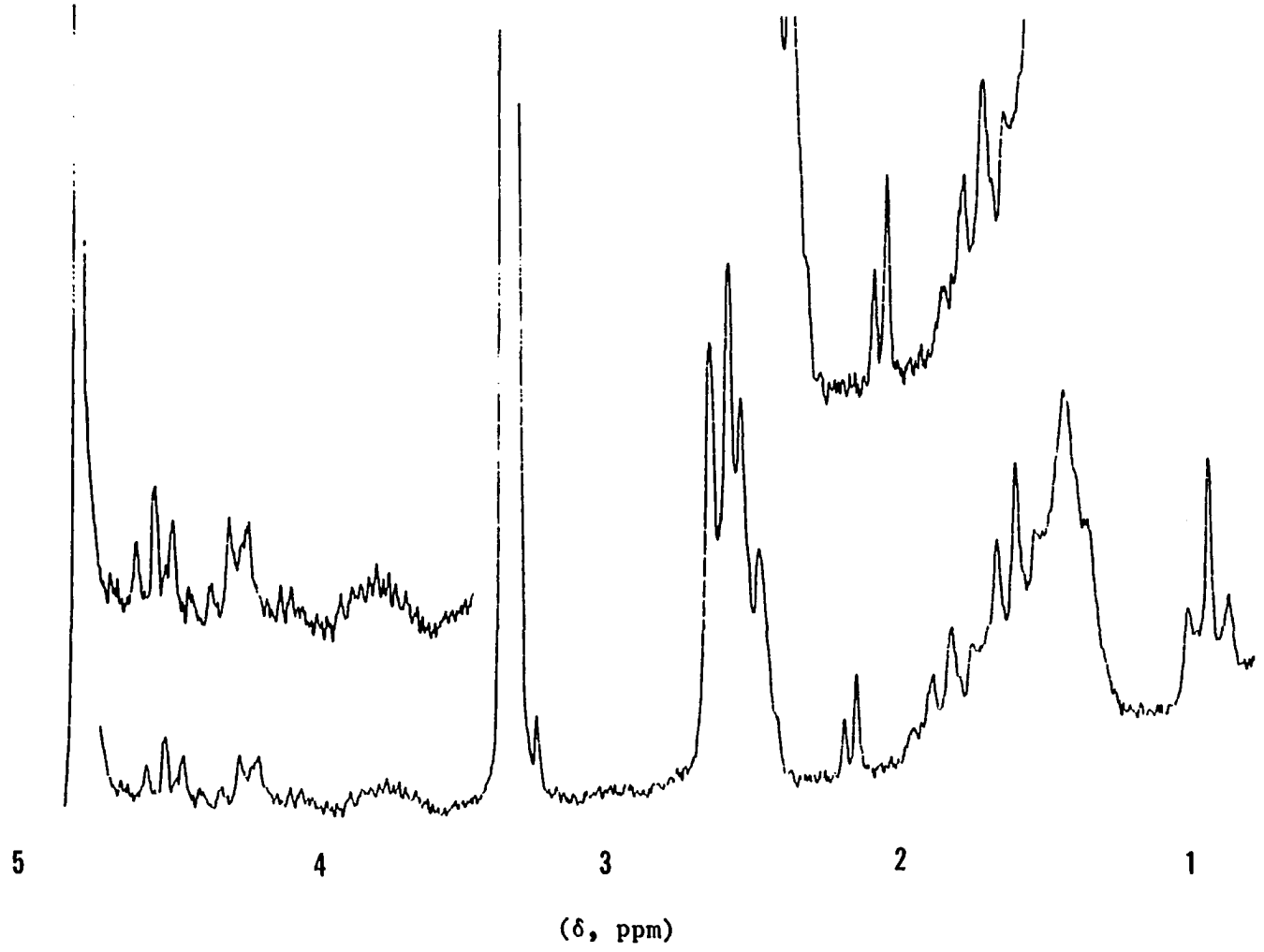
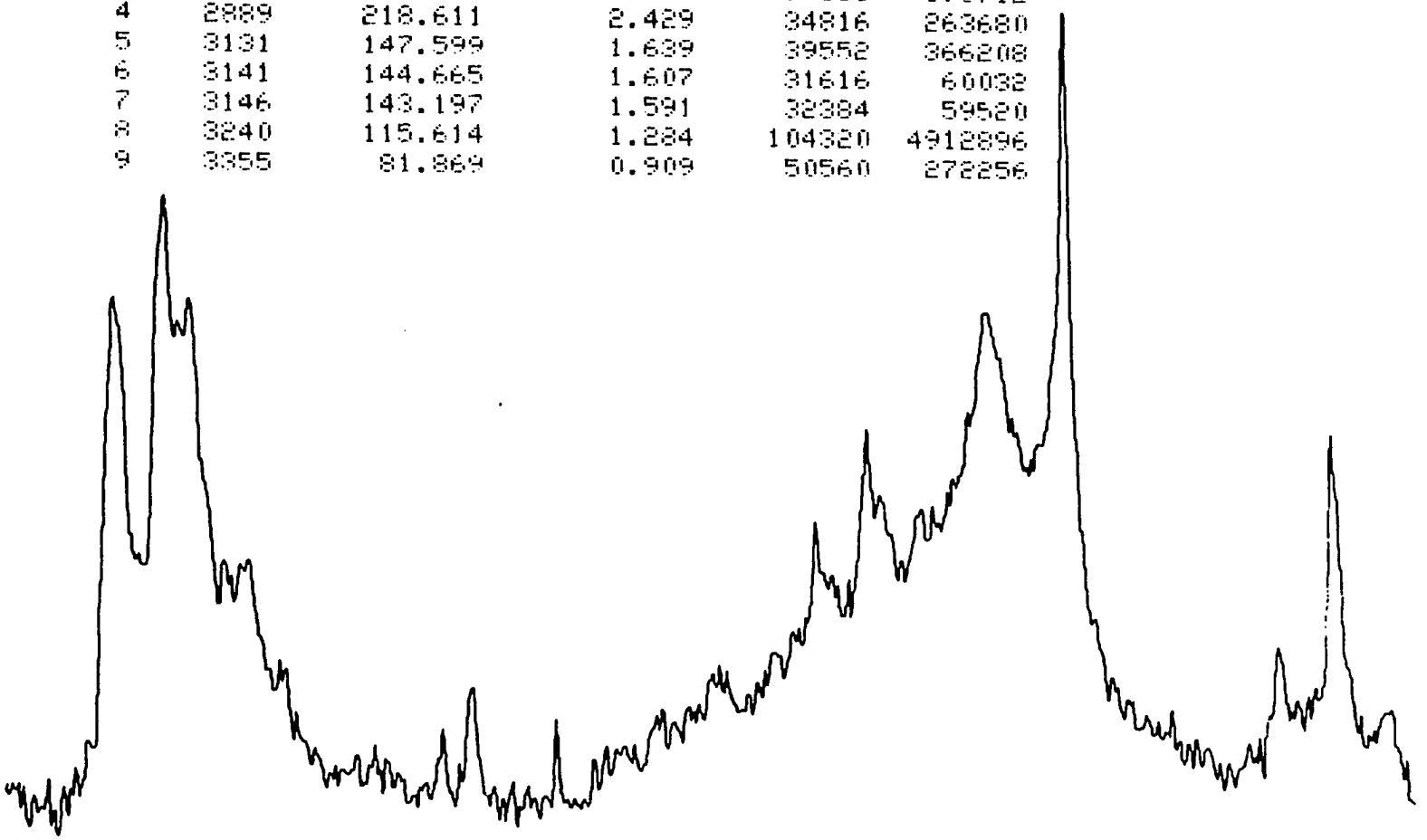

5
4
3
2
1
(δ, ppm)

Figure 23. 90 MHz proton ft-NMR spectrum of P2 after second purification

%XP
PB P2 IN CD3OD+TMS 29JUN78 B92601

| NO. | CURSOR | FREQ. | PPM | INTENS. | AREA |
|---|---|---|---|---|---|
| 1 | 2832 | 235.337 | 2.614 | 67840 | 911360 |
| 2 | 2854 | 228.881 | 2.543 | 80896 | 1513600 |
| 3 | 2878 | 221.839 | 2.464 | 34816 | 195712 |
| 4 | 2889 | 218.611 | 2.429 | 34816 | 263680 |
| 5 | 3131 | 147.599 | 1.639 | 39552 | 366208 |
| 6 | 3141 | 144.665 | 1.607 | 31616 | 60032 |
| 7 | 3146 | 143.197 | 1.591 | 32384 | 59520 |
| 8 | 3240 | 115.614 | 1.284 | 104320 | 4912896 |
| 9 | 3355 | 81.869 | 0.909 | 50560 | 272256 |



Analytical TLC of this sample after NMR analysis revealed P2, (Rf .55) and 2 new components with higher Rf values.

HMT toxin reduction The HMT toxin mixture (24.5 mg) was dissolved in 10 ml MeOH. 66 mg sodium borohydride was added and the mixture stirred at room temperature overnight. The reaction mixture was cooled on ice and 2 ml of 3% $H_2SO_4$ was added. Ten ml $H_2O$ was added and the mixture was extracted 3 times with 15 ml $H_2O$ saturated 1-butanol. The butanol was taken to dryness under vacuum and the residue taken up in 10 ml toluene and again taken to dryness. Three ml MeOH was added and salts were removed by centrifuging. The methanol soluble material was taken to dryness and reconstituted in 0.6 ml MeOH and applied to the small Sephadex LH-20 column. After fractionation, organic material was noted in mls 19.5 to 30 which when taken to dryness yielded 16.1 mg white material, 100 MHz $^1H$ NMR (MeOH-$d_4$) $\delta$ 0.86 (t, $-CH_2CH_3$ methyl), 1.5 broad s, 1.59 s, 1.64 s, 3.76, 3.98. IR (KBr) complete disappearance of signal at 1710 $cm^{-1}$ (See Figs. 24 and 25).

Polyiodide 16.1 mg polyol was dissolved in 6 ml of 58% HI. 50 mg of red phosphorous and 0.5 ml acetic acid were added and the mixture was refluxed for 48 hours. The reaction was cooled, 25 ml $H_2O$ added, and the mixture extracted with 30 ml of $Et_2O$ 3 times. The ether phase was washed with 100 ml $H_2O$, 100 ml 3% sodium thiosulfate twice and taken to an orange oil by evaporation under vacuum, which when dried over $CaSO_4$ yielded 20.7 mg polyiodide. 100 MHz $^1H$ NMR ($CDCL_3$) $\delta$ 0.89, 1.22, 1.75, 4.15 (See Fig. 26).

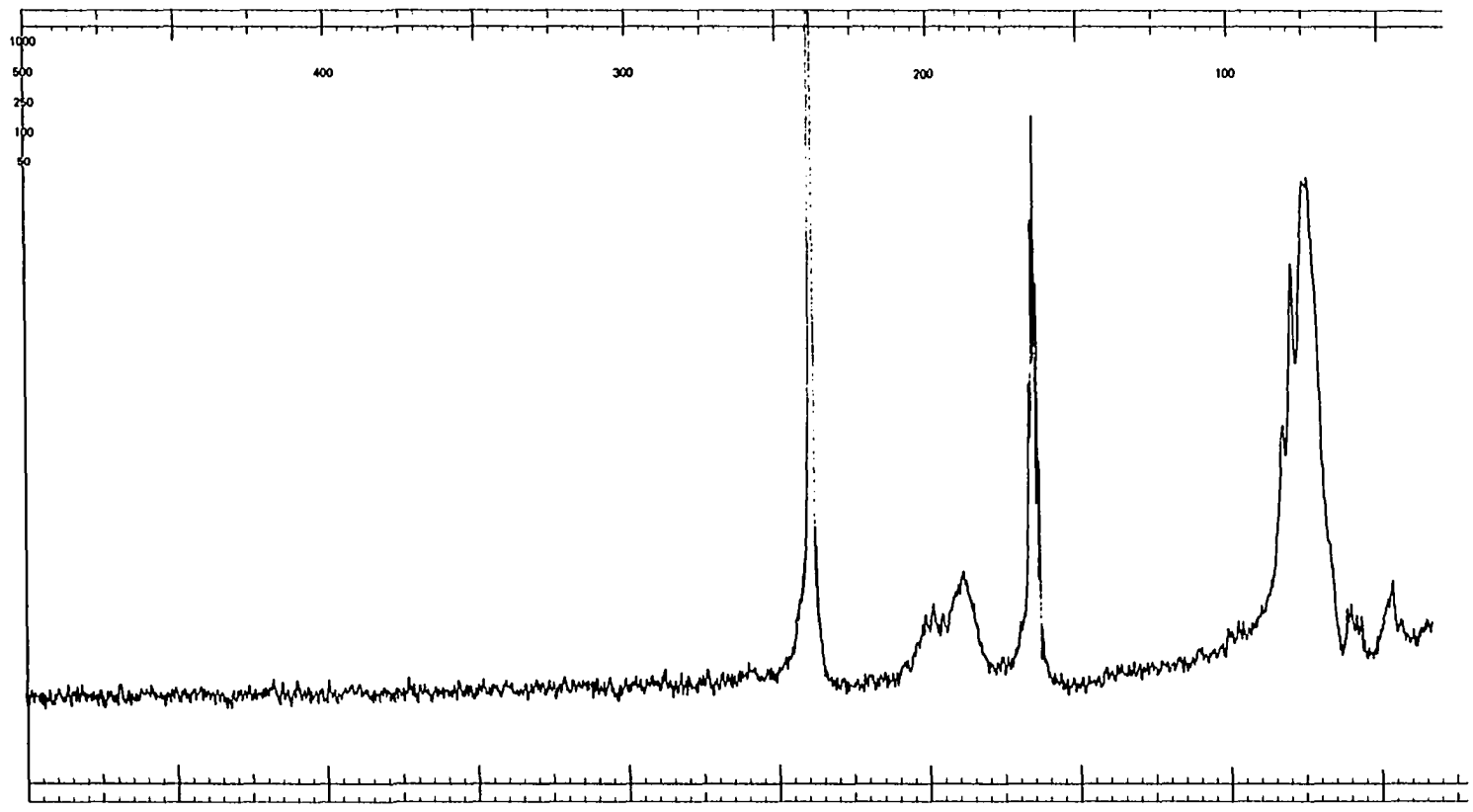



Figure 24. 100 MHz proton NMR spectrum of HMT polyol. The scan width was 1000 Hz

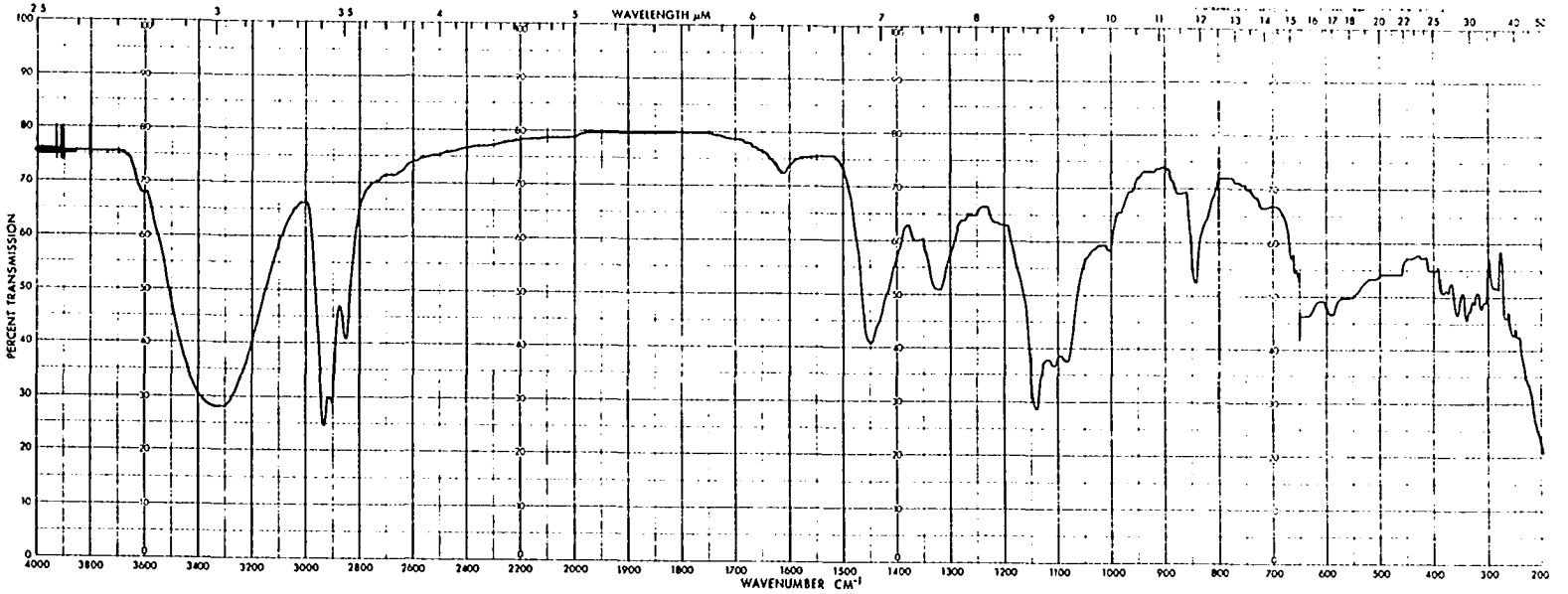



Figure 25. Infrared spectrum of HMT polyol (KBr)

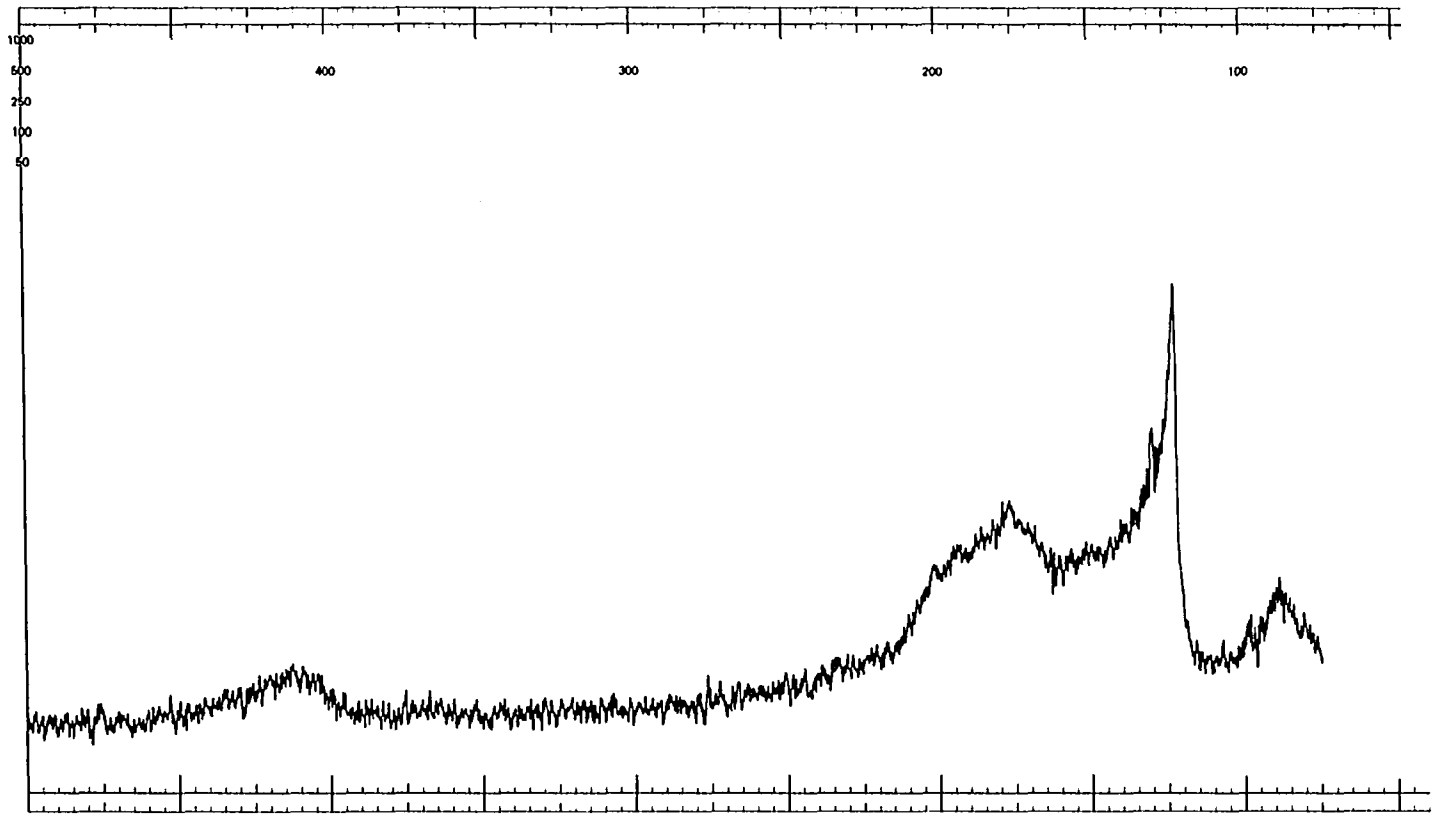



Figure 26. 100 MHz proton NMR spectrum of polyiodide. The scan width was 500 Hz

Attempted reduction of the polyiodide The polyiodide (20.7 mg) was dissolved in 6 ml dry $Et_2O$ and 40 mg lithium aluminum hydride was added. This material was refluxed for 66 hours, cooled, and 2 ml ethyl acetate was added. The mixture was cooled over ice, mixed with 30 ml cold 7% $H_2SO_4$ and extracted with 30 ml hexane three times. The hexane was washed with 50 ml of 5% sodium carbonate 2 times and finally washed with 100 ml $H_2O$. The washed hexane was evaporated under vacuum to give 1.25 mg clear oil. The oil was dissolved in 5 ml $Et_2O$ and 1 mg $PtO_2$ added. This mixture was stirred under $H_2$ at approximately 3 atm for 24 hours, filtered through a micro column of sodium sulfate and taken to dryness. GLC and GC/MS analysis were conducted on this material dissolved in acetone or hexane (See Figs. 27, 28, and 29).

Bioassay of HMT polyol The dried, white polyol (0.35 mg) was dissolved in 0.35 ml warm MeOH. A portion of this solution (0.02 ml) was added to 2 ml distilled $H_2O$ to give a stock solution of 10 μg/ml. The leaf streak assay was used. See Table 9 for results.

Table 9. Leaf streak assay of HMT polyol

| Sample | Concentration (μg/ml) | Response | Lesion size (cm) |
|---|---|---|---|
| $H_2O$ | | 000 | |
| Polyol | 0.1 | ++00 | .3, .3, .1 |
| | 1.0 | +++++ | all lesions >3 cm (to leaf tip) |

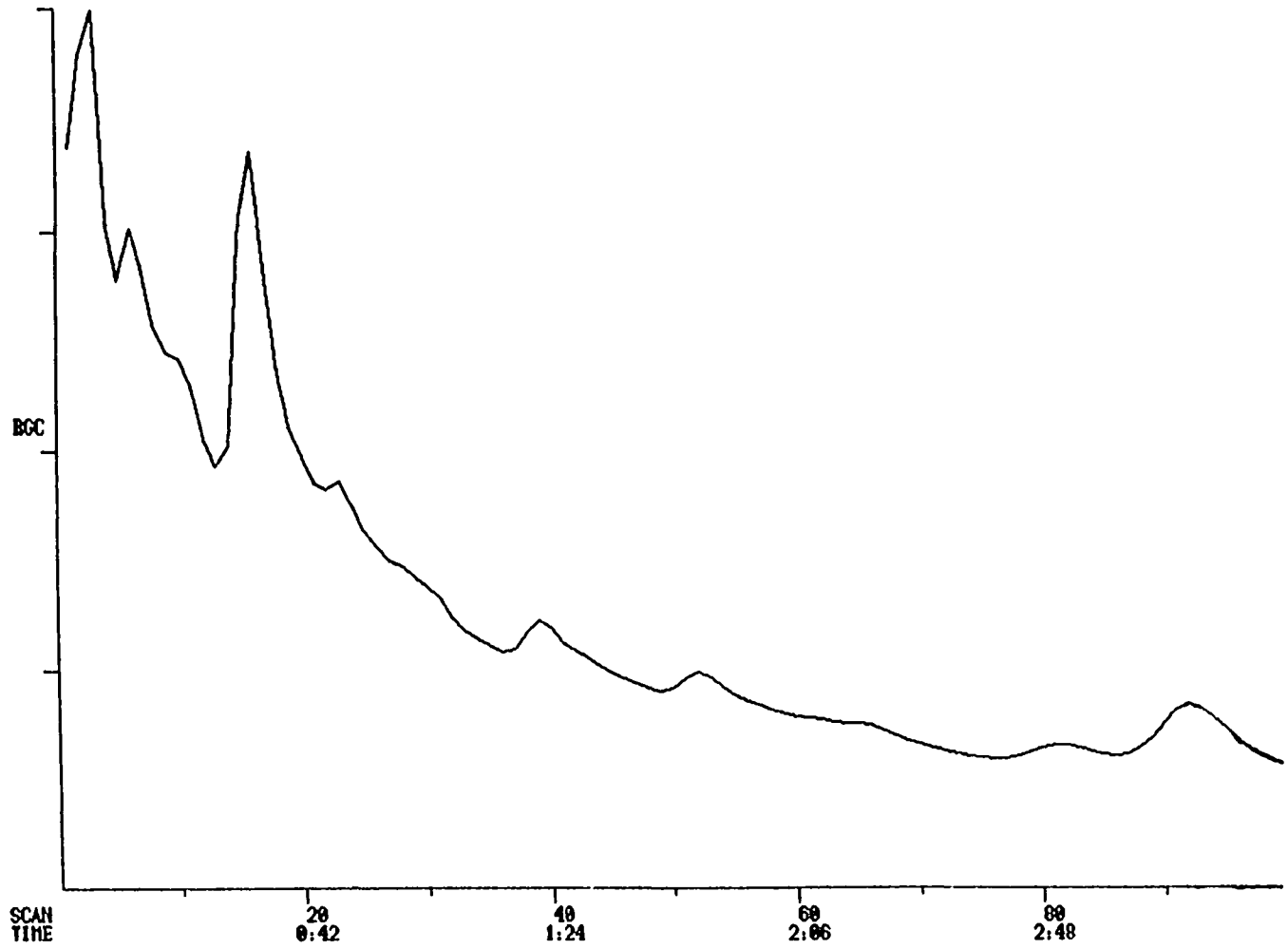



Figure 27. Reconstructed gas chromatogram from total ion currents of HMT hydrocarbon GC/MS

Figure 28. 70eV electron impact mass spectrum of scan #39

Figure 29. 70eV electron impact mass spectrum of scan #52

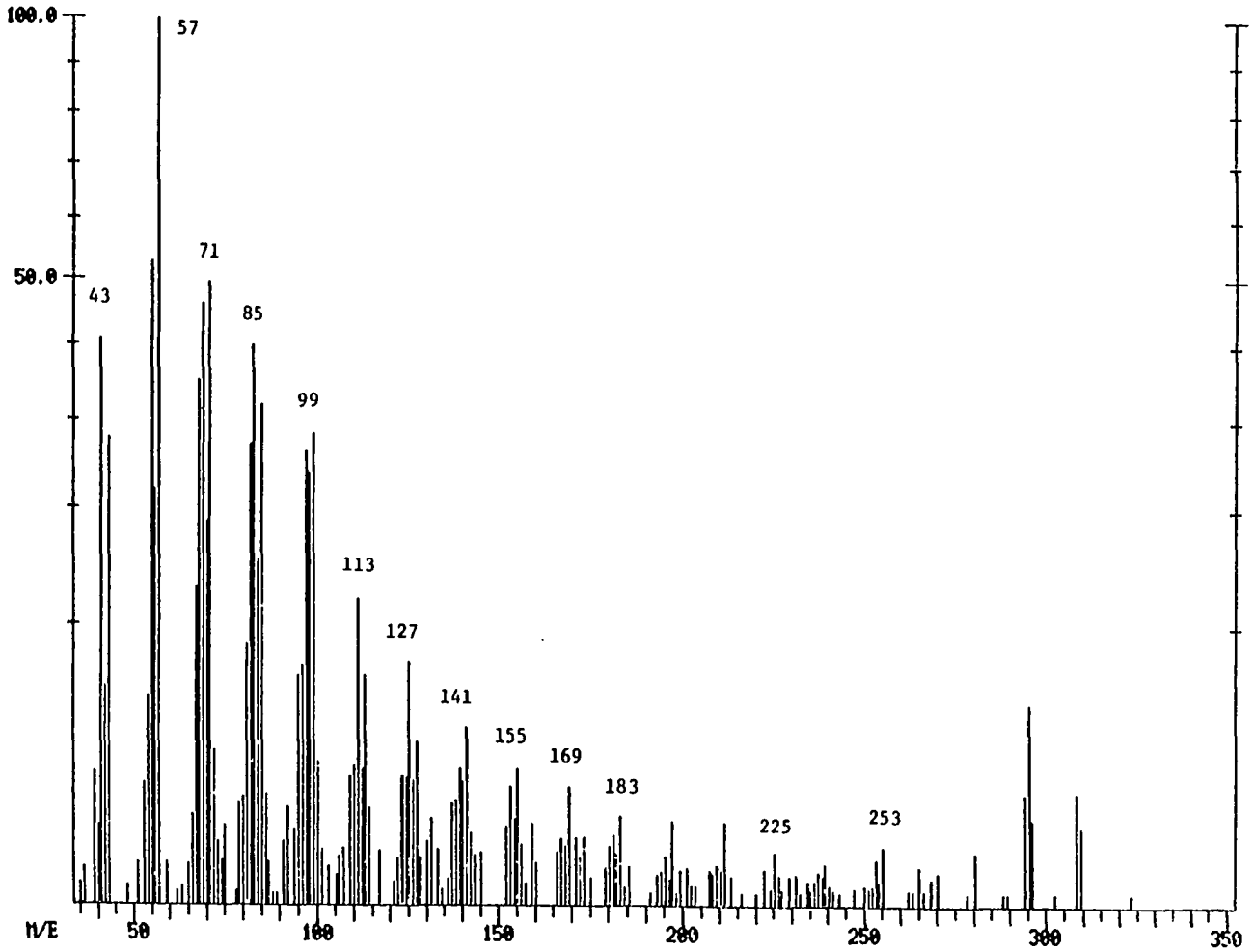
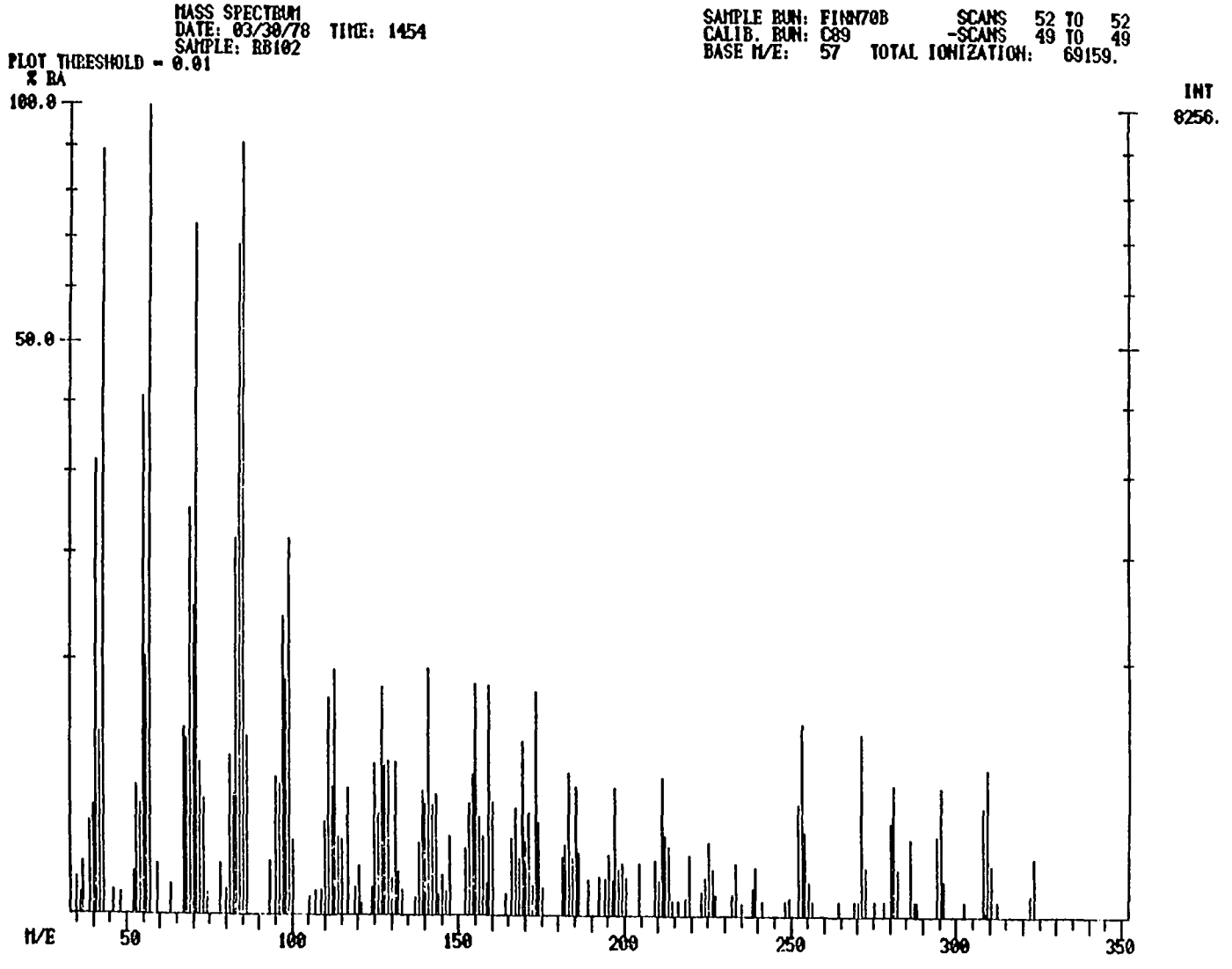

100.0
50.0
M/E
57
71
43
85
99
113
127
141
155
169
183
225
253
50
100
150
200
250
300
350

Figure 29. 70eV electron impact mass spectrum of scan #52


MASS SPECTRUM
DATE: 03/30/78 TIME: 1454
SAMPLE: RB102
PLOT THRESHOLD = 0.01
SAMPLE RUN: FINN70B SCANS 52 TO 52
CALIB. RUN: C89 -SCANS 49 TO 49
BASE M/E: 57 TOTAL IONIZATION: 69159.
% BA
100.0
50.0
INT
8256.
M/E
50
100
150
200
250
300
350

Phyllosticta maydis toxin Five 2800 ml Frenbach flasks each containing 300 ml modified Fries medium were inoculated with Phyllosticta maydis. Two flasks were placed in a growth chamber operating at 25° C with continuous lighting. Three flasks were placed in a room with approximately 8 hours in darkness per day. After 10 days growth, the cultures in the growth chamber appeared much darker brown than the other cultures. At day 10, samples were removed from each culture, dilutions were made, and the leaf streak bioassay conducted. This procedure was also followed at day 16 (See Table 10).

The chemically defined medium A was also tested for the ability to produce P. maydis toxin. Ten Fernbach flasks each containing 400 ml of this medium were inoculated and grown at approximately 26° C in the light. After 19 days, a portion of growth medium was removed. Leaf streak bioassay indicated that toxin activity was slightly less than that obtained from Fries medium. After 23 days of growth, the flasks were pooled and worked up as usual to give 3300 ml of culture filtrate. The solution was extracted with 1650 ml EtOAc four times, the extracts pooled and taken to dryness by evaporation under vacuum. The residue was dissolved in 0.6 ml hot MeOH and a small portion removed, diluted with $H_2O$, and bioassayed. Considerable toxin activity was noted at this point. The rest of the extract was placed on the small LH-20 column. A flow rate of 0.46 ml/min. was used to collect 15, 1.4 ml fractions. A total of 12 ml was collected before tube #1 was collected. For the leaf

Table 10. *Phyllosticta maydis* bioassay results

| Sample | Dilution | Response | Lesion size (cm) |
|---|---|---|---|
| **T cytoplasm maize** | | | |
| $H_2O$ only | 0 | 000 | |
| Day 10, continuous light | 1/100 | 000 | |
| | 1/10 | +++ | 0.1, 0.3, 0.2 |
| | 1 | +++ | 0.9, 0.4, 0.7 |
| Day 16, continuous light | 1 | ++++++ | 3.5, 3.5, 2.5, 0.3, 4.5, 0.5 |
| Day 10, 8 hours darkness | 1/100 | 000 | 0.1 |
| | 1/10 | +00 | 0.3, 0.3, 0.2 |
| | 1 | | |
| Day 16, 8 hours darkness | 1 | ++++++ | 0.4, 0.4, 0.5, 0.4, 1.5, 2.5 |
| **N cytoplasm maize** | | | |
| $H_2O$ only | 0 | 000 | |
| Day 10, continuous light | 1/100 | 000 | |
| | 1/10 | 000 | |
| | 1 | 000 | |
| Day 10, 8 hours darkness | 1/100 | 000 | |
| | 1/10 | 000 | |
| | 1 | 000 | |

streak bioassay, 5 µl were removed from each tube and dilutions to approximately 8 times the original culture filtrate were made with $H_2O$. Tubes 6-11 contained material causing leaf streaks with most of the activity in tubes 7, 8, and 9. Tubes 6, 7, and 8 were pooled and the solvent removed under a stream of $N_2$. Drying over $CaSO_4$ yielded 1.25 mg material. Tubes 9 and 10 were not included because they contained quantities of brown, oily material. A small amount of the material from tubes 6, 7, and 8 was applied to a precoated silica gel TLC plate, developed with solvent system B, and visualized with $H_2SO_4$/ MeOH, 1:1 (v/v). The materials turned a yellow-brown with considerable streaking over the lower 1/2 of the plate.

HPLC analysis of the P. maydis toxin Thirty ml of culture filtrate from a 12 day old P. maydis culture grown on media A were extracted twice with 30 ml portions of EtOAc. The organic fractions were pooled and taken to dryness under vacuum. The extract was dissolved in 0.5 ml MeOH and 7 µl was injected into the liquid chromatograph equipped with the same column, solvent system, flow rate, and detector sensitivity as used in Ophiobolin chromatography. Figure 30 shows the results.

90 MHz $^1H$ Ft-NMR and IR data 1.25 mg material from the biologically active fraction after gel filtration was dissolved in 150 µl MeOH-$d_4$, 100.0 atom % D. $\delta$ 0.906 (t, $-CH_2CH_3$ methyl), $\delta$ 1.291, 1.568 (broad sharp multiplets; aliphatic regions), 2.500, 2.565 (protons adjacent to -CO-) (See Fig. 31). IR (KBr) 1100, 1150, 1700, 2845, 2920, 3400 $cm^{-1}$ (See Fig. 32).

Figure 30. High performance liquid chromatogram of Phyllosticta maydis medium extract

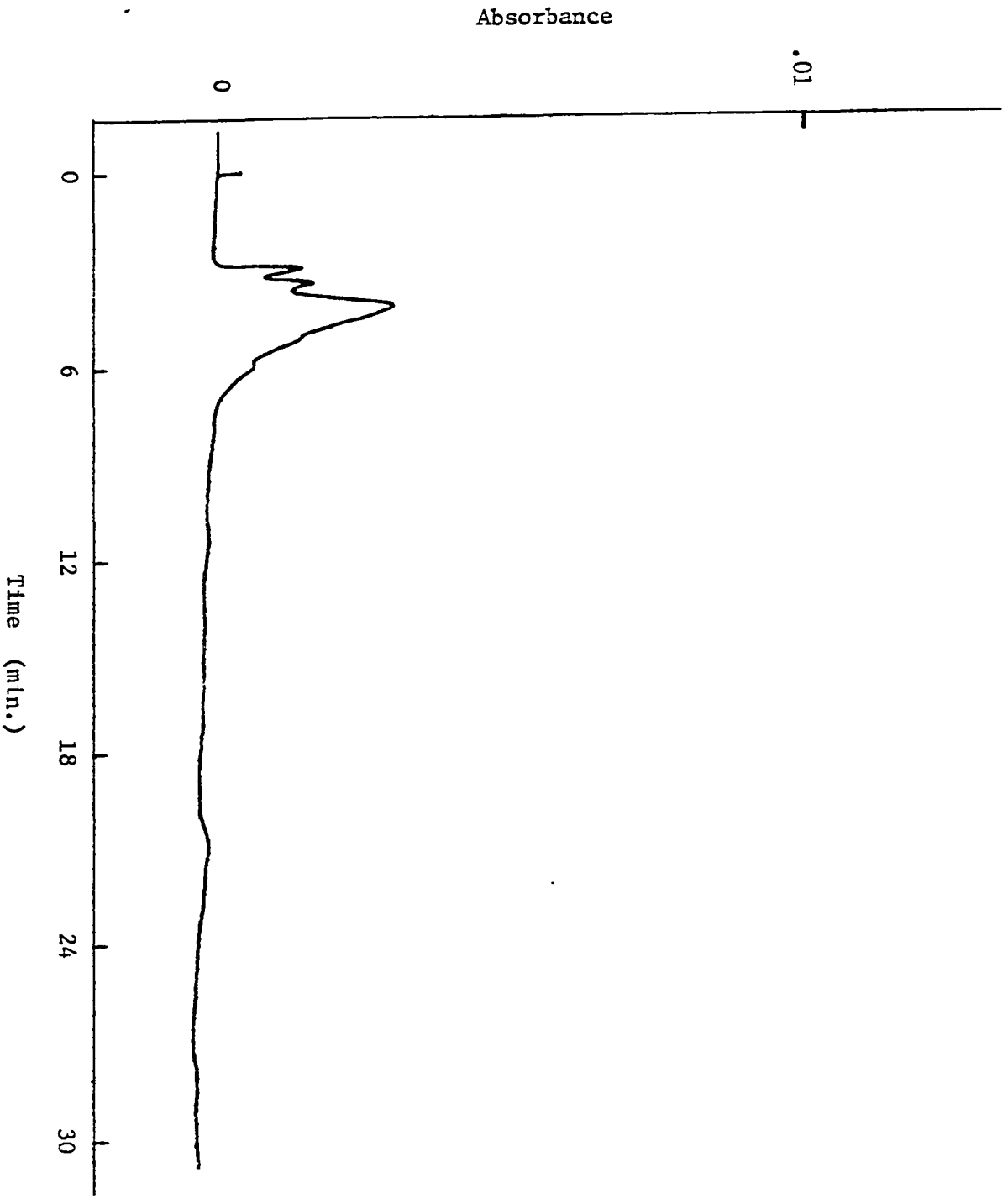
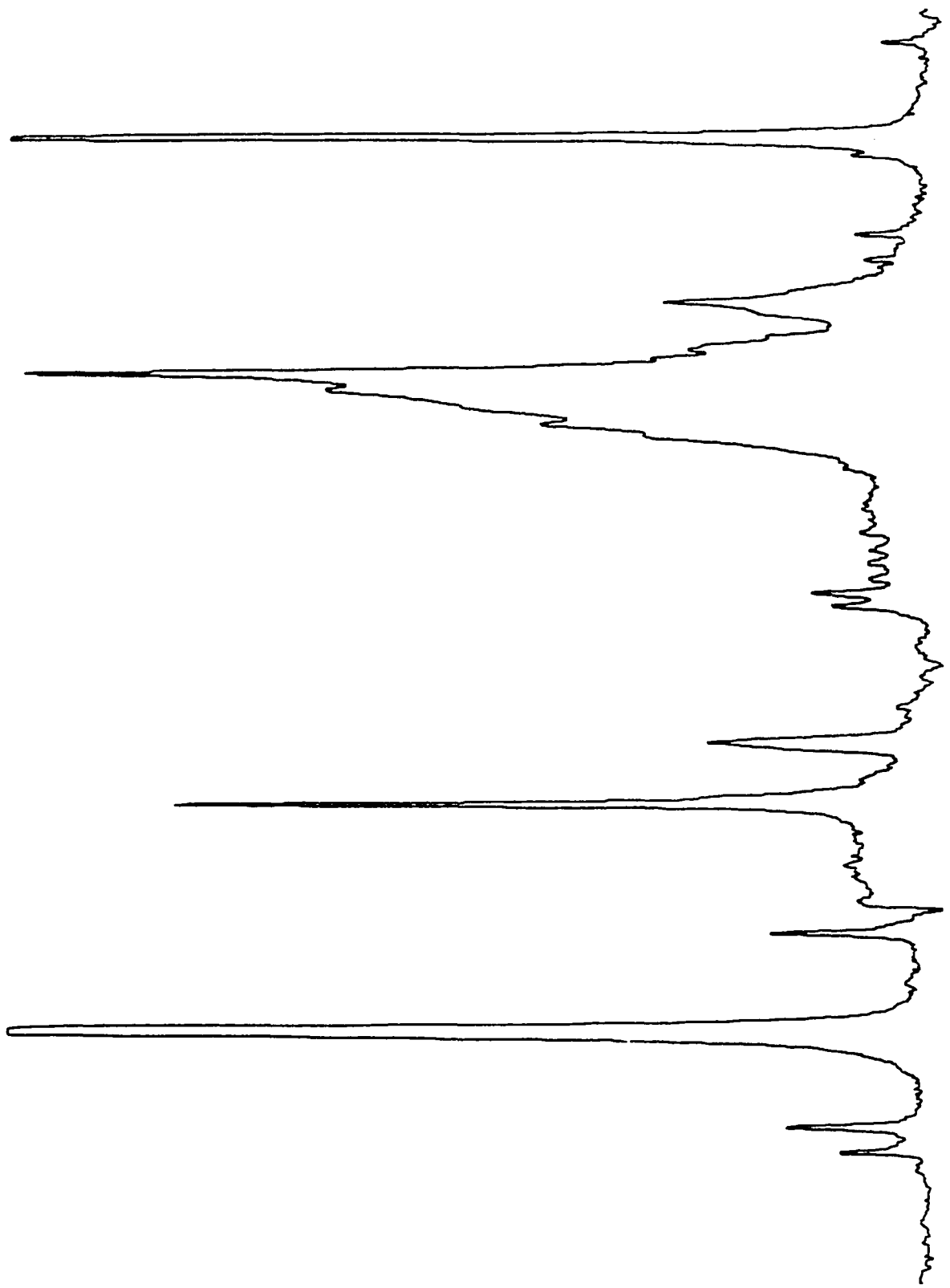

Absorbance
0
.01
0
6
12
18
24
30
Time (min.)

Figure 31. 90 MHz proton ft-NMR spectrum of Phyllosticta maydis toxin(s)

PM TOXIN IN MEOH(100%)D4 31JAN78 B65501

| NO. | CURSOR | FREQ. | PPM | INTENS. | AREA |
|---|---|---|---|---|---|
| 1 | 1687 | 497.671 | 5.529 | 6112 | 68384 |
| 2 | 1730 | 465.053 | 5.389 | 10016 | 120512 |
| 3 | 1890 | 438.103 | 4.867 | 340352 | 4085856 |
| 4 | 2052 | 390.566 | 4.339 | 11152 | 148784 |
| 5 | 2269 | 326.890 | 3.632 | 53760 | 1763600 |
| 6 | 2370 | 297.252 | 3.302 | 15744 | 369264 |
| 7 | 2596 | 230.935 | 2.565 | 6976 | 116448 |
| 8 | 2616 | 225.067 | 2.500 | 8432 | 111440 |
| 9 | 2902 | 141.143 | 1.568 | 27808 | 1733696 |
| 10 | 2987 | 116.201 | 1.291 | 64592 | 4307504 |
| 11 | 3105 | 81.575 | 0.906 | 19232 | 917024 |
| 12 | 3180 | 59.567 | 0.661 | 4864 | 91744 |
| 13 | 3222 | 47.243 | 0.524 | 5568 | 58752 |
| 14 | 3383 | 0.000 | 0.000 | 138992 | 1459216 |
| 15 | 3546 - | 47.830 - | 0.531 | 3760 | 23712 |



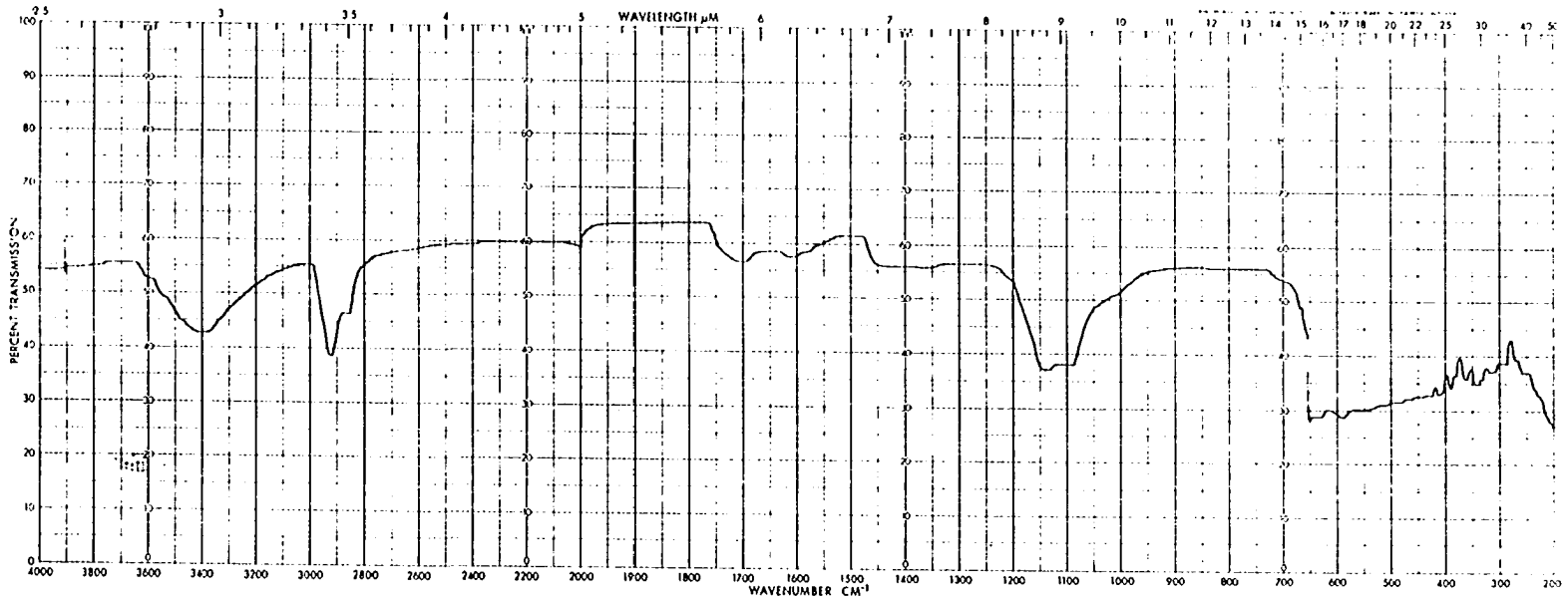



Figure 32. Infrared spectrum of Phyllosticta maydis toxin(s) (KBr)

Production of HMT toxins by several isolates of H. maydis Race T

The purpose of this study was to investigate the possibility that different isolates of H. maydis Race T produce different host-specific toxins. Isolates were obtained from:

Dr. J. M. Daly, Dept. of Agricultural Biochemistry, the University of Nebraska, Lincoln, Nebraska.

Dr. C. J. Arntzen, Department of Botany, University of Illinois, Urbana, Illinois.

Dr. O. C. Yoder, Department of Plant Pathology, Cornell University, Ithaca, New York.

Dr. G. A. Strobel, Department of Plant Pathology, Montana State University, Bozeman, Montana.

Various isolates were obtained as lesions on corn leaves, agar slants, or in one case spores deposited on silica gel. All isolates were subcultured once to sterile slants of potato-dextrose agar. Sterile water was added to the 6-10 day old slants to produce spore suspensions for culture flask inoculation. Four Fernbach culture flasks were prepared for each isolate, 2 containing 400 ml medium B to test for ophiobolin biosynthesis and 2 containing 400 ml medium A to compare HMT toxin production. Two additional flasks containing 400 ml of the defined medium of Mertz and Arntzen (49) (Medium E) and inoculated with the Arntzen isolate were prepared. At the end of 10-12 days of growth under continuous light, 30 ml samples were removed from flasks containing medium B. These were extracted with EtOAc and the extracts examined for ophiobolins by HPLC or TLC. The entire culture filtrate

from each isolate grown on medium A was extracted with EtOAc and worked up as usual for HMT toxin isolation. It was found that all isolates produced similar HPLC or TLC profiles when grown on medium B, furthermore solvent precipitation of extracts from every isolate grown on media A and E resulted in formation of the usual white precipitate. TLC examination of these precipitates revealed that each had chromatographic patterns qualitatively and quantitatively similar to the Iowa isolate used in all earlier work.

In a separate experiment, 300 ml of medium E was placed in each of four, 1 l Erlenmeyer flasks and inoculated as usual with the Arntzen isolate. Each flask was placed on a rotary shaker which was then operated at 240 rpm for 7 days under continuous laboratory light. At the end of 7 days, the thick mycelial mass and medium had turned black but EtOAc extraction and workup produced the usual white precipitate. TLC examination showed this material to be identical to the HMT toxin complex produced in still culture.

# DISCUSSION

## Isolation of Ophiobolins

Literature reviews of the chemistry of H. maydis secondary metabolites indicated that very little was known about the HMT toxin. Since speculations on the chemical nature of this toxin ranged from it being a polypeptide to a tricyclic terpenoid, initial experiments delt with ways to extract effectively any toxic materials from the growth medium or mycelia. It was found that organic solvents such as $CHCl_3$, EtOAc, and to a lesser extent $Et_2O$ would extract the toxin from aqueous media. Interpretation of the effectiveness of various solvents was initially difficult due to the lack of a good quantitative bioassay. It was decided to use EtOAc because of its cost, lower toxicity, and good recovery of toxin activity. Modified Fries growth medium was used throughout the first part of the research. This rich medium produced quick growth of dense fungal mycelial pads along with high levels of toxin activity. EtOAc extracts of the growth medium after removal of all mycelium, hereafter called the culture filtrate, was examined by silica gel TLC chromatography and found to contain several major as well as many minor metabolites (See Fig. 33). Several attempts were made to identify and isolate individual zones of toxin activity on preparative TLC plates. These experiments were always unsuccessful, the results indicating toxin activity was spread throughout the entire plate. This could indicate problems with quantitation of the bioassay end point, more than one toxin with widely different TLC mobilities, or streaking of one or

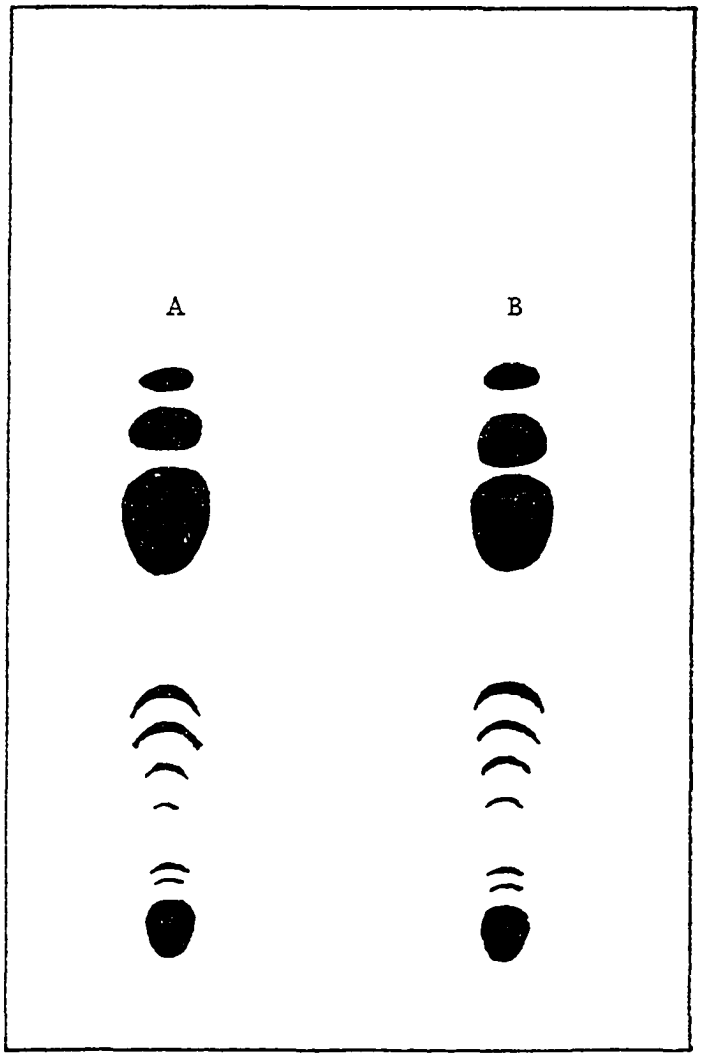



Figure 33. Thin layer chromatogram of ethyl acetate extracts of H. maydis grown on medium B. Solvent system A was used

A = Race O
B = Race T

more toxic components throughout the plate. Isolation of each of the 3 major metabolites separated by solvent system A was undertaken to determine if they were individually active and also to get some idea of their chemistry. On one occasion an EtOAc extract of the culture filtrate was taken to dryness and redissolved in a small volume of MeOH. A syringe was filled with this solution but before it could be applied to a preparative TLC plate, crystallization occurred to the extent that the plunger was frozen. Resolubilization in warm MeOH followed by TLC analysis indicated this to be one of the major metabolites in very pure form. Recrystallization from $CH_2Cl_2/Et_2O$ resulted in a white, crystalline product. Low resolution mass spectrometry of this material revealed an apparent molecular ion at M/e = 400 as well as M-$H_2O$ at M/e = 382 (See Fig. 2 pages 25 and 26). High resolution analysis of this ion gave a measured M/e of 400.2584. The formula $C_{25}H_{36}O_4$ has a calculated M/e of 400.2614. A prominent M-18 ion as well as ions at M/e = 273, 176, and 165 were observed on the high resolution scan. $^1H$ NMR analysis revealed a strong singlet at $\delta$ 9.24, apparent olefinic resonances at $\delta$ 5.17 and 7.20, and several aliphatic resonances including 5 methyl resonances (See Fig. 1, page 24). This data is consistent with the known sesterterpene ophiobolin A 1 (28,29). In addition, the metabolite co-comatographs with authentic ophiobolin A in TLC and HPLC systems. The melting point is 178-181° C (lit. required 181° C). The second compound studied appeared to be produced in amounts smaller than ophiobolin A and had an Rf value of 0.53 as compared to 0.46 for ophiobolin A.

Preparative TLC followed by gel filtration gave a small quantity of the pure compound. $^1$H NMR analysis revealed a singlet at δ 9.20, 5 methyl resonances, and the disappearance of the prominent AB quartet that characterizes ophiobolin A. This data coupled to the greater TLC mobility indicates the compound could be anhydroophiobolin A 3, the dehydration product of ophiobolin A. Dehydration would shift the C3 methyl group from the observed value of δ 1.37 ($CDCl_3$) to the observed value of δ 2.05 ($CCl_4$). The appearance of a singlet at δ 5.90 is probably that of the C4 proton which would be found further upfield in ophiobolin A (See Fig. 34). All other NMR data is consistent with published values for anhydroophiobolin A. To further substantiate the identity of the metabolite, a quantity of ophiobolin A was dehydrated with acid. $^1$H NMR of the metabolite and the dehydration product were identical (See Fig. 3 and 4, pages 27 and 28).

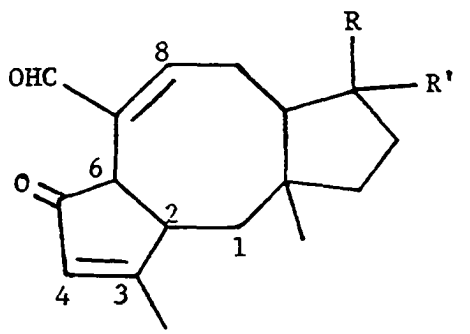
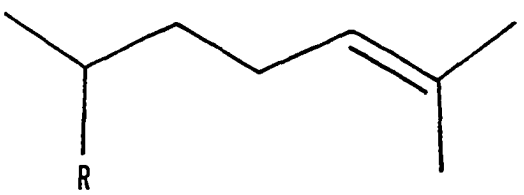



Fig. 34. Dehydration product

Similar isolation procedures were used to isolate a very small amount of material having an Rf value of .60, which is higher than either ophiobolin A or anhydroophiobolin A. $^1$H NMR ($CCl_4$) indicated a probable ophiobolane ring system with a single proton at δ 9.36 and 5 methyl resonances. Since one of the methyl signals is located at δ 1.24 and no signal is found near δ 2, the C3 hydroxyl appears to be intact. Methyl groups at δ .90 (d, J=7), 1.56, and 1.62 indicate the structural characteristics of 11.




11

High resolution mass spectrometry indicated an apparent molecular ion at M/e = 380.2344. The formula $C_{25}H_{32}O_3$ requires M/e = 380.2352. The metabolite co-comatographed with authentic ophiobolin C 5 but this compound requires a M/e = 386 ($C_{25}H_{38}O_3$). Due to the paucity of compound no further structural work was attempted.

Biological activity of ophiobolins

Purified ophiobolin A was bioassayed using the leaf streak bioassay on Tcms and Normal plants and was found to produce none of the yellow streaking associated with EtOAc extracts of the culture filtrate. However, it was discovered that ophiobolin A at 50 µg/ml was an effective inhibitor of hexose uptake in maize and carrot roots.

Stimulation of ion leakage from these tissues was also noted after ophiobolin A treatment (50). This observation is significant since it had been reported that partially purified preparations of HMT toxin caused a nonhost-specific carbohydrate leakage with maize roots (51).

Isolation of HMT toxins

Analytical HPLC was used to fractionate the ophiobolins from the HMT toxin activity. Since earlier work had indicated the possibility of toxic metabolites having wide polarity differences, a reverse-phase system was used to insure that extremely polar components would easily be eluted for bioassay. The leaf streak assay used was only semi-quantitative, but it was clear that toxin activity could be separated from ophiobolin fractions. Also, since the toxin activity eluted from the reverse phase column before the ophiobolins there was a high probability that the toxin was more polar. Another very important discovery was made at this time. Bioassays of HPLC fractionated extracts from two completely defined media, A and C, indicated considerable toxin activity, but UV monitoring at 254 nm showed that no ophiobolins were present, and moreover, few of the minor components detected in extracts of different media had similar retention times (See Fig. 7, page 36). The toxin clearly was an extremely toxic component or its UV properties were such that it was not detected at 254 nm. Later work proved the latter hypothesis to be correct. Medium induced changes in metabolite profiles are common in fungi (33) and studies were done in an attempt to determine what factors

in the defined media were responsible for suppression of ophiobolin biosynthesis. Modified Fries medium (B) was used with substitutions made in the carbon and nitrogen source. Deletion of the yeast extract or tartaric acid were also made. Ophiobolin production was monitored by HPLC. These changes had no or only small effects on qualitative and quantitative production of ophiobolins. The same experiments were done with addition of micronutrients common to media A and C but not originally contained in medium B unless addition occurred through yeast extract. Emphasis was placed on zinc and manganese levels. Addition of manganese at 1.95 ppm, similar to levels found in media A and C, had no observable effect. Addition of 0.9 ppm zinc as $ZnSO_4$ resulted in a 68% reduction of ophiobolin A production as judged by relative peak height. When both zinc and manganese were added at 0.9 and 1.95 ppm, respectively, there was an 84% reduction. Addition of zinc and manganese at 5.65 and 8.13 ppm, respectively, resulted in an 86% reduction of ophiobolin A (See Fig. 6, page 33). Zinc at levels as low as 0.9 ppm clearly has some inhibitory effect on ophiobolin A production. When media A and C were made without zinc, fungal growth was very slow and HMT toxin activity low. Similarily, when yeast extract (the only possible source of zinc) was deleted from modified Fries medium, the growth was slow. Analysis of the yeast extract revealed it to contain 107 ppm zinc, thus the vigorous fungal growth and ophiobolin production observed with medium B occurs with 0.11 ppm zinc. Since all three media had high levels of HMT toxin activity, production of these

compounds is evidently not under control by zinc at levels needed for optimum mycelial growth. Ophiobolin A is also produced when the fungus is grown on potato-dextrose agar. Clearly, more research is needed along these lines but to avoid divergence from the main research project, no further biosynthetic experiments were attempted.

Since HPLC only resolved the HMT toxin into a band of activity and detection problems loomed imminent, an attempt was made to preparatively separate the toxin using open column gel filtration. EtOAc extracts of culture filtrate from Fries medium were dried under vacuum, dissolved in MeOH, and applied to a Sephadex LH-20 column. The column was developed with MeOH and individual fractions were monitored by analytical TLC and the leaf streak bioassay. Materials responsible for leaf streaking activities were easily separated from the major ophiobolins. The HMT toxin activity was found in fractions near the void volume while the ophiobolins were retained until later fractions. Analytical TLC of the toxic fractions was attempted using $CHCl_3$/MeOH solvent systems. When visualized with $H_2SO_4$ the only material observed was a brown spot at the plate origin. As more MeOH was added to the solvent system, streaking made any conclusions impossible. It became apparent that while this method could be used to produce quantities of HMT toxin devoid of ophiobolins for biological studies, it was probably not the method of choice for isolation of toxin for chemical characterization. Toxic fractions from Fries medium were always a light yellow color. Rechromatography reduced the coloration and TLC smearing without affecting biological activities

but complete removal of the contaminating material was never achieved. Because of this difficulty and since it had been determined that the defined medium A did not support ophiobolin biosynthesis, attention turned to the use of this medium. Initial fungal growth was slower but after 6 to 8 days growth, a thick mycelial pad had covered the top of the liquid. The cultures were grown under continuous light because of reported light stimulation of toxin production (52). Harvest of the culture usually was done after 12 to 15 days. Toxin produced in this manner was extracted and fractionated by the same method used with Fries medium. Active fractions from gel filtration were allowed to evaporate to dryness. Unlike active fractions from the Fries medium, these fractions contained quantities of a white, waxy-like substance. One hundred µg of this material was injected into the analytical HPLC system operating at maximum UV detector sensitivity. Only 3 very minor peaks were observed. A UV spectrum revealed only a very minor absorbance at 269 nm. The material was soluble only in hot MeOH, reprecipitation occurred upon cooling. The substance was highly active in the leaf streak bioassay, giving host specific yellowing of tissue at .01-.05 µg/ml. Concentrations of 10 µg/ml had no effect on corn plants with N cytoplasm.

TLC of HMT toxin

Because of reported multiple toxin components, considerable effort went into development of a good analytical TLC system. All solvent systems reported by Karr et al. (19) for resolution of multiple

toxin species were tried and found to be unsuccessful. Materials purified by gel filtration always migrated as one diffuse spot, usually at or near the solvent front, as did the internal standard, cholesterol. Fifteen to twenty different basic, neutral, and acidic solvent combinations were tested for their ability to resolve the material when applied to silica gel plates. Also tested were 2 solvent systems on reverse phase TLC, and systems compatible with polyamide and cellulose TLC plates. The various systems indicated the possibility of multiple species but results were usually difficult to interpret due to streaking of the compounds. Eventually, it was discovered the the use of E. Merck silica gel 60 F-254 pre-coated TLC plates and a solvent system of $CHCl_3$/MeOH 50:5 (v/v) (solvent system B) would resolve the toxin mixture into at least 8 components, three of them in major amounts. Visualization was usually by spraying with $H_2SO_4$/MeOH followed by heating. A good internal standard, 2-deoxy-D-ribose, would appear as a black spot but the toxin components would turn pink (P), brown (B), or violet. P1, P2 refers to pink compounds; B1, B2 to brown compounds; and L1, L2, L3 to compounds with Rf values below all the rest (See Table 2, page 16).

Later it was learned that solvent precipitation of the toxin could be done with EtOAc extracts of culture filtrates from the defined media A and C. Evaporation of the solvent until it appeared cloudy resulted in the precipitation of large amounts of white, non-crystalline material. TLC comparisons of the precipitate and material fractionated by gel filtration showed that they were nearly identical,

the only difference being a small amount of material at the plate origin with the precipitated sample (See Fig. 8, page 42). Paper chromatography indicated this material to be glucose, probably co-precipitated from the media. Production of purified HMT toxin by this method avoids gel filtration and results in a highly active preparation suitable for most biological experiments. Most toxin preparations for chemical analysis were prepared by this method followed by preparative TLC of the resulting mixture.

Bioassays of HMT toxin components

Considerable effort was expended to determine if all of the resolvable components were active in each of two bioassays. Since H. maydis Race 0 is inactive in both assays, extraction and purification of Race 0 culture filtrates from defined media was done exactly as for Race T. TLC analysis showed that Race 0 contained no materials having Rf values similar to the toxic components of Race T nor were any compounds found that gave the characteristic coloration when visualized with $H_2SO_4$. Evidently the 8 compounds found in Race T were unique to that race. Clearly, new natural products, one, several, or all could be responsible for the reported biological responses of the Tcms cytoplasm. It was also possible that each one of the various molecular species was responsible for different toxic effects. Since preparative TLC was to be used to separate individual compounds for bioassays it was essential to determine if any loss of biological activity or chemical decomposition occurred. Two dimensional TLC as described by Stahl (43) indicated that no decomposition had

taken place during chromatography. In addition, TLC could be followed by elution of the silica gel with MeOH, concentration, and then rechromatography, repeating the entire operation for a total of 3 times without altering Rf values or color upon visualization. There was also no apparent loss of biological activity after this extensive chromatography. This result was important since initial attempts at preparative TLC were unsuccessful until multiple elution was employed to successfully separate small amounts of the 3 major molecular species, one minor species, and a mixture of 3 other minor compounds. The completed plates were visualized by spraying them with distilled water until the silica was soaked. The individual bands appeared opaque against the translucent background. After the purified compounds had been eluted from the silica gel, each one was passed through a column of Sephadex LH-20. This effectively removed dissolved silica gel and other contaminants. Analytical TLC of each compound was finally done to insure a homogenous preparation. Each compound rechromatographed with reproducible Rf values. Conversion of a pure component into other components of the mixture was never observed, but in some cases conversion to materials migrating to the top of the TLC plates (Rf>.90) was observed. If this was noted that particular sample was not bioassayed. Results of two different bioassays were the same, on a weight basis each of the purified compounds and the mixture of 3 minor compounds were equal in activity (See Fig. 9, page 53 and Table 7, page 52).

On some occasions it was noted that none of the toxic components (Rf<.55) were present, instead materials having high TLC mobility (Rf>.90) were found. This material was clearly less polar as indicated by Rf values and its solubility in $CHCl_3$. Unaltered toxin components are not soluble in $CHCl_3$. Leaf streak bioassays revealed this material to have good biological activity (See Table 8, page 52). Recovery of unaltered toxin from preparative TLC was initially low (8%), evidently most of the toxin was converted to nonpolar fractions. Later it was found that higher yields of unaltered materials could be recovered if the toxins were never taken to complete dryness from MeOH under vacuum. A study of the chemical nature of this material was initiated in an attempt to understand and avoid this transformation. See in the next section, Chemical characterization of HMT toxins).

Chemical characterization of HMT toxins

Proton NMR was initially conducted on the toxin mixture. The results were consistent with the lack of aldehydes, aromatic, or olefinic structural features. Because of limited solubility of the toxins in most organic solvents, NMR analysis was limited to the use of MeOH-$d_4$ and as a result of proton exchangeability signals corresponding to carboxyl, hydroxyl, or amino functionality were not seen. Obvious NMR features were: a triplet signal at $\delta$ 0.90 probably due to a terminal methyl adjacent to a methylene, broad multiplets from $\delta$ 1.40 to 1.85 due to aliphatic functionality, a doublet centered at $\delta$ 2.13, and a large broad multiplet from $\delta$ 2.43 to 2.61. Small diffuse signals were at $\delta$ 3.74 and 4.29. There appeared to be a quintet

at δ 4.47 (See Fig. 10, page 57). IR data (KBr) indicated a saturated carbonyl; 1720 $cm^{-1}$, aliphatic functionality; 2830, 2870, 2880 $cm^{-1}$, and hydroxyl groups; 3420 $cm^{-1}$ (broad) (See Fig. 16, page 68). $^{13}C$ NMR signals at 211 to 212 ppm substantiated the presence of saturated carbonyl functionality. $^{13}C$ NMR spectra of the toxin mixture were devoid of signals from 102 to 211 ppm. This was useful to eliminate the presence of aromatic and olefinic carbon atoms, esters, lactones, and anhydrides, as well as acids and enolized β-diketones (53,54). Spectra obtained on toxin samples prepared directly from solvent precipitation showed two small signals at 98 and 93 ppm and several signals from 63 to 78 ppm. The downfield signals could result from the α and β anomeric forms of carbon #1 from contaminating glucose. The upfield signals from 63 to 79 ppm could arise from glucose as well as toxin carbon atoms containing hydroxyl groups (54). Paper chromatography had indicated the presence of glucose in toxin samples precipitated from the extraction solvent. Glucose was never found to be present after the precipitated material was further purified by gel filtration. $^{13}C$ NMR of glucose-free toxin indicated loss of signals at 93 and 98 ppm and an altered appearance of the region from 63 to 78 ppm (See Fig. 15, page 67). Signals for a terminal methyl group (14.4 ppm) and several aliphatic carbon atoms (18 to 44 ppm) were also seen. $^{13}C$ NMR spectral studies were difficult since low toxin solubility in all solvents tested necessitated long spectral accumulation times. Twenty-five mg/ml was found to be the highest toxin concentration obtainable when dissolved in methanol.

At this concentration at least 12 hours was required and up to 60 hours was sometimes used to obtain a usuable spectrum. The resonances found at 99 and 102 ppm in glucose-free toxin are particularly interesting. When toxin samples were prepared using techniques known to minimize any transformations and spectral accumulation times shortened the number and intensity of signals in this region were reduced. Resonances from 95 to 105 ppm are diagnostic for acetal and ketal functionality (54). If ketals are being formed in solution, it is probable that they are intramolecular since their elution volume during gel filtration is unchanged.

Fourier transform proton NMR spectra were obtained at 90 MHz from each of the four purified materials: P1, B1, P2, and B2. These spectra were striking in their similarity (See Figs. 17 to 20, pages 70 to 76). One point of interest was the signals at $\delta$ 2.12 and $\delta$ 2.16 which in the mixture exists as a doublet but in the case of P1 and B1 are clearly singlets. Small shoulders are present on each resonance, probably corresponding to signals from a small amount of cross contamination. Preparative isolation of toxins B1 and P2 was done twice. The second time they were examined at 100 MHz using a computing signal averager. Results were comparable with data obtained at 90 MHz (See Figs. 21 and 22, pages 78 and 80). With toxin P2, the signals at $\delta$ 2.12 and 2.16 were especially interesting. The area of the signal at $\delta$ 2.16 seems to be reduced but not eliminated after purification from the mixture. In the case of toxins P1 and B1, one or the other of the signals $\delta$ 2.16 and 2.12 is reduced to a small

shoulder. TLC analysis indicated P1 contained a small amount of B1 and vice versa; however, after NMR analysis of P2, TLC examination showed that the sample was free of contaminating toxins. The entire sample was rechromatographed and examined again at 90 MHz with ft-NMR. The signal at δ 2.16 had not been reduced in size compared to the signal at δ 2.12 (See Fig. 23, page 82). Finally, analytical TLC of the sample indicated it did not contain any P1, B1, or B2. It was noted that 2 new components with Rf values greater than .85 were present. This was not unexpected since the sample had stood several days in MeOH-$d_4$.

Examination of the literature suggested that NMR signals in the region δ 2.10 to 2.20 could be due to terminal methyl ketones (55). There was also strong support for the assignment of signals from δ 2.4 to 2.6 as being from protons adjacent to carbonyl groups. Sodium borohydride reduction of the toxin mixture also suggests these assignments. NMR analysis of the reduction product showed complete disappearance of all signals from δ 2.10 to 3.30, an altered appearance of signals in the aliphatic region of the spectra, and the appearance of a new unfield quartet at δ 1.18 (See Fig. 24, page 84). These changes are expected when the electron withdrawing properties of carbonyl groups are removed by reduction to the alcohol. Examination of the quartet at δ 1.18 in the reduction product is diagnostic and suggests two doublets at δ 1.125 and δ 1.150, J=6. This is shown in Fig. 35. The location of these signals and magnitude of the coupling constant are

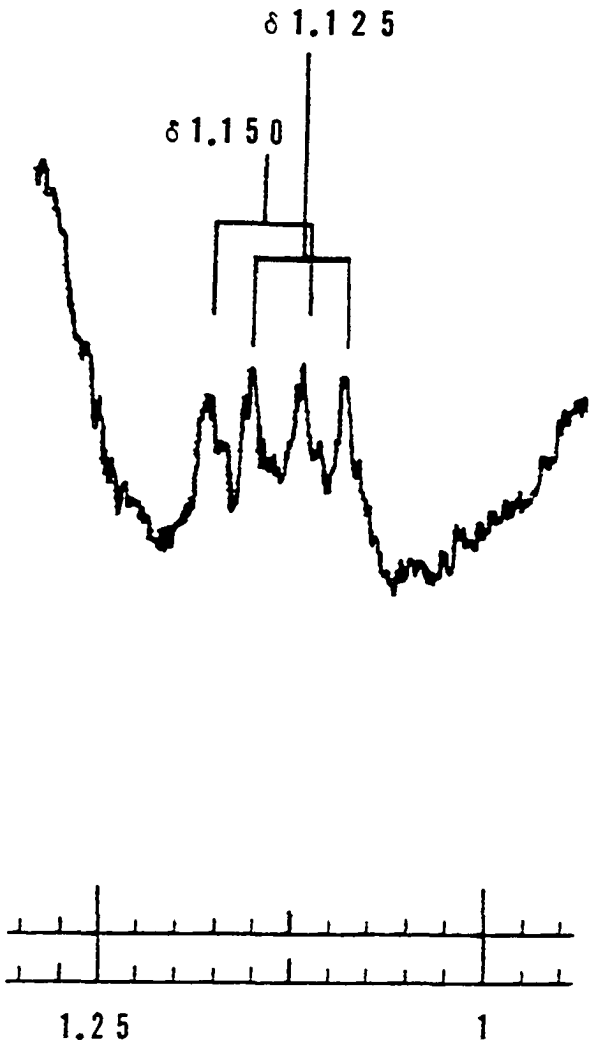



Figure 35. 100 MHz proton NMR spectrum of HMT polyol, δ 0.95 to 1.30. The scan width was 250 Hz

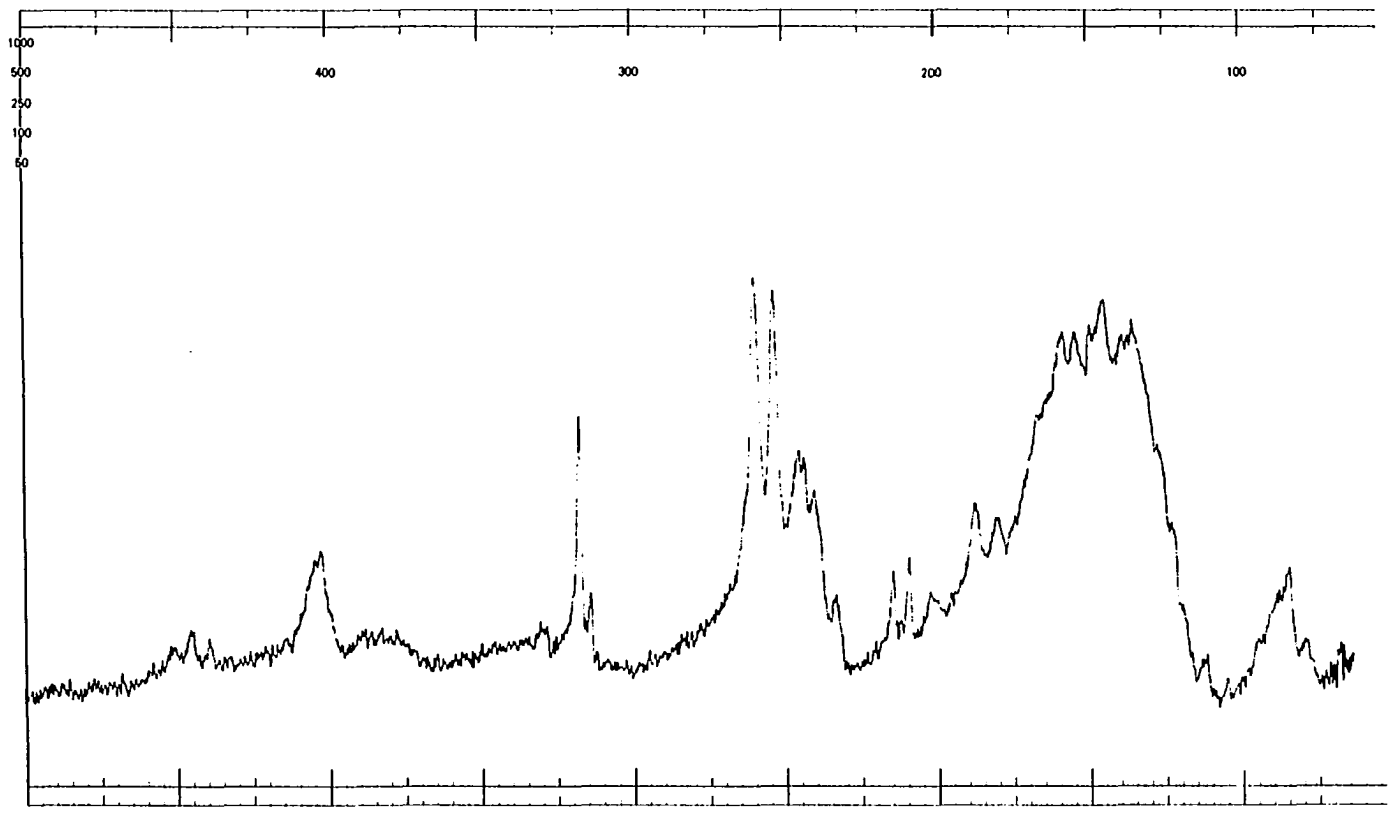



Figure 36. 100 MHz proton NMR spectrum of "decomposed" HMT toxin. The scan width was 500 Hz

consistent for two terminal methyl groups adjacent to carbon atoms containing one hydroxyl group and one proton each (56). The methyl group at δ 0.90 is unaltered by the reduction. At this point, the following chemical functionalities emerge: $-CH_2CH_3$, $-CH_2COCH_3$, and $-CH_2CHOHCH_2-$.

Since P2 apparently contains 2 different methyl ketones in unequal amounts it is possible that this component actually consists of 2 unresolved species. Further work is needed along these lines.

Chemical studies on "decomposed toxin"

$^1H$ NMR examination of this material showed the presence of terminal methyl group resonances and unaltered appearance of the methyl ketone functionality (See Fig. 36). New signals at δ 3.14 and δ 3.175 were observed. It is interesting to speculate that these resonances could be assigned to the methoxyl portion of an intramolecular hemi-ketal followed by ketal formation from the solvent, methanol (See Fig. 37).

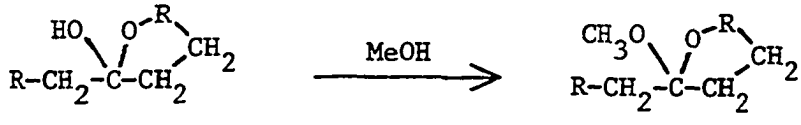



Figure 37. Proposed ketal formation

Thorough drying of the sample under vacuum did not result in the removal of these signals. Another area of the spectrum having noticeable changes is from δ 2.40 to 2.60. This would be expected if some of the carbonyl groups were tied up as ketals. Protons adjacent to ketal carbon atoms are shifted upfield (57). $^{13}$NMR resonances at 99 and 102 ppm in some of the samples also support this theory. It is possible that rapid removal of the solvent under vacuum promotes formation of the methyl ketals, however, this transformation was not always noticed and one attempted reversal of the reaction by treating the isolated transformation product with acid failed. The native HMT toxins could be dissolved in hot MeOH and reprecipitated by slow solvent evaporation repeatedly without effect. After it was learned how to avoid this condition, no further work on the problem was done.

Mass spectral studies on HMT toxin

Electron impact mass spectrometry was repeatedly conducted as various toxic chromatographic fractions and purified compounds became available. Chemical ionization mass spectrometry of the toxin complex was also done by Finnigan Corporation. Very little usable data was obtained from these studies. There never was an indication of formation of molecular ions and numerous ions in the low mass range gave little structural information. Field desorption mass spectrometry done by Dr. K. L. Rinehart, Jr. was also inconclusive, but analysis of a mixture of P1 and B1 gave strong ions at 794 and 724 with weak ions at 792,796 and 722,726, respectively.

To obtain more information on the carbon skeleton, complete reduction of the toxin mixture was undertaken. This had previously been done by Cope and Rinehart to obtain the exact carbon skeleton of polyene antibiotics (58,59). In the case of HMT toxin reduction, no materials were produced that matched GC retention times of a series of standard linear paraffins from C22 to C30. GC/MS analysis of the reduction products indicated several hydrocarbon fractions had been produced. These can be identified by groups of peaks 14 mass units apart corresponding to $C_nH_{2n+1}$ and are accompanied by ions at $C_nH_{2n}$ and $C_nH_{2n-1}$ (60). Figures 27, 28, and 29, pages 88 to 92, show a reconstructed gas chromatogram from total ion currents and a normalized spectrum from scans 39 and 52. Scan 39 shows an especially good hydrocarbon fragmentation pattern from M/e = 43 to M/e = 253 corresponding to $C_{18}H_{37}$. No evidence of branching is seen in this fragment. The presence of high masses at M/e values inconsistent with usual hydrocarbon fragmentation may indicate incomplete reduction.

Isolation of a host specific toxin from Phyllosticta maydis

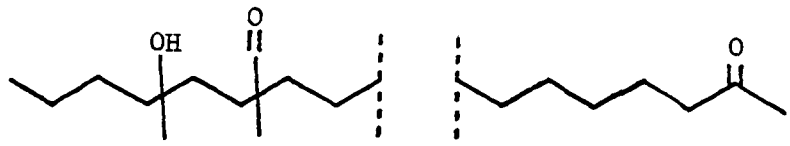
Because of the ease with which HMT toxin can be isolated using the new procedure, isolation of the P. maydis toxin was investigated. Initial experiments indicated that although both media A and B supported fungal growth and toxin production, extractions from the defined medium A contained less yellow pigment that interfered with TLC examinations. As with HMT toxin production with H. maydis, P. maydis responded to continuous light with increased toxin activity. Toxic material from the culture filtrate was extractable with EtOAc.

Dried extracts were dissolved in MeOH and chromatographed over Sephadex LH-20 in a procedure similar to that used with H. maydis extracts. Results from gel filtration were very interesting in that the toxic component eluted from the column at the same point as did the HMT toxin, indicating that the two may have similar chemical characteristics and/or molecular size. $^{1}H$ NMR of the material from active fractions indicated striking chemical similarities to the HMT toxins with a triplet at $\delta$ 0.906 and a region of aliphatic signals from $\delta$ 1.2 to 1.6. One obvious difference was the lack of a large number of signals for protons adjacent to carbonyl groups. The apparent lack of carbonyl groups was also supported by IR data. The large absorption at 1720 $cm^{-1}$, which is so evident with the HMT toxin, is replaced with a much smaller absorption at 1700 to 1710 $cm^{-1}$ (See Fig. 32, page 100). Small NMR signals from approximately $\delta$ 1.8 to a larger doublet centered at $\delta$ 2.53 could also indicate some carbonyl functionality although they are certainly not as numerous as compared to the HMT toxin (See Fig. 31, page 99). There are some interesting structure-activity-relationships with the host specific toxins from H. maydis and P. maydis. Toxins from both species are very active and somewhat similar chemically. Bioassay of the polyols produced by reduction of the HMT toxins indicated that carbonyl functionality is not needed for leaf streaking activity (See Table 9, page 87). In view of these results, it is not surprising to find that P. maydis toxin almost devoid of carbonyl functionality. It is not known if the active material is one or multiple components.

HPLC was used to examine a crude EtOAc extract of the culture filtrate from medium A. Very little material was detected with the UV detector operating at 254 nm (See Fig. 30, page 97). TLC examination of the active fractions from Sephadex chromatography was not successful since extensive streaking occurred. Upon visualization, the material appeared yellowish brown with none of the pink coloration of the HMT toxins noted.

Structure of the toxins

A proposed structure for any of the HMT toxins is premature at this time. The availability of only small amounts of individual components, limited solubilities, noncrystalline nature, and troublesome mass spectral characteristics have made study of these compounds extremely difficult. Information obtained is, however, consistent for a group of toxin species having polyketide-like structures. They appear to contain only carbon, hydrogen, and oxygen. There is no evidence of chelated metals in the isolated toxins. A common feature of all isolated toxin species appears to be a terminal methyl group adjacent to a methylene group. Two different methyl ketone $^1$H NMR signals of nearly equal size in the toxin mixture may indicate 2 main molecular species with other small differences which were unobservable in the various spectra. Generalized toxin structures are illustrated with 12 and 13. A hydroxyl group close to the methyl ketone is included in 13 since this could account for the observed $^1$H NMR singlet at $\delta$ 2.16. The singlet at $\delta$ 2.12 is consistent with an isolated methyl ketone as in 12. There is some evidence for $\beta$-hydroxy ketone functionality but no evidence for $\beta$-dicarbonyl groups.



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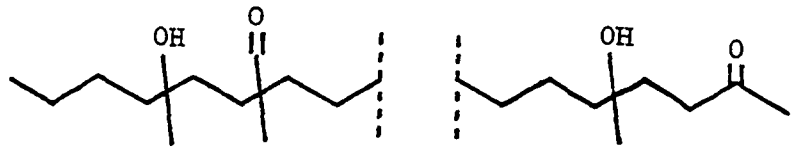


13

Polyketides are formed by condensation of an acyl unit with malonyl units and concomitant decarboxylation as in fatty acid biosynthesis; however, with polyketides there is no obligatory reduction of the β-dicarbonyl intermediate 14.

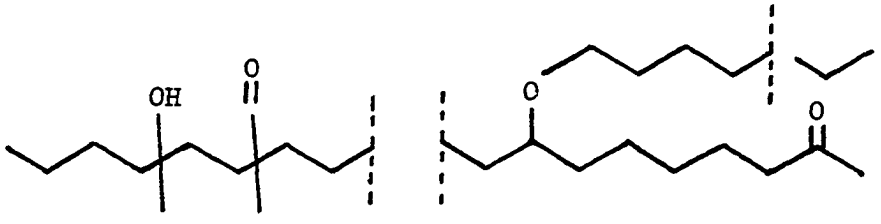
$$CH_3COCH_2COCH_2COCH_2COR$$

14

Structural variety in the HMT toxin complex could result from loss of ketones from different positions by reduction to alcohols, dehydration of different alcohols followed by reduction of resulting olefins, and formation of species having different chain lengths.

One problem has been interpretation of proton NMR spectral integrations. The proposed carbon skeletons would require equal

areas for the regions comprising the terminal methyl and methyl ketone functionality; however, actual integrations of the mixture as well as compounds P1 and B1 indicated ratios from 1 to 2 to as high as 1 to 3 for the region $\delta$ 2.13 and $\delta$ 0.90. This may indicate the presence of more than one $-CH_2CH_3$ group per toxin molecule as in 15.




15

Future work must certainly include attempts at accurate mass measurement of the individual toxin molecules. Initial field desorption (FD) mass spectral attempts appeared promising with some of the purified toxins. It is also possible that laser-assisted FD mass spectrometry could be of use for these complex, involatile materials. Several workers have reported loss of biological activity in base-treated toxin preparations. Chemical data now suggests that base inactivation may be due to retro-aldol cleavage. Controlled degradation of the toxins with base followed by GC/MS analysis of the

fragments and/or their derivatives may be of value. Formation of derivatives for preparation of crystals suitable for x-ray analysis also would be of value.

## SUMMARY

A structurally similar group of host-specific toxins manifesting two biological activities against Tcms maize tissues has been isolated from Helminthosporium maydis Race T. Extraction of culture filtrates from defined growth medium with ethyl acetate followed by solvent precipitation or gel filtration resulted in a mixture of at least 8 components. Further purification by preparative silica gel TLC afforded 4 individual components having approximately equal biological activities. The closely related structures appeared to be polyketide-like, completely different from the terpenoidal ophiobolins also produced by H. maydis under certain conditions. Comparison of several different isolates of Race T from various research laboratories indicated that similar toxin species were produced from all. The toxins are chemically similar to a toxin(s) having the same host specificity isolated and partially characterized from Phyllosticta maydis.

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