ISOLATION AND IDENTIFICATION

OF

CONSTITUENTS OF PROPOLIS

Magdolna Koltay

A Thesis

in

The Department

of

Chemistry

Presented in Partial Fulfillment of the Requirements

for the degree of Master of Science at

Concordia University

Montréal, Québec, Canada

July 1981

-i-

ABSTRACT

ISOLATION AND IDENTIFICATION

OF

CONSTITUENTS OF PROPOLIS

Magdolna Koltay

Propolis, a material obtained from bee hives, has been considered as a bacteriocide in empirical medicine, and was used in the Boer War and the First World War as a disinfectant. Since then, many constituents, mainly flavones and other phenyl derivitives, have been isolated from propolis and identified, and have been found to have antibacterial properties.

The present work reports the isolation of a phthalate diester, which has high anti-mildew activity, from the chloroform extract of propolis. Column chromatography was used for separation, and infrared, ^1H and ^{13}C nuclear masnetic resonance, and mass spectra were employed for identification. The proposed formula is $C_{24}H_{38}O_4$, molecular weight 390.

Further work on propolis resulted in six separated and purified wax esters, with long alcohol chains containing variable numbers of carbon atoms and, possibly, double bonds.

These compounds were separated and subsequently purified by
thin layer chromatography from the hexane extract of propo-
lis. Melting points of these esters ranged from 55 to 70°C.

One aliphatic hydrocarbon was separated from the hexane
extract of propolis by thin layer chromatography. The pro-
posed elemental formula is $C_{31}H_{64}$.

The above mentioned isolated compounds are only a few
of the many components present, most of which are unident-
ified due to the complexity of propolis.

Acknowledgements

I am most indebted to the late Professor Jacques A. Lenoir, whose interests in natural products made this project possible, and to Dr. L.D. Colebrook for his time and help in enabling me to finish my thesis.

I am deeply grateful to my late brother-in-law, Dr. Kiss Dezsö, who awakened my interest in bees and in propolis, and who provided the sample of Hungarian propolis.

I wish to thank Drs. Z. Hamlet, O.S. Tee, and P.H. Bird for helping me to proceed with my work following the death of my supervisor.

Dr. Orville Mamer and Dr. Jeremy Everett, of McGill University were helpful in providing mass spectra and NMR spectra of the isolated compounds, while Dr. R.T. Rye gave invaluable help in reviewing my interpretations of the mass spectra.

1. INTRODUCTION

1.1 Origin and General Characteristics of Propolis.

Propolis is a natural material found in bee hives. Bees use propolis in a thin layer on the internal walls of the hive or any other cavity they inhabit. It is used to block holes and cracks, to repair combs and for making the hive weathertight. It is also employed to narrow the entrance for easier defence of the hive.

Apart from the purely mechanical use of the gluelike and cementing properties of propolis, its use may also have biological significance. The embalming of an intruder may serve to contain putrefaction and thus to prevent the spread of infection and diseases. The present author has seen a brown mass glued in the corner of a hive at the spring inspection and the examination of the core revealed the presence of the corpse of a mouse. It was well preserved though it had been there since the previous fall. It seems that propolis is responsible for the lower incidence of bacteria and moulds within the hive than in the atmosphere outside. The thin layer of propolis provides an impervious lining for the hive which limits the escape of water vapour needed by the developing brood and maintains a constant humidity to keep the honey at the desired concentration.

Early observers of bee behaviour have been aware of the

plant origin of propolis (1,2,3,4). The plant origin of propolis was asserted by Rosch (5) in 1927 and again by Vansell and Bisson (6) in 1940. It is now generally accepted that bees collect propolis from various plant sources in the north temperate zone, particularly species of poplar, birch, elm, alder, beech, conifer and horse-chestnut. Rather little is known about these trees as the source of propolis and even less about the sources of propolis in the tropics.

Characteristically, propolis is a hard and brittle material when cold, but soft, pliable and very sticky when warm. Its colour varies from greenish yellow to reddish brown. It is difficult to remove from the human skin by rubbing. It develops a fine, shiny layer and it seems to interact with the oils and proteins in the skin. Its pleasant smell remains long after it has been washed off. Solubility tests show that it is almost insoluble in cold water but that some volatile material separates in steam distillation. It is partially soluble in cold alcohol, in cold and hot ligrion, and in benzene. Propolis is much more soluble in chloroform, ether, acetone and ammonia.

1.2 Composition of propolis

Early attempts to determine the composition of propolis were concerned with simple fractionation. The alcohol extract was used mostly for further investigation. One of the earliest reports is that of Dieterich and Helfenberg (7,3)

describing their extraction method and the constituents of propolis separating in alcohol, chloroform and ether. The wax and resin soluble in chloroform could not be separated further, but that portion soluble in alcohol was separated into wax (mp. 65-66°C.) and a brown resinous residue, having an aromatic odour, resembling the cinnamin of Peru balsam. The resin contained constituents of conifer resin and large amounts of aromatic substances which varied with the locality from which the resin was obtained. Other workers found various amounts of resin and waxes in propolis (4,5,6). Dieterich (9), in later work, described vanillin as a trace constituent of propolis and Kustenmacher (2), identified cinnamyl alcohol and cinnamic acid. Some sixteen years later, in 1927, Jaubert (4) found that the colour of beeswax was due to the presence of the flavonoid pigment chrysin which could be isolated from propolis. In 1957, Uskhalova in the USSR (8) found 4 types of waxes in propolis which varied in colour, mp., acid content and iodine test results according to the collecting place of bees.

No further identification of any new compound in propolis appeared until 1969, but the pharmacological effects of extracts of propolis were investigated during these years. In 1969 Popravko et al. (10,11) identified six flavonoid pigments and they separated and identified two flavanones and isovanillin. Evidence was obtained for the presence of derivatives of another flavone, quercetin, but the comp-

ounds were not further characterized. These flavone and fl-
avanone compounds were also found in dormant buds of Betula
varrucosa, which was frequented by the bees from where the
samples were taken for investigation.

Lavie (12,13) observed that propolis showed significant
antibacterial activity towards Bacillus substilis and Prot-
ius vulgaris. He and his co-workers showed that this activ-
ity was due partially to the flavone galangin, which they
separated from the resin fraction. Later work by the same
group (14) resulted in the isolation of a flavone,
pinocembrin, with similar activity to galangin. In addition,
the flavones chrysin, tectochrysin and isalpin were also
isolated and identified. In 1970 Cizmarik and Martel (15)
described the separation and identification of 3, 4-dihydro-
xycinnamic acid present in propolis. These workers have also
separated ferrulic acid. These compounds are known for their
antibacterial activity on some gram positive and gram
negative microorganisms.

Metzner and his collegues (16,17) identified more com-
ponents of propolis in 1975. Their technique consisted in a
combination of thin layer chromatography and bacterial incu-
bation on the same plate. It led to the identification of
seventeen constituents, including nine previously identified
compounds. The newly identified compounds were two flavones,
pectolinarigenin, quercetin-3, 3'-dimethyl ether, three flav-

anones,. p-cumaric benzyl ester and an ester of caffeic acid.
Australian investigators (18) in 1977 have shown the similar
composition of propolis found in Western Australia. They
separated and identified four flavanones and chrysin and 3,
5-dimethoxybenzyl alcohol.

The fatty acid constituents of propolis was investigat-
ed by W. Heinen and H. Linskens (19). The total fatty acid
content of propolis is approximately 5%. The fatty acid
fractions contain C_7-C_{18} acids, the major component of
which is myristic acid (C_{14}), (approximately 70%, figures
obtained by gas chromatography). Similar fatty acids were
found in the poplar buds from which the bees collect the
sticky material.

The presence of small amounts of vitamins such as B_1,
B_2, B_6, C and E has been shown in propolis from the USA.
(20, 21,22,23,24). Nicotinic acid and pantothenic acid have
also been detected in variable amounts.

The ash residue of propolis has been shown to contain
the elements: iron, calcium, aluminium. vanadium, strontium,
manganese and silicon (24, 25).

The early investigator Helfenberg (3) did not detect
any alkaloids or volatile oils in his samples. However
Janes and Bumba (26) later identified benzoic acid, sorbic

.acid and vanillin in the steam distillate.

The largest group of compounds isolated so far from propolis are flavonoid pigments which are ubiquitous in the plant kingdom. The series of flavonoids isolated from propolis correlates reasonably well with those pigments present in plants but relatively few of the many flavonoids found in the buds are detectable in propolis. Russian investigat ors (27) suggested, that some of the flavones are modified by an enzyme in the honeybee during collection of the material. It was suggested that honeybees collect selectively flavonoid compounds or alter them for their own purpose to achieve the maximum activity of propolis. It must be emphasized that the many compounds already isolated represent only a small portion of the total. Most came from the fraction soluble in organic solvents or water. A proper delineation of the constituents will require much more investigation and they will doubtless vary with the plant source used by the bees.

Identified Constituents of Propolis

No.	COMMON NAME	CHEMICAL NAME OR FORMULA
1	cinnamyl alcohol	$C_6H_5CH=CHCH_2OH$
2	cinnamic acid	$C_6H_5CH==CHCO_2H$
3	vanillin	4-hydroxy-3-methoxybenzaldehyde
4	isovanillin	3-hydroxy-4-methoxybenzaldehyde
5	caffeic acid	3,4-dihydroxycinnamic acid
6	ferulic acid	4-hydroxy-3-methoxycinnamic acid
7	chrysin	5,7-dihydroxyflavone
8	tectochrysin	5-hydroxy-7-methoxyflavone
9	acacetin	5,7-dihydroxy-4'-methoxyflavone
10	--	5-hydroxy-4'-7-dimethoxyflavone
11	quercetin	3,3',4',5,7-pentahydroxyflavone
12	kaemferide	3,5,7-trihydroxy-4'-methoxyflavone
13	rhamnocitrin	3,4',5-trihydroxy-7-methoxyflavone
14	--	3,5-dihydroxy-4',7-dimethoxyflavone
15	galangin	3,5,7-trihydroxyflavone
16	isalpinin	3,5-dihydroxy-7-methoxyflavone
17	--	5,7-dihydroxy-3,4'-dimethoxyflavone
18	pectolinarigenin	5,7-dihydroxy-4',6-dimethoxyflavone
19	pinostrobin	5-hydroxy-7-methoxyflavanone
20	--	5-hydroxy-4',7-dimethoxyflavanone
21	pinocembrin	5,7-dihydroxyflavanone
22	sacuranetin	4'5-dihydroxy-7-methoxyflavanone

Identified Constituents of Propolis - continued

No.	COMMON NAME	CHEMICAL NAME OR FORMULA
23	isosacuranetin	5,7-dihydroxy-4'methoxyflavanone
24	quercetin-3,3' dimethyl ether	4',5,7-trihydroxy-3,3'-dimethoxy- flavone
25	pinobanksin	3,5,7-trihydroxyflavanone
26	3-acetyl pino- banksin	5,7-dihydroxy-3-acetylflavanone
27	pterostilbene	$(CH_3O)_2C_6H_3.CH--CH.C_6H_4OH$
28	xanthorrhoeol	
29	--	3,5-dimethoxybenzyl alcohol
30	myristic acid	tetradecanoic acid
31	benzoic acid	benzoic acid
32	benzyl alcohol	benzly alcohol
33	sorbic acid	hexa-2,4-dienoic acid
34	eugenol	4-allyl-2-methoxyphenol
35	--	3,5-dimethoxybenzyl ester
36	p-cumaric acid benzyl ester	

No.	COMMON NAME	CHEMICAL NAME OR FORMULA
37	Vitamin B_1	thiamine
38	Vitamin B_2	riboflavin
39	Vitamin B_6	pyridoxine
40	Vitamin C	ascorbic acid
41	Vitamin E	tocopherols
42	Vitamin A	retinol

Identified Constituents of Propolis - continued

No.	COMMON NAME	CHEMICAL NAME OR FORMULA
43	Pantothenic acid	
44	Nicotinic acid	
45	Copper, manganese, iron, calcium, aluminium, vanadium strontium, magnesium, silicon. (61).	

1.3 Biological Activity of the Constituents of Propolis

Although much work has been done to determine the spectrum of biological activity of propolis, very little information is available as to which compounds present in propolis extracts are responsible, entirely or in part, for its reported activity.

In 1960 Lavie (12) found that propolis showed antibacterial activity towards Bacillus substilis, Proteus vulgaris and E. alvei. Less effect was found on E. coli, Salmonella gallinarum, S. pullorum and S. dublin. Separation of galangin and pinocambrin by the same workers, by further fractionation, showed that those compounds were partially responsible for the activity. The first systematic investigation of the antibacterial activity of propolis on a range of bacteria appears to be that of Kivalkina (28), who found that propolis showed bacteriostatic activity against Streptococcus aureus, the typhoid bacillus, and other bacteria. In a detailed study of the antimicrobial activ-

ity of propolis, Lindenfeiser (29) tested fifteen samples, collected from different parts of the USA, at various seasons. Extracts showed strong inhibitory activity in vitro in 25 of 39 bacterial species tested. Bacillus larvae were most strongly inhibited and 24 others were sensitive including gram positive cocci and acid fast groups. Of 39 fungus species, 20 were inhibited. Resistance was shown by two yeast cultures. Recently, in a study of the constituents of propolis, Metzner et al (16) detected compounds which are active against B. subtilis, Staphilococcus aureus, E.coli and Candida Albicans. The authors concluded that out of many compounds detectable in propolis, only pinobanskin-3-acetate, pinocembrin, p-cumaric acid benzyl ester and a caffeic acid ester showed significant antimiocotic activity. Ferrulic acid, separated and identified by Cizmarik et al. (15), showed anti-bacterial and fungistatic activity. In vitro tuberculostatic activity was also found. Sacuranetin is active against wood rotting fungi (30).

A number of flavones isolated from propolis have been shown to have papaverin-like spasmolitic activity in a test carried out on the small intestine of mice in vitro (31). Quercetin has been shown to enhance the histamin-fixing capacity of several protein fractions in vitro (particularly gamma-globins) (32).

A number of flavonoid compounds exert an anti-inflam-

matory effect on joints, skin and mucous membranes. It has
been suggested that some flavonoids stabilize connective
tissue and vessels, protect vitamin C against oxidation, and
decrease the activity of the mucopolysaccharide-destroying
enzyme (33). Recent evidence (34,35) shows polymethoxylat-
ed flavonoid compounds may inhibit erythrocyte aggregation
in vitro and in vivo.

These activities mentioned are but a small part of the
known biological activities of flavones, flavanones and
flavonols. Whether these compounds are directly or indir-
ectly responsible for some of the observed effects of propo-
lis remains an open question. However, since the major con-
stituents of propolis are flavonoids and propolis appears to
be widely used in certain countries, it is useful to consid-
er the known pharmacological properties of these compounds.

Reports of the clinical use of propolis appeared in the
early 1900's. A preparation of propolis and vasilin "pro-
polisin vasogen" was used as a medication during the Boer
war (36). Other reports refer to the use of propolis for
inhalations and as a non-irritant dressing for wounds (37).
There are many recent reports in the literature describing
different clinical uses of propolis and preparations, con-
taining propolis, such as: dermatological diseases (38,39,
40), inflammation treatment in surgery (40,41,42), immunol-
ogy (43) anaesthesis (44,45), dentistry (40), lung tubercol-

osis (64), synthesis of agglutinins (47,48,49).

Several reports have referred to the phytoinhibitory and phytotoxic activity of propolis extracts. Potato tubers kept in a hive did not sprout (50) and after storage in the bee hive for an extended period they suffered permanent inhibition. An aqueous extract of propolis was shown to be responsible for inhibiting the germination. Growth of lettuce and rice grains was also inhibited. Alcoholic extracts of Russian propolis were found to inhibit the germination of Cannabis sativa seeds (51).

Unfortunately, most of the experiments and treatments were carried out with crude extracts giving no information of components responsible for activity, and different experiments gave contradictory results. A consideration of the chemicals separated until 1979 does not explain the various effects of propolis, which leaves us with the conclusion that either many active ingredients are still obscure or the different chemicals present exhibit a-synergistic effect. The properties of propolis-extracts depend upon the methods of separation used and upon the origin of the propolis. This variety may partially explain the contradictory findings in the literature. In any event more systematic research needs to be conducted to establish the composition and properties of propolis.

1.4 Techniques used in the identification of components of propolis

The early studies mostly used extractions to measure physical properties of propolis. Extractions were carried out in alcohol, chloroform and methanol. With the advent of modern physical methods, research and knowledge about propolis has multiplied. In general, the extractions were separated by different chromatographic methods such as paper, column, gas and thin layer chromatography. Other methods include bioautographic methods, distillation, combined with different chemical methods. For identification purposes UV, NMR, and mass spectra were often used.

Metzner (16) described a combined method for separation, biological activity detection, and identification: TLC plates were prepared holding an ethanol extract and were developed in two directions. One of the two identically treated plates was coated with agar and bouillion medium. Bacterium spores were then spread evenly over the plate which was kept at a proper temperature for maximal growth of the colonies. Over the spots containing the active compounds no development of colonies could be detected. This method gave good, reproducible results. The R_f values of the other plate were used for identification.

Lindenfelser (29) used a similar method to determine

biological activity, but he used paper chromatography.

Cizmarik and his co-workers (15) separated caffeic acid from a water-ether extract. TLC plates were used for purification. For identification they performed elemental analysis, bromine and $KMnO_4$ tests for unsaturation, Tollens and Fehling reactions for aldehyde and ketone recognition, R_f values and UV measurements.

Researchers of the Univesity of Western Australia (18) used extraction methods, and fractionation at different pH's. The aqueous alcohol extract was further partitioned into 5% HCl, saturated $NaHCO_3$, 10% $NaCO_3$ and 5% NaOH solutions. For further separation, isolation and purification of the constituents of each fraction, column, analytical and preparative TLC techniques were used in the usual way. For structure determination they used NMR and mass spectra.

Heinen and Linskens (19) used gas chromatography for identification of the fatty acid content of propolis.

2. OBJECTIVES

The aim of this work was to isolate compounds with active antimildew characteristics. Secondly, that these compounds should be pure as determined by TLC with different eluents and their purity determined conclusively by IR, NMR, and MS. Thirdly, the functional groups or chemical formulas of the purified compounds should be established as accurately as the available physical methods permit.

3. EXPERIMENTAL

3.1 Methods, Material, and Equipment Used

Sources of Raw Material

The propolis used in this study came from Green Valley (Ontario) and from Hungary.

Extraction Procedures

Extracts from propolis were obtained with organic solvents using a Soxhlet continuous extraction apparatus, with clay or paper thimbles. Details are given in Section 3.2.

Thin Layer Chromatography

In order to monitor separation of the components obtained by the extraction procedures, thin layer chromatography (TLC) was performed using prepared fluorescent silica-gel coated sheets (Eastman Chromatogaphy Sheet, 13181 Silica Gel, with Fluorescent Indicator #6060). The 200 x 200 mm sheets were cut into four 100 x 100 mm squares, each capable of providing 5 - 7 spaces for application of the mixtures to be separated. Twelve inch, screw cap, wide mouth jars were used as developing chambers. The 100 x 100 mm sheets were folded, and held in rolled form by paper clips for insertion into the jar. A 10 ml volume of elution liquid was used in the developing chamber, the elution time being 8 - 10 minutes. Ultraviolet light from a UVS II

Mineralight was used to make the separated spots visible on the fluorescent TLC sheets.

For preparative scale separations, 200 x 200 mm glass plates, coated with a 0.5 mm layer of Silica Gel G (prepared with a Delaga thin layer spreader, Model DS 200/0-2), were used. The pre-dried plates were activated at 110°C for two hours before use. Spots were made visible by spraying the developed, (dried) plate with a 0.2% solution of 2',7' -dichlorofluorescein in 96% ethanol, or with 40% sulphuric acid.

For reversed-phase partition chromatography the plates were rendered hydrophobic using Kaufman's method (55). The impregnating material used was tetradecane (5%) in petroleum ether.

Column Chromatography

Unless otherwise specified, a 140 ml column (440 x 28 mm) was used for column chromatography. Absorbent materials used were silica gel 60-200 mesh, acid washed silica gel, alumina, and Sephadex LH-20.

Preparation of silica gel:

Silica gel was washed before the column was packed by stirring 500 g with a mixture of 500 ml concentrated hydro-

chloric acid and 500 ml distilled water. The suspension was allowed to stand overnight so that most of the silica gel could settle. The supernatent liquid was then decanted, and the solid phase was re-suspended in 1000 ml distilled water. When the solid had settled, the supernatent liquid was again decanted. This procedure was repeated until the solid had been washed with distilled water three times. The silica gel was then filtered by suction, and was washed with distilled water until the filtrate was neutral. It was then further washed with ca. 300 ml ethanol, then with ca. 300 ml distilled benzene. The purified silica gel was dried in an oven at 120°C for 24 hours.

An LKB fraction collector (type 3406B Turntable disc) was used for column chromatography requiring collection of many fractions.

Detection of Biological Activity

Czapek's test (57) was used for biological activity in the fractions isolated. The ingredients which make up Czapek's solution are:

Water (distilled)	1000 ml	KCL	0.5 g
$NaNO_3$	3.0 g	$FeSO_4,7H_2O$	0.01 g
K_2HPO_4	1.0 g	Sucrose	30.0 g
$MgSO_4,7H2O$	0.5 g	Agar	15.0 g

The solid ingredients were dissolved in the water, and the solution was boiled for five minutes. Upon cooling to 60°C, the solution was poured into disposable petri dishes with 4 divisions. The dishes were covered and the contents allowed to solidify.

Other containers which were economical and practical were ice cube trays with 18 sections, covered with Saran wrap. Before re-use, these trays were sterilized by flame.

Procedure for testing activity

When a fraction was to be tested for activity, a sample was spread over the surface of the agar, or a drop was placed at the centre. After the solvent had evaporated, spores of green mould (from mouldy white bread), Eurotiales aspergillus (61) were inocculated (with a bent needle for colonies, or a glass rod for spores) into the agar in the centre of the area covered by the sample being tested. The inocculated plate was then developed for varying periods (see below) at room temperature. The growth of the mould was assessed by macro visual methods, and the results were classified as:

Strongly positive: when the inocculated mould died

Positive: when the inocculated mould survived, but did not develop further

Active: when the mould grew on the agar but not

on the surface covered by the sample
under test

Negative: when the mould developed over the entire
surface of agar at the same rate as a
blank control

Strongly negative: when mould developed faster than on the
blank control, and increased growth rate
was easy to recognize visually.

. The plates were examined at 3, 7, and 15 day intervals.
The test was repeated at least twice if enough material was
available for testing. In each case a blank control was
prepared. Organic solvents used in the experiments were
also tested for activity (see Figs. 6 & 7). The mould used
for testing was grown on Czapek's medium, having been
isolated from mouldy bread.

Tests for Enzyme Activity

Biuret assays (58) were performed to check if the
activities of active fractions were due to enzyme activity.
The results were considered to be positive if the spectro-
photometer reading at 540 nm was 0.09 (absorbance) or above.
Further quantitative determination was considered
unnecessary.

Spectroscopic Measurements

Spectroscopic measurements were made using a Unicam S.P.

800-B or S.P. 800-2 spectrophotemeter with solutions in carbon tetrachloride.

Infrared spectra were taken (as CCl_4 solutions) using a Perkin-Elmer 599B spectrophotometer.

Proton nuclear magnetic measurements were made at 60 MHz using a Varian T-60A instrument, at 200 MHz using the Varian XL-200 instrument at McGill University, and at 400 MHz using the Bruker WH-400 instrument at the Regional High-Field Nuclear Magnetic Resonance Laboratory (University of Montreal). $CDCl_3$ solutions were used in all cases. Since the maximum available dispersion was essential for the interpretation of the spectra, only the 400 MHz proton data are reported in this thesis.

Typical operating parameters for the proton n.m.r. spectra taken the on Bruker WH-400 instrument were as follows:

Operating frequency: 400.00 MHz   Deuterium lock on solvent

Spectral width: 2000 - 4000 Hz   Pulse width: 6.5 usec (90°)

Data block size: 16 or 32 K   No. of transients: 24 - 64

Line broadening: 0.1 - 1.0 Hz   Quadrature detection

Typical operating parameters for the carbon-13 spectra taken on the Bruker WH-400 spectrometer were as follows:

Operating frequency: 100.48 MHz   Deuterium lock on solvent

Spectral width: 20,000 Hz   Pulse width: 10 - 15 usec

Data block size: 16 or 32 K   No of transients: 5100-72464

Line broadening: 1 - 2 Hz   Quadrature detection

Spectra were taken with the same sample (in 5 mm tubes) used for the proton spectra ($CDCl_3$ solvent).

Mass Spectra

Mass spectra were taken on the LKB-9000 instrument in the Mass Spectrometry Laboratory at McGill University. A heated direct inlet system was used, with an ion source temperature of approximately 290°C, and an accelerating potential of 70 e.v.

Miscellaneous

Melting points (uncorrected) were taken on a Gallenkamp melting point apparatus.

Removal of water from samples was achieved using a Virtus (Research Equipment, Gardiner, New York) freeze-drying apparatus.

A clinical centrifuge was used.

Identification of materials was based on Refs. 59 and 60.

3.2 Working Extracts

The components of prop. is were separated into seve al
fractions on the basis of fferent solubilities in various
organic solvents and water . The "working solutions" were
obtained by extraction in a Soxhlet apparatus, using a
charge of 15 - 20 g of propolis, and successive extractions
with 500 ml of hot solvent, in a sequence of increasing or
decreasing polarity.

Solvents employed were:

Ligroin (bp 60 - 70°C), benzene, chloroform, ethanol,
water used in this or the reverse sequence, i.e. starting
with either ligroin or water. Each step took about 24
hours. The resulting solutions were evaporated to dryness,
then re-dissolved in cold solvents. In general, the solids
obtained in this manner were not fully soluble in the
original (cold) solvent, so that a second solvent was
required to dissolve the insoluble material (see Flow Chart
#1, Fig. 1)

These procedures resulted in the preparation of ten
"working solutions" (see Flow Chart #1, Fig. 1). All of the
working solutions showed biological activity, biuret tests
were positive, and TLC showed that all of the extracts were
very complex mixtures. The dried extracts were weighed and
the following distribution was determined:

Ligroin extract: 21% by weight

Benzene extract: 9%   "

Chloroform extract: 42%   "

Ethanol extract: 18%   "

Water extract: 3%   "

Residue: 7%   "

Appearance of the working solutions:

Ligroin extract

The ligroin extract was a light yellow emulsion which, upon cooling, separated into two layers, the upper layer being a clear yellow liquid, and the lower layer being a yellow material of creamy consistency. The upper layer gave a negative biuret reaction, and TLC showed fewer than ten spots on pre-prepared silica plates.

Benzene extract

The benzene extract had a darker yellow appearance than the ligroin extract and, upon cooling, three layers separated. The top and middle layers were similar to the two layers of the ligroin extract (by TLC analysis). The residue (lowest layer) was a brown material, soluble in acetone.

Chloroform extract

The chloroform extract was a brown, sticky material which separated into two layers on cooling. The upper layer

had very strong anti-mould activity. TLC indicated more than 20 components.

Ethanol extract

The ethanol extract separated into two layers on cooling. The upper layer, which prevented mould growth, was a clear, reddish liquid, whereas the residue (lower layer) was a dark brown, sticky material. This material arrested mould growth during the first three days of development, but after this period, mould growth was promoted. TLC of both layers showed very complex mixtures.

Water extract

The water extract was a greyish liquid, which deposited a black precipitate after a couple of weeks. The extract had a pleasant smell, resembling that of vanillin. The black residue was soluble in an acetone-water mixture (60:40).

Residue

After completion of the extraction sequence, a greyish powder in which fibre and white waxy material could be detected, remained.

3.3 Separation of Components from Working Extracts

Samples from the working extracts were further investigated. Separation of the components of the extracts was

carried out by using (a) column chromatography, (b) agar coated TLC plates, (c) steam distillation followed by simple distillation, and (d) extraction and precipitation.

(a) Column Chromatography

Samples from all working extracts were subjected to column chromatography, on silica gel. The ligroin and benzene extracts gave poor separations when the collected eluate fractions were larger than 2 ml. One apparently pure, biologically active oily material was separated from the chloroform extract (Compound 5, see later). Column chromatography of the ethanol extract yielded several biologically active fractions, but each was shown (by TLC) to contain more than 10 compounds. Elution with different polar solvents yielded active fractions, suggesting that more than one compound was responsible for the biological activity. One apparently pure (by TLC) biologically active, crystalline material was separated from the water extract.

Detailed descriptions of the three column chromatography procedures which resulted in the separation of pure compounds are as follows:

Column chromatogram #1

Stationary phase: Silica gel, 60 – 200 mesh

Liquid phase: Benzene

Sample used: Chloroform sample from the chloroform extract, 2.5 ml (650 mg)

The flow rate was maintained between 16 - 20 drops/minute. Eluate fractions of 15 - 20 ml were collected. The colour of the eluant served as a guideline for the collection of fractions. The elution solvent ('initially benzene) was changed gradually to a mixture of higher polarity by adding carbon tetrachloride, chloroform, chloroform-methanol (2:1), chloroform-methanol (1:1), and finally methanol, in sequence.

Altogether nine fractions were collected. Fractions 2 and 3 gave positive Czapek's tests. Only these two fractions gave spots with Rf values of 53 and 67% on TLC plates. These spots appeared to be responsible for the activity. Fractions 2, 3 and 4 were further separated by column chromatography (Column Chromatogram #2).

Column Chromatogram #2

Stationary phase: Silica gel 60-200 mesh

Liquid phase: Chloroform

Sample used: Combined fractions 2, 3 and 4 from Column Chromatogram #1 in benzene

Flow rate: 15-16 drops/minute

Volumes collected: 10 ml

Elution solvents:

Carbon tetrachloride: fractions 1-4

Benzene: fractions 5-10

Benzene/chloroform (1:1): fractions 11-17

Chloroform: fractions 18-20

TLC analysis of the fractions showed that #5, the first benzene fraction, was apparently pure. In four different TLC elution liquids only one spot, with an Rf value of 60%, was consistently observed. Fraction 2 had a spot with a similar Rf value, but it was accompanied by a weak overlapping second spot. All of the other fractions showed two or more spots on TLC plates. Czapek's test confirmed that the fraction, #5, which contained the compound with an Rf value of 60%, was biologically active.

Fraction 5 was evaporated to yield about 1.0 ml of a yellow oil. The identification of this material, designated as Compound 5, is described later.

Column Chromatogram #3

    Stationary phase: Silica gel 60-200 mesh

    Liquid phase: Cyclohexane

    Sample used: Water extract, in ethanol solution

Thirty-four fractions were collected. The first six fractions contained only a single compound; the subsequent fractions were more complex. Czapek's tests were negative, except for the first six fractions, which were strongly positive. On evaporation of the solvent from fractions 1-6, a crystalline solid which decomposed at 219°C, was obtained. The remaining material was insufficient for further investigation.

Further column chromatography.

In order to obtain better resolution of the working
solutions, a fraction collector was used to obtain 400-500 2
ml fractions from each column. This method showed that each
working extract contained more than one active compound, but
the quantities remaining after Czapek's tests had been
carried out were insufficient for further identification.
TLC, in conjunction with Czapek's test, showed that there
was more than one active component in each working extract.

(b) Agar-coated TLC plates

Agar-coated preparative TLC plates, a modification of a
method developed by Metzner (16), were used for an alterna-
tive method of separation. Glass plates (200 x 200 mm) were
coated with a 0.5 mm layer of silica gel D-5. The plates
were activated for one hour at 120°C. In each case, two id-
entical plates were prepared with samples from active frac-
tions, and were developed. A 2 cm column at the left side of
the plates was spread with 2', 7'-dichlorofluorescein re-
agent (prepared as described above, p.17) in order that
separation of the components could be detected. One of the
two identical plates was immersed in warm Czapek's medium to
coat the developed surface of the plate. After the Czapek's
medium had solidified, it was sprayed evenly with spores of
green mould. To prevent the plates from drying out, they
were covered with moist filter paper and were kept at room
temperature for mould development. After three days, the

growing mould showed a distinguishable pattern. Between 90
and 92% Rf values a clear stripe, where no mould grew,
remained on the plate. The same stripe was scraped off the
other TLC plate, and 2 mg of yellowish-white powder was
recovered. TLC showed that two compounds were present. No
further identification was carried out because of the small
quantity available.

(c) Distillation

Additional separation methods employed were steam dis-
tillation and fractional distillation. A non-volatile oily
material with an Rf value similar to that of the pure
compound separated by column chromatography (from the
chloroform extract, Column Chromatogram #2, p.   ) was
obtained by these methods. A further three apparently pure
compounds separated by steam distillation showed strongly
negative biological activity. Gas chromatography showed
that each of these fractions contained a single component.
Insufficient materials were available for further investi-
gation.

(d) Extraction and Precipitation

Ligroin Extract (Flow Chart #1, Fig. 1)

On standing, the ligroin extract separated into two
layers, the upper being a clear yellow liquid, and the lower
a yellowish-white sediment. Both layers showed anti-mould
activity. When the two layers had been separated and the

solvent removed from the liquid layer, the resulting
sediment was extracted with acetone, leaving a white
residue. On centrifugation of the upper layer, a white,
flaky wax sedimented. This was washed six times with
acetone, then extracted with chloroform and cyclohexane.
The chloroform-soluble material gave a positive Czapek's
test. The chloroform-insoluble but cyclohexane-soluble
material also gave a positive Czapek's test. The original
white solid gave two or three spots on TLC, with a bluish
ring around the spots. On exposure to air, the chloroform-
soluble material became yellow and lost its ability to
dissolve in chloroform.

Extraction of the sediment (i.e. the lower layer) from
the ligroin extract was next performed stepwise at room
temperature by shaking the solid material with solvents of
differing polarity, using hexane, cyclohexane, benzene,
carbon tetrachloride, chloroform, ethanol and acetone. The
solvent was removed from the extracts by using a stream of
nitrogen gas, and the resulting solids were dissolved in the
original solvents to similar concentrations and were tested
by TLC and on Czapek's medium. The cyclohexane and the
carbon tetrachloride extracts were biologically active.
When the extraction procedure was started with more highly
polar solvents such as acetone, ethanol, and chloroform, the
chloroform extract was the most active fraction. TLC of
this fraction showed five or six travelling spots, and some

material which did not move.

In order to obtain an extract with a smaller variety of components, extraction with cold solvents was carried out (Flow Chart #2, Fig. 2). 'Hungarian propolis was extracted by shaking for three minutes with cold hexane. The extract was filtered and the solvent removed with a stream of nitrogen. The resulting yellow solid was partially soluble in acetone, the insoluble material being separated by centrifugation. Removal of the solvent from the supernatent liquid (nitrogen gas stream) yielded an oily material. TLC showed seven spots in three groups. The Czapek's test showed strong biological activity.

The acetone-insoluble material (m.p. 68-70°C) was soluble in carbon tetrachloride and was biologically active. On TLC it showed one spot which did not move, and two diffuse spots at the solvent front. On addition of ethanol to the carbon tetrachloride solution a biologically active, white precipitate formed; this showed only an unmoved spot on TLC. This precipitate was dissolved in carbon tetrachloride, and methanol was added to precipitate a white, waxy material, which was partially soluble on addition of chloroform. White, waxy material (m.p. 64-65°C), designated as Compound #1, was recovered by centrifugation. This gave a positive Czapek's test and a single spot on TLC. Elemental analysis was performed on this hexane-soluble wax

(see later for details, p. 47)

. The left-over supernatent liquid was concentrated, but
no further precipitation occurred. When cyclohexane was
added dropwise a biologically active precipitate, m.p.
63-64°C, formed. The remaining supernatent liquid was
cooled to 4°C, yielding further biologically active preci-
pitate, m.p. 63-64°C, apparently pure by TLC. Elemental
analysis was carried out but the sample was later found to
be impure.

The procedures described above used polarity, concentra-
tion and temperature differences to separate waxy materials
with different melting points and Rf values. Further
separation of wax esters from propolis was carried out by
using the working extracts (see earlier) and solvents of
differing polarity (see Flow Chart #3, Fig. 3). Each
extract fraction was tested using Czapek's medium, and
showed strongly positive activity.

Hexane #1 Extract

The supernatent liquid from the hexane #1 extract was
evaporated, and the resulting solid was dissoled in benzene
(see Flow Chart #4, Fig. 4). As the hexane extraction (by
shaking at room temperature) proceeded, the amount of
material extracted with successive extractions decreased,
and the colour of the solutions changed from white to gray-

ish-yellow. The hexane fractions extracted were labelled as Hex #x (Flow Chart #4, Fig. 4), and were stored in a desiccator at room temperature. Each extract was tested on preparative TLC plates; none was found to be pure. Each fraction showed spots not present in other fractions. Czapek's tests have positive results, and the resulting solution when no further precipitation by polar solvents was possible gave a strongly positive test.

The benzene and the chloroform extracts were processed with similar procedures, and different wax esters were obtained (Flow Chart #5, Fig. 5). The ethanol working extract did not lend itself to good separation using extraction procedures.

Further procedures

Two samples obtained from the hexane #1 extract (Precipitates Hex #1 and Hex #3) weighed more than 3 g., and were selected for further separation and purification. The method described by Kaufman (55) was tried, using preparative TLC plates. Kieselguhr plates impregnated with dodecane, as described by Kaufman, did not prove to be superior to plain Kieselguhr plates activated at 120°C., using a developing mixture of trichloroethylene-chloroform (3:1). The Hex #1 sample separated into five layers, plus one immobile layer on preparative plates. Each layer was further purified, in successive stages, on preparative

plates. Czapek's test showed that each fraction had lost its biological activity. Under microscopic examination, the materials were seen to be crystalline.

The layer which failed to move was recovered and tested; it had strong biological activity. The spots which were present on TLC plates were separated on preparative plates, but the recovered samples were in the microgram range, making further investigation impossible.

The Hex #3 sample was also separated on preparative plates, and yielded three pure compounds. These had identical infrared and n.m.r. spectra, but different melting points. Further identification was not possible.

Steam distillation

Four approximately equal fractions were collected on steam distillation of propolis. Each of these fractions was then subjected to fractional distillation, water was removed from the subsequent fractions, and the residues were injected into a gas chromatograph, yielding a single peak in three cases. Identification of these compounds (which had different retention times) was not pursued.

An oily material (#33) failed to distill below 120°C.

Summary

The differing separation methods used each resulted in
the isolation of pure compounds. Of these, nine compounds
were selected for further identification:

From column chromatography: #5 oily material
From distillation: #33 oily material
From extraction: Hex #1, Chlo, #1/1, 1/2,
                               1/3, 1/4, and 1/5

3.4   Identification of the isolated compounds

Since only small quantities of materials were isolated,
degradation and preparation of derivatives were not
practical methods for their identification. Limited
chemical tests for functional groups could be carried out,
e.g. tests for unsaturation by bromination in carbon tetra-
chloride solution, for halogens by reaction with silver
nitrate and by the Beilstein test, for nitrogen by sodium
fusion, and for carbonyl groups by reaction with 2,4-dini-
trophenylhydrazine. In general, spectroscopic methods (mass
spectrometry (MS), infrared spectroscopy (IR), and proton
and carbon-13 nuclear magnetic resonance (NMR) spectroscopy,
were employed. In one case, Compound 1, elemental analysis
was carried out but, because of the high molecular weights,
elemental analysis was not considered a sufficiently sensit-
ive technique, particularly as mass spectra were available.

Chemical Tests

All of the compounds isolated gave negative chemical
tests for halogen, nitrogen, and carbonyl groups. Tests
for unsaturation were inconclusive, presumably because of
the high molecular weights of the compounds.

Infrared Spectra

Infrared spectra were obtained for all of the isolated
compounds, with the exception of #1/1. The presence of an
ester function was indicated by the characteristic strong
carbonyl bands in the 1730 cm^{-1} region, and by two $C=O$
absorptions in the 1300-1050 cm^{-1} region (Fig. 12). In
the case of compound 5 a more detailed identification based
on the infrared spectrum was possible. For this compound,
the presence of an aromatic ring was indicated by the band
at 1575-1600 cm^{-1} (C=C stretch). A broad band in the
fingerprint region of the spectrum (750 cm^{-1}) indicated
a disubstituted aromatic ring system, and the characteristic
phthalate C=O frequency (1280 cm^{-1}) was observed (Fig.
13).

Nuclear Magnetic Resonance Spectra

Because of the high molecular weights of most of the
compounds isolated, and the lack of functional groups to
induce chemical shifts, low field (i.e. conventional) NMR
spectra revealed little detail (Figs. 14 & 15). In order to
obtain as much information as possible from the spectra, the

Bruker WH-400 spectrometer at the Regional High Field
Nuclear Resonance Laboratory (located at the University of
Montreal) was employed. Some proton NMR spectra were also
taken using the Varian XL-200 spectometer (200 MHz) located
at McGill University. These spectra lacked sufficient dis-
persion to be very useful.

The operating frequencies employed for the spectra
reported were:

Proton spectra   - 400.00 MHz

Carbon-13 spectra   - 100.48 MHz

$CDCl_3$ solutions were used.

Mass Spectrometry

Mass spectrometry gave convincing proof of the presence
of ester functional groups in the isolated compounds, and
also helped to establish their molecular weights. The mass
spectra of esters in which the alcohol moiety is C_2 or
greater are usually more complicated than the spectra of the
methyl esters, because the alkoxyl group may participate in
additional fragmentation and rearrangements. Rearrangements
are particularly important in the mass spectra of esters:
frequent rearrangements involving one- or two-electron
transfers give rise to the carboxylic acid cation and the
analogous protonated species, respectively.

$$(RCOOR')^{\overset{+}{\bullet}} \longrightarrow (RCOOH)^{\overset{+}{\bullet}} + (R'-H)$$

$$(RCOOR')^{\overset{+}{\bullet}} \longrightarrow (RCOOH_2)^{+} + (R'-2H)$$
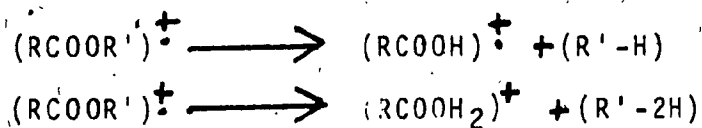
Fairly abundant rearrangement ions at m/z 60 and m/z 61 are observed in the spectra of C_2 (or higher) esters of butyric acid or higher acids (54). The fragmentation mechanism suggested for their formation is as follows:

1H Transfer   2H Transfer

m/z 60   m/z 61
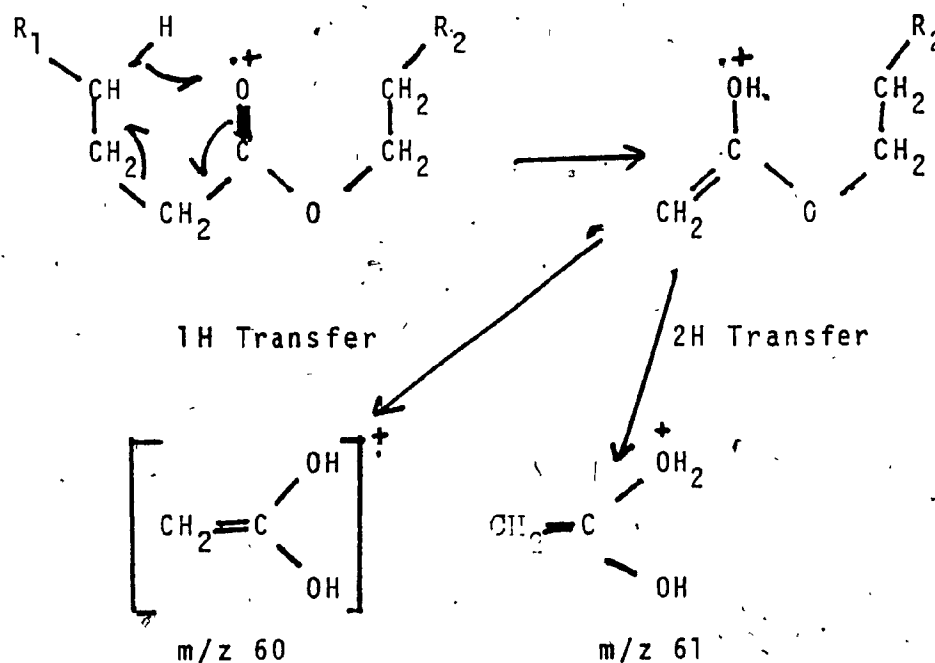
Cleavage alpha to the oxygen of the alcohol moiety followed by elimination of neutral formaldehyde may lead to additional rearrangement ions in the ester mass spectra. (54)
Interpretation of fragmentation peaks from alkyl chains follow the rules established for hydrocarbons (54). The mass spectra of normal hydrocarbons exhibit clusters of peaks with the formula (C_nH_{2n-1}), with the peak intensity decreasing as m/z for the fragment increases. Other

prominent peaks of composition (C_nH_{2n-1}), increase in intensity relative to the (C_nH_{2n-1}) peaks, at lower values of m/z. Chain branching causes a sharp break in the trend established for the normal paraffins owing to the better stabilization of a secondary carbonium ion. Monomethyl alkanes under electron impact fragment by cleavage on either side of the methyl branch to give two ions with odd m/z values, (C_nH_{2n-1}). Subsequent loss of a hydrogen atom from these two fragment ions gives rise to the analogous even m/z ions, (C_nH_{2n}). The ratio of relative abundances (odd m/z)/(even m/z) appears to be a function of chain length and of any additional branching present. For fragment ions containing 7 to 18 carbon atoms, the ion peak of even mass has a higher relative abundance than the odd mass peak; the situation is reversed for fragment ions with more than 18 carbon atoms. The presence of a second methyl branch leads to an odd mass peak with a higher relative abundance than the even mass peak (54).

In unsaturated compounds, hydrogen rearrangements are prevalent, resulting in the migration of radical sites along the chain. Thus, mono-olefins which differ only with respect to the position of the double bond generally have very similar mass spectra. The spectra are dominated by peaks corresponding to (C_nH_{2n-1}) fragments, with peak intensity decreasing with increasing mass.

In addition to peaks of odd mass, fairly abundant peaks corresponding to C_nH_{2n} were observed. These must have been formed by elimination of an olefin (53, 54).

Compounds Isolated

Seven compounds were separated and subsequently purified by thin-layer chromatography (TLC). Five of these compounds (1/1 - 1/5) were extracted from the hexane solution. Their Rf values decreased from 1/1 to 1/5, and their melting points varied from 55 to 70°C. Two other compounds, ident-ified as #1 and #Chlo, were purified from a hexane solution, and were similar in appearance and physical/chemical prop-erties. The crystalline form of these compounds was ob-served using a low power microscope.

Compound #5

This compound was obtained from the benzene extract by chromatography on silica gel (p. 28).

Infrared Spectrum

The infrared spectrum (CCl_4 solution, Fig. 13) shows a strong band typical of an ester carbonyl group at 1725 cm^{-1}, together with a C-O stretching band of as ester near 1270 cm^{-1}.

Carbon-13 Spectrum

The broad-band proton-decoupled spectrum contains 13 re-

solved signals, of varying intensities (Fig. 16). Four

signals are shifted substantially downfield (below 120 ppm),

typical of aryl and carbonyl carbon atoms, whereas the other

signals appear in the range 10-70 ppm, typical of aliphatic

carbon atoms.

The chemical shift of the lowest field signal (166.83

ppm) is consistent with a carbonyl group, most probably in

an acidic or ester function (59, 60). The three signals at

higher field are consistent with the presence of an ortho

di-substituted benzene (symmetrically substituted). Two of

these signals have intensities characteristic of carbon

atoms carrying protons (128.04 and 129.96 ppm), whereas the

remaining signal (132.02 ppm) has the low intensity charac-

teristic of quaternary carbon atoms. These signals are well

resolved, and the presence of only three signals in the

aromatic region strongly suggests an ortho substitution

pattern with identical, or very similar, substituents.

A signal at 67.42 ppm suggests the presence of a carbon

atom carrying an oxygen function (59, 60). The combination

of this signal and the signal at 166.83 ppm suggests the

presence of an ester group.

The remaining seven signals, in the 10-38 ppm region,

are characteristic of two methyl groups (12.91 and 9.99

ppm), a beta methihe carbon atom (38.17 ppm) and four

methylene carbon atoms. (29.70, 28.15, 23.11, and 22.02 ppm) (59, 60).

Thus the carbon-13 spectrum is consistent with an ortho phthalate ester, with identical alcohol moieties having branched structures with two methyl groups.

The solvent (CDCl$_3$) signal appears (as a triplet) at 76.17 ppm.

Proton NMR Spectrum

The proton NMR spectrum (CDCI$_3$ solution) (Fig. 17) shows a characteristic AA'BB' (4H) multiplet, typical of the aromatic protons of an ortho di-subsituted benzene, with the A and the B components centred at 7.5 and 7.7 ppm, respectively. A complex multiplet (4H) centred at 4.23 pp, has a chemical shift typical of protons on a carbon atom adjacent to oxygen. The complexity of this multiplet indicates that it arises from non-equivalent methylene protons, suggesting the presence of a chiral centre. Thus the substituents on the aromatic system must be branched so as to contain an a-symmetric carbon atom. This finding is consistent with the carbon-13 NMR spectrum, in which two non-equivalent methyl signals, at 12.91 and 9.90 ppm, appear. The methyl proton signals near 0.9 ppm, which integrate for 12 protons, are not fully resolved, but clearly indicate the presence of two tyes of methyl groups. A methine proton signal at 1.7 ppm

has the same integral as each of the aryl proton signals
(two protons), indicating that there is one methine proton
on each alcohol moeity. The remaining signals in the proton
spectrum appear (near 1.3 ppm) as complex multiplets due to
methylene protons, with integrals corresponding to 16 or 18
protons. The overall integral suggests that each alcohol
moeity contains 15 to 19 protons, corresponding to 7 to 9
carbon atoms. This analysis is consistent with the carbon-
13 spectrum, which shows 8 resolved carbon signals for the
alcohol moiety.

Mass Spectrum

The mass spectrum (Fig. 18) showed the base peak at m/z
149, characteristic of phthalates, with abundant peaks at
m/z 167 and m/z 279. A peak due to the molecular ion could
not be positively identified, but a weak peak at m/z 390 can
be considered as being probably due to the molecular ion,
based on NMR evidence.

These spectral data suggest that Compound 5 has the
following structures (Fig. 19):

Since iso-octyl phthalates of these types are employed
extensively as plasticizers by the plastics industry, and
have become widely distributed, no further attempt was made
to elucidate the structure.

Compound #1

This compound was isolated from the hexane extract (p.33
, Flow Charts 3 and 4).

Infrared Spectrum

This compound has a simple infrared spectrum (CCl_4
solution) (Fig. 12) typical of a compound with long hydro-
carbon chains. The strongest bands (2850-2930 cm^{-1}) are
due to C-H stretching vibrations. A medium intensity band
at 1765 cm^{-1} may be attributed to an ester carbonyl
group.

Carbon-13 NMR Spectrum

The broad-band decoupled carbon-13 spectrum ($CDCl_3$
solution) (Fig. 20) exhibits 18 resolved carbon signals,
with most of the intensity concentrated into a group of
unresolved, or incompletely resolved, signals at 28-29 ppm.
The spectrum is consistent with an aliphatic ester struc-
ture. A relatively weak signal at 173.112 ppm has an
intensity corresponding to a carbon atom with no attached
proton, and a chemical shift indicating an ester carbonyl
carbon (59, 60). The next signal to higher field appears at
63.46 ppm, suggesting a carbon atom adjacent to oxygen; the
intensity indicates that the carbon atom has attached
protons. A signal at 33.50 ppm is attributed to the beta
carbon of the acid moiety of the ester (59, 60). These
findings are consistent with the interpretation of the mass

spectral data for this compound. Although two (at least)
methyl carbon signals at high field would be anticipated for
a long chain aliphatic ester, only one signal (at 13.07 ppm)
clearly attributable to a methyl group can be identified.
The chemical shifts of the remaining signals in the high
field region correspond to methylene carbon atoms. It seems
likely that the aliphatic chains of the acyl and the alcohol
moeities are sufficiently long that accidential chemical
shift equivalence of the methyl groups results in the
appearance of only one signal, even at the high operating
frequency (100.48 MHz) employed.

Proton NMR Spectrum

The proton NMR spectrum (Fig. 21) consists of a triplet
(2H, J = 6.7 Hz) at 4.06 ppm (CH_2O methylene protons with
a methylene neighbour), a triplet (2H, J = 7.5 Hz) at 2.29
ppm ($CH_2C=O$ with a methylene neighbour), and an unresolved
multiplet (4H) at 1.61 ppm (methylenes displaced to low
field by oxygen functions), an intense, unresolved methylene
envelope (78 - 80H) near 1.3 ppm, and a well resolved trip-
let at 0.88 ppm (J - 6.8 Hz), with an integrated intensity
corresponding to 6 protons, i.e. two methyl groups (with
methylene neighbours). The methyl proton signal is thus
consistent with the single signal observed in the carbon-13
spectrum (see above).

Mass Spectrum

The mass spectral data (Fig. 22) indicate a molecular weight of 732 daltons, and identify this material as C_{16} acid ester of a saturated C_{34} alcohol. The proposed molecular formula, based on the mass spectrum, is

$$CH_3-(CH_2)_{14}-CO_2-(CH_2)_{33}CH_3.$$
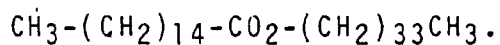
Elemental Analysis

Calculated for $C_{50}H_{100}O_2$: %C 81.96, %H   %O 4.38
13.66, Found: %C 82.17, %H 14.14, %O 3.69

Compound #1/1

All of the compounds in this series were isolated from the hexane extract (Flow Charts 3 and 4).

Proton NMR Spectrum

This compound exhibits a spectrum (Fig. 23) typical of an aliphatic hydrocarbon, with no signals at lower field than 1.5 ppm. The only well resolved signal is a methyl triplet (J = 6.8 Hz) at 0.88 ppm, indicating that the compound contains a terminal $-CH_2CH_3$ fragment. The remainder of the spectrum consists largely of a methylene envelope near 1.3 ppm. Thus, the spectrum is consistent with a linear alkane structure.

Mass Spectrum (Fig. 24)

On the basis of the observed molecular ion peak at m/z 436, and a fragmentation pattern consistent with a saturated aliphatic hydrocarbon, the proposed elemental formula is $C_{31}H_{64}$. The mass spectrum further suggests that mono-methyl branching occurs at C-4.

Compound #1/2

Proton NMR Spectrum

The spectrum of this compound differs in only minor respects from that of Compound #1, the general appearance of the spectra being the same, and the chemical shifts of the resolved signals identical (Fig. 25). The only differences are in the region of the methylene envelope, and it is not clear that they are significant.

If the highest m/z peak in the mass spectrum (m/z 676) (Fig. 26) is assumed to be the molecular ion, then the base peak at m/z 257 can be rationalized in terms of a double McLafferty rearrangement from a C_{30} ester of palmitic acid. Other peaks in the mass spectrum are consistent with a regular sequence of two-carbon fragment eliminations.

Hence this compound appears to be an ester of a long chain carboxylic acid and a linear alcohol.

Compound #1/3

.This compound was recovered from the third band of the
preparative TLC, and was identified as an ester by its
infrared and proton NMR spectra. The mass spectra (Fig. 27)
showed that this material is a mixture of 1/2 and an unknown
substance. If the highest peak in the mass spectrum (m/z
730) is taken as being due to the molecular ion of the
impurity, and the spectrum is compared with that obtained
from #1/2, the impurity may be tentatively identified as an
unsaturated C_{34} ester of palmitic acid.

Compound #1/4

Proton NMR Spectrum

The spectrum of this compound (Fig. 28) has a strong
resemblance to those of #1 and #1/2, e.g. it contains a
methyl triplet at 0.88 ppm (J - 6.8 Hz), indicating a term-
inal ethyl group, a methylene triplet at 4.05 ppm
(J = 6.8 Hz), an envelope near 1.7 ppm due to methylene
and/or methine protons shifted to lower field, and an in-
tense methylene envelope near 1.3 ppm. However, the signal
near 2.3 ppm, which appears as a triplet in #1 and #1/2 and
is attributable to a methylene group adjacent to a carbonyl
group appears as four lines in this spectrum. The spectrum
contains resolved signals on the high field side of the main
methylene envelope which are absent from the spectra of the
other compounds. In additin, there is a quartet at 4.9 ppm,
suggestive of a vinyl proton. The intensity of this signal

is lower than that of the $-CH_2O-$ signal, suggesting a single vinylic proton.

The most probable structure indicated by the proton spectrum is that of a long chain ester of an unsaturated carboxylic acid. An alternative explanation that this sample consists of a mixture of a saturated long chain ester and some unsaturated material cannot be ruled out from proton spectral data.

Mass Spectrum

The mass spectrum (Fig. 29) in the region m/z 700-800 suggests that the molecular weight of 1/4 is greater than 800, but an exact value cannot be assigned. The mass spectrum displayed features common to the spectra of all of the long chain esters: abundant peaks at m/z 257 (McLafferty rearrangement) and m/z 236 (loss of water and hydrogen re-arrangement), and the peak at m/z 446 which can be ration-alized in terms of fission alpha to the oxygen of the alcohol moiety. These peaks are consistent with a C_{16} unsaturated ester. Variations in the mass spectra among the wax esters may result from differing sites of unsaturation and/or branching.

Compound #1/5

The mass spectrum (Fig. 30) of this compound showed that fragmentations due to the acid chain were similar to

those observed for 1/4. The identity of the alcohol moiety
is uncertain, and hence the molecular weight cannot be
estimated. The highest m/z peak in the mass spectrum is at
m/z 745, and the spacing of fragment ion peaks suggests loss
of methyl radical and olefinic groups.

Compound #Ch1o

This sample, isolated from the hexane solution (p.33)
proved to be a mixture, one component of which was identical
to #1/2. The impurity was identified by mass spectrometry
(Fig. 31) as a hydrocarbon of molecular weight 464 daltons,
having a single methyl branch at the end of the chain.

Compound #33

This compound was recovered by distillation of material
obtained by steam distillation. It was shown by mass spec-
tometry (Fig. 32) to be a mixture, one component of which
was a phthalate.

4. DISCUSSION

There is a high percentage of wax in propolis. In the early days, when research was mainly directed towards neutral products, Helfenberg and Dieterich (1, 9) and others determined the wax content of propolis. Since then some studies have appeared on the subject of beeswax (52), but no detailed research has been undertaken to separate and identify the complete array of wax esters present in bee glue (propolis).

The present work did not start out directly to study the ester constituents of propolis, but rather to separate any compounds which had not been identified previously, and were biologically active. The chromatographic method resulted in one pure compound with active anti-mildew characteristics (Compound 5). It was identified as a bis-isooctyl phthalate. Its isolation and identification raises the question ofwhether it is a genuine constituent of propolis or an artifact which contaminated the eluent during chromatography. A second phthalate mixture (#33) was isolated one year later by another method (steam distill-ation). The second phthalate has the same base peak at m/z 149 in its mass spectrum as the first phthalate, but it has a higher molecular weight. We may assume that the two compounds originated in propolis.

Separation of consituents by precipitation from the

working extracts proved to be successful, and provided a
framework for a more organized study. The wax content of
propolis was divided according to its solubility into four
fractions (hexane, benzene, chloroform, and ethanol); each
fraction was subsequently precipitated into subfractions.
The four main groups included almost the total wax content
of propolis. Each main group was biologically active. Sub-
fractions 9, 7, 6, and 4 of the hexane extract were then
separated and identified. It was only possible to carry out
a small section of the comprehensive plan; this resulted in
the isolation of five pure compounds. Six wax ester frac-
tions (#1, #Chlo, 1/2, 1/3, 1/4, 1/5) and one paraffin (1/1)
were isolated from the hexane subfraction.

A white crystalline material was isolated from the
water extract and was purified. It melted, with decomposi-
tion, at 194°C. Cizmarik and Matel (15) have described a
similar material recovered from an ether extract. It was
identified as 3,4-dihydroxycinnamic acid (caffeic acid)
(m.p. 195°C) by these authors. Too little material was
available to permit further investigation.

Steam distillation of propolis, followed by fractional
distillation and gas chromatography, resulted in the sepa-
ration of three compounds, which were not identified because
of the small quantities available. These compounds had the
interesting property that their biological-activity was

reversed. Instead of inhibiting mould formation, they pro-
moted growth three times faster than that of the control,
under the same conditions.

A search of the literature revealed that the compounds
described above have not been reported previously by other
workers who have isolated components of propolis. Thus,
this study has provided a further contribution to the under-
standing of the nature of the complex character of
propolis.

5. CONCLUSION

The objective of this study was to separate and
identify components of propolis which were not described in
the literature. Furthermore, separate compounds which also
show anti-mildew characteristics were looked for. The
present work resulted in the isolation of seven pure
compounds. Only one compound retained its biological
activity on purification. The bis-isooctyl phthalate
prevented the growth of moulds on agar agar. The biological
activity of the other separated compounds diminished as the
purification proceeded, to the point that the pure compounds
lost their activity entirely. It is not clear whether the
impure fractions were active biologically or whether a
synergistic effect was operating. Testing the removed
impurity showed only weak anti-mildew characteristics. No
further work could be undertaken to settle the question
which arises about the lost activity because of lack of
time.

It is evident from the work described that propolis is
a very complex mixture, and the present work is only a small
contribution to the many questions asked by people who are
acquainted with this natural product.

6.   SUGGESTIONS FOR FUTURE WORK

The diverse properties of propolis deserve a more systematic investigation. To complete the determination of the wax ester content of propolis, gas chromatography (analytical and preparative scale) would be a valuable technique. Hydrolysis of the isolated esters, followed by preparation of volatile derivatives and their identification by gas chromatography/mass spectrometry, would establish the structures of the esters.

It would be useful to establish the sources of biological activity in the crude wax extracts, since these materials might have useful properties, e.g. in food preservation.

REFERENCES

1. P.W. Philipp, Bidl, Zentralbl., 48, 705 (1928).

2. H. Kustenmacher, Ber. dt. pharm.ges.,, 21, 65-92 (1911).

3. K.D. Helfenberg, Chemikerzeitung, 31, 987-988 (1908).

4. G.F. Jaubert, C.r.hebd.Séanc.Acad.Sci.,184, 1134 (1927).

5. G.A. Rosch, Biol Zbl., 47 (2), 113 (1927).

6. G.H. Vansell; C.S. Bisson, U.S. Bur.Ent., E495 (1940).

7. K. Dieterich, Phar. Post., 40, 369 (1908).

8. R. Uskhalova, Phelovodstvo, 93 (6), 31 (1973).

9. K. Dieterich, Pharm. Ztg. 52, 1019-1027 (1911).

10. S.A. Popravko, et al., Chim. prirodnych Soedin, 5, 476 (1969).

11. S.A. Popravko, Rev. franc. Apicult.,315, 494 (1973).

12. P. Lavie, Annls, Abeille, 3, 103-183 (1960).

13. P. Lavie, C.r. Acad. Sci. Paris, 244 2653-2655 (1957).

14. P. Lavie, Bee World, 39, 323 (1958).

15. J. Cizmarik; I. Matel, Experientia, 26(4), 713 (1970).

16. J. Metzner, et al., Pharmazie, 30, 799 (1975).

17. E.M. Schneidweind, et al., Pharmazie, 30, 803 (1975).

18. E.L. Ghisalberti, et al., Experientia, 15, 157-158 (1978).

19. W. Heinen, et al., Port. Acta biol., Ser. A., 12, 65-76 (1972).

20. M.H. Haydak et al., J. econ. Ent.., 33, 396 (1941).

21. -----ibid., 34, 37-38 (1941).

22. -----ibid., 31, 576-77 (1938).

REFERENCES (Continued)

23. -----ibid., 35, 319 (1942).

24. A. Caillas, C.r. hebd Séanc. Acad. Sci., 177, 1145 (1923).

25. A. Nikiforov, Uchen, Zap. kazan. yet. Inst. 108, 180 (1971).

26. K. Janes; V. Bumba, Pharmazie, 29, 544-545 (1974).

27. S.A. Popravko, et al., 22 Int. Beekeep. Cong. Summ. 163-164 (1970).

28. V.P. Kivalkina, et al., Antibiotiki (Moscow), 18, 261, (1973).

29. L.A. Lindenfelser, Am. Bee, 107, 90 (1967).

30. R.M.V. Assumpcao, et al., Anais Acad. bras, Cienc., 40, 297 (1968).

31. S. Shibata, et al., Yakugaku Zasshi., 80, 620 (1960).

32. G. Di Maggio, et al., Archo. ital, Sci. farmac., 11, 191 (1961).

33. P. Niebes, et al., Angiologica, 8, 297 (1971).

34. R.C. Robbins, et al.,Clin. Chem., 17, 1109 (1971).

35. R.C. Robbins, J. clin. Pharmac., 1, 271 (1973).

36. V.R. Villanueva, et al., Annals Inst. Pasteur, 106, 292 (1964).

37. J.F. Thorpe; M.A. Whiteley, Thorpe's dictionary of applied chemistry 4th ed. London: Longmans, Green and Co.

   Vol. 5, 449 (1924).

38. V.F. Bolshakova, et al., Nauch Zap. Gor.

REFERENCES (continued)

Naucho Isled.Inst.Derm. Vener., 24, 148 (1964).

39. Pharmacuetical preparations for treatment of dermatol-
ogical diseases. Patent No.: 109511.

40. H.W. Dunnborg, Archives of Dermatology, 95, 381 (1967).

41. N.F. Chernyak, Antibiotiki, 18, 259 (1973).

42. A. Chvidchenko, Apiculteur, 94, 213 (1950).

43. V.P. Kivalkina, et al., 22 Int. Beekeep, Cong. Summ.
136 (1969).

44. N.N. Prokopovich, Vrach. Delo, 10, 1077 (1957).

45. -----ibid, 1, 41-44 (1956).

46. Z. Karimova, Pchelovodstva, 40, 36-37 (1963).

47. I.I. Teterev, Veterinariya (Moscow), 48, 41 (1972).

48. R.C. Robbins, Int. J. Vit. Nut. Res. 45, 163 (1975).

49. -----ibid, 46, 338 (1976).

50. M. Gonnet, Annls, Abeille, 11, 41 (1968).

51. A. Derevici, et al., Annls. Abeille, 7, 191 (1964).

52. A.P. Tulloch, Chem. Phys. Lipids, 6, 935 (1971).

53. P.E. Kolattukudy, "Chemistry and Biochemistry of Natur-
al waxes." American Elsevier Publishing Company, Inc.
New York (1976).

54. H. Budzikiewicz, C. Djerassi, D.H. Williams, "Mass
Spectrometri of Organic Compounds." Holden-Day, Inc.
San Francisco, California (1967).

55. H.P. Kaufmann and B. Das, Fette-Seifen-Anstrichmittel,
65(5), 398-402 (1963).

56. H.R. Bollinger, M. Brenner, H. Ganshirt, H.K. Mandold,

REFERENCES (Continued)

H. Seiler, E. Stahl and D. Waldi, "Thin-layer Chromatography" Edited by E. Stahl, Springer-Verlag, Berlin, Academic Press Inc. Publishers 1-11 Fifth Avenue, New York 1003 pg 475.

57. K.B. Raper and D.I. Fennel, "The genus Aspergillas.", Williams and Wilkins, Baltimore, Maryland. (1965).

58. O.H. Lowry et al., J. Biol. Chem. 193: 265 (1951).

59. J.B. Strothers, "Carbon-13 NMR Spectroscopy". Academic Press, Inc. 111 Fifth Avenue, New York, New York 10003 (1972).

60. F.W. Wehrli, and T. Wirthlin, "Interpretation of Carbon-13 NMR Spectra." Heyden & Sons Inc. 225 Park Avenue, New York, N.Y. 10017, U.S.A. (1976).

61. E.L. Ghisalberti: Propolis: A Review, Annual report of the International Bee Research Association (1979).

APPENDIX

LIST OF FIGURES   PAGE

63

Flow Chart # 1

3.2 Working extracts.

Propolis
|
Ligroin soxhlet

Residue   Ligroin extract
| |
| dissolved
| Acetone   Ligroin
|
Benzene soxhlet

Residue   Benzene extract
| |
| dissolved
| Chloroform   Benzene
|
Chloroform soxh.

Residue   Chloroform extract
| |
| dissolved
| Ethanol   Chloroform
|
Ethanol soxhlet

Residue   Ethanol extract
| |
| dissolved
| Acetone   Ethanol
|
Water soxhlet

Residue   Water extract
|
dissolved
Acetone-   Water
|
Water

Flow Chart #2

FIGURE 2.

3.3 Separation.

Propolis

| cold Hexane

Hexane extract

| dried

Yellow material

Acetone

Residue   Extract

CCl_4 | | dried

CCl_4 extract   Oily material

Ethanol

Ppte   Filtrate

CCl_4 |

CCl_4 extract

Methanol

Ppte   Filtrate

Chloroform |

Ppte   Filtrate
mp. 64-65 C. | concentrate
(#1)   Cyclohexane

Ppte   Filtrate
mp. 63-64 C.

| cooled

Ppte   Filtrate
mp. 63-64 C.
(# Chlo)

Flow Chart # 3

3.3 Separation.

Dry Ligroin Soxhlet

Hexane

Residue   Supernatent
                (Hexane # 1)

Benzene

Residue   Supernatent
                (Benzene # 2)

Chloroform

Residue   Supernatent
                (Chloroform # 3)

Ethanol

Residue   Supernatent
                (Ethanol # 4)

Flow Chart # 4

3.3 Separation .

Flow Chart # 5

3.3 Separation.

Benzene # 2 extract
|
evaporate
|
Yellow material
|
Cyclohexane
/ \
Filtrate   Ppte
/ \ |
Acetone   Chloroform+Benzene
/ \ |
Ppte   Filtrate   Solute
Be #1 |
                 Acetone
                 / \
            Ppte   Filtrate
            Be #2 |
                     concentrate
                     Acetone
                     / \
                Ppte   Filtrate
                Be #3 |
                         concentrate
                         cool
                         / \
                    Ppte   Filtrate
                    Be #4 |
                             Methanol
                             / \
                        Ppte   Filtrate
                        Be #5 |
                                 evaporate
                                 Ether
                                 |
                                 Solute
                                 |
                                 Methanol
                                 / \
                            Ppte   Filtrate
                            Be #6   Be #7

Ten day old mould culture on Czapek's medium

with organic solvents. 1: Ethanol; 2: Chloro-

form;   3: Benzene; 4: Control.
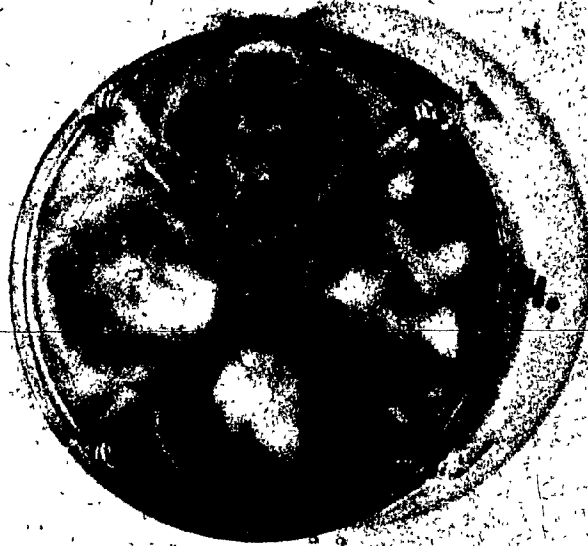
Ten day old mould culture on Czapek's medium

with organic solvents. 1: Ligroin;   2: Hexane;

3: Carbon tetrachloride;   4: Control.

Seven day old mould culture on Czapek's medium
with partially purified wax esters. 1: Hexane
precipitate; 2: Hexane precipitate; 3: Hexane
precipitate; 4: Control.
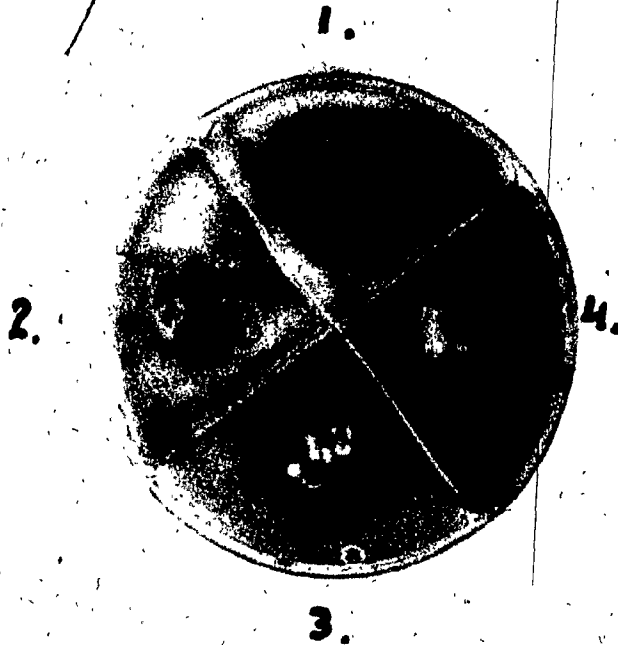
Three day old mould culture on Czapek's medium
with working solutions prepared from propolis.
1: Hexane extract;   2: Benzene extract: 3:
Chloroform extract; 4: Control.
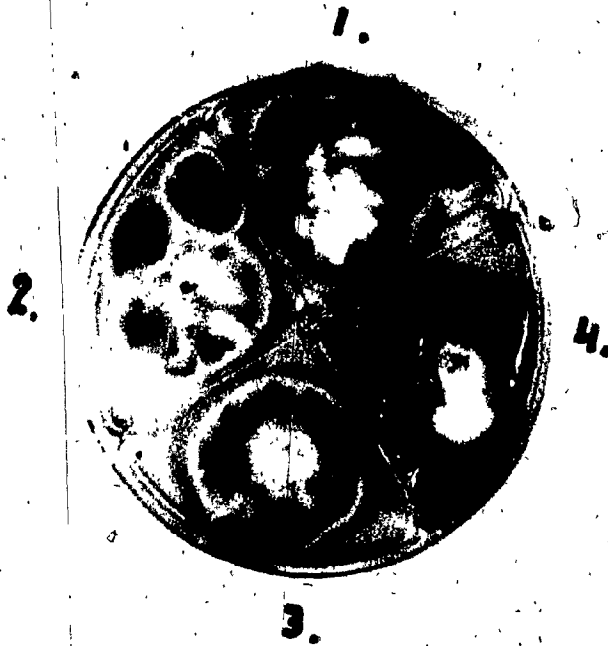
Three day old mould culture on Czapek's medium
with fractions of steam distillate.
1: Distillate fraction at 75-85 C; 2: Distillate
fraction at 86-95 C; 3: Distillate fraction at
96-106 C; 4: Control.

Ten day old mould culture on Czapek's medium
with isolated compounds. 1:, 2:, 3:, 9:,
10:, 11: Distillate fractions; 4: # 33 oily
material; 12: # 5 oil; 5: # 1/2 Wax ester;
6: # 1/4 Wax ester; 7: #1/5 Wax ester; 14:
1 Wax ester; 13: Unmoved layer on TLC plate
purifying Wax esters; 8:, 9: Controls.
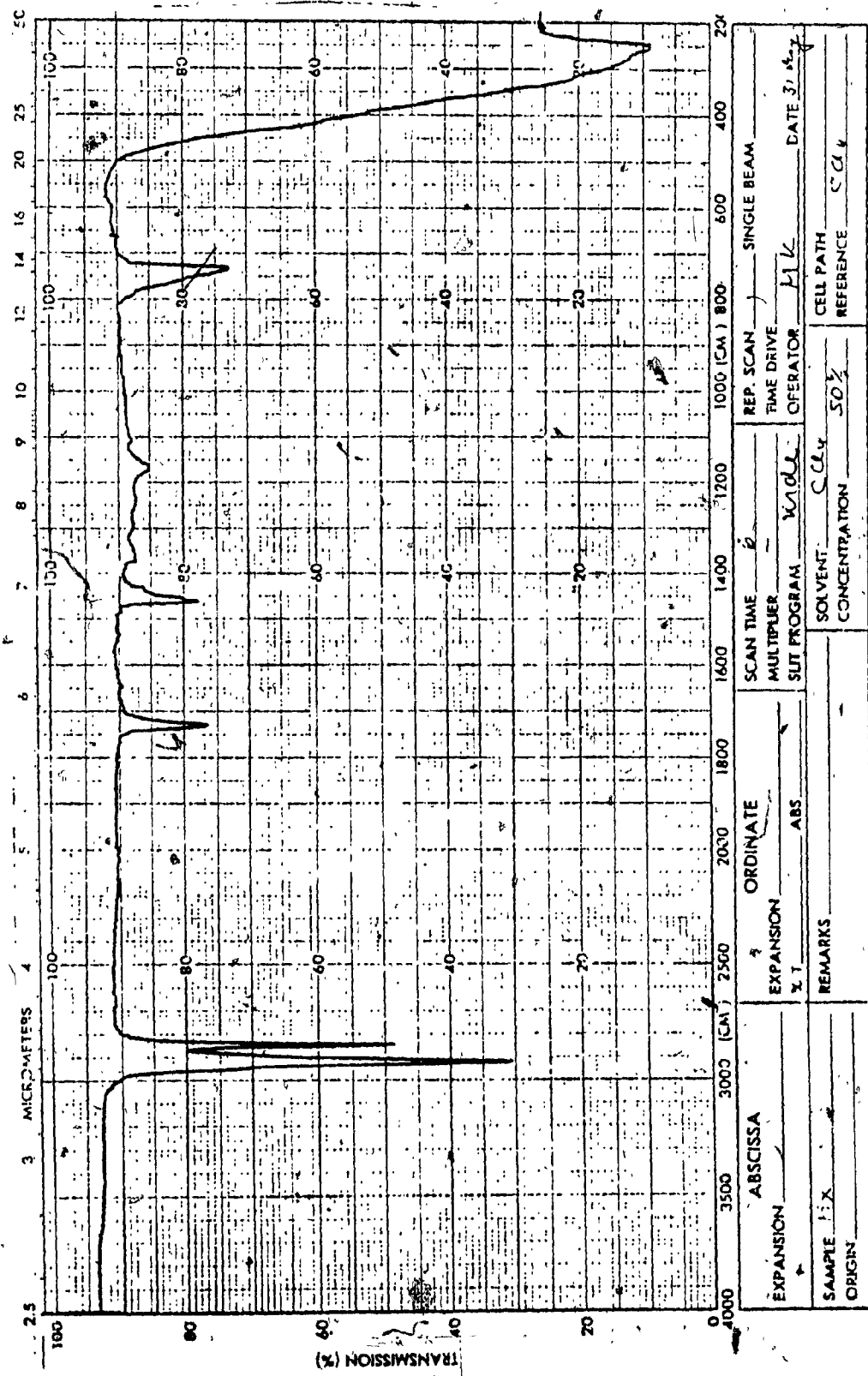
F I G U R E 12 . Infrared Spectrum of #1 compound, separated from Hexane extract.

Solvent: Carbon tetrachloride.
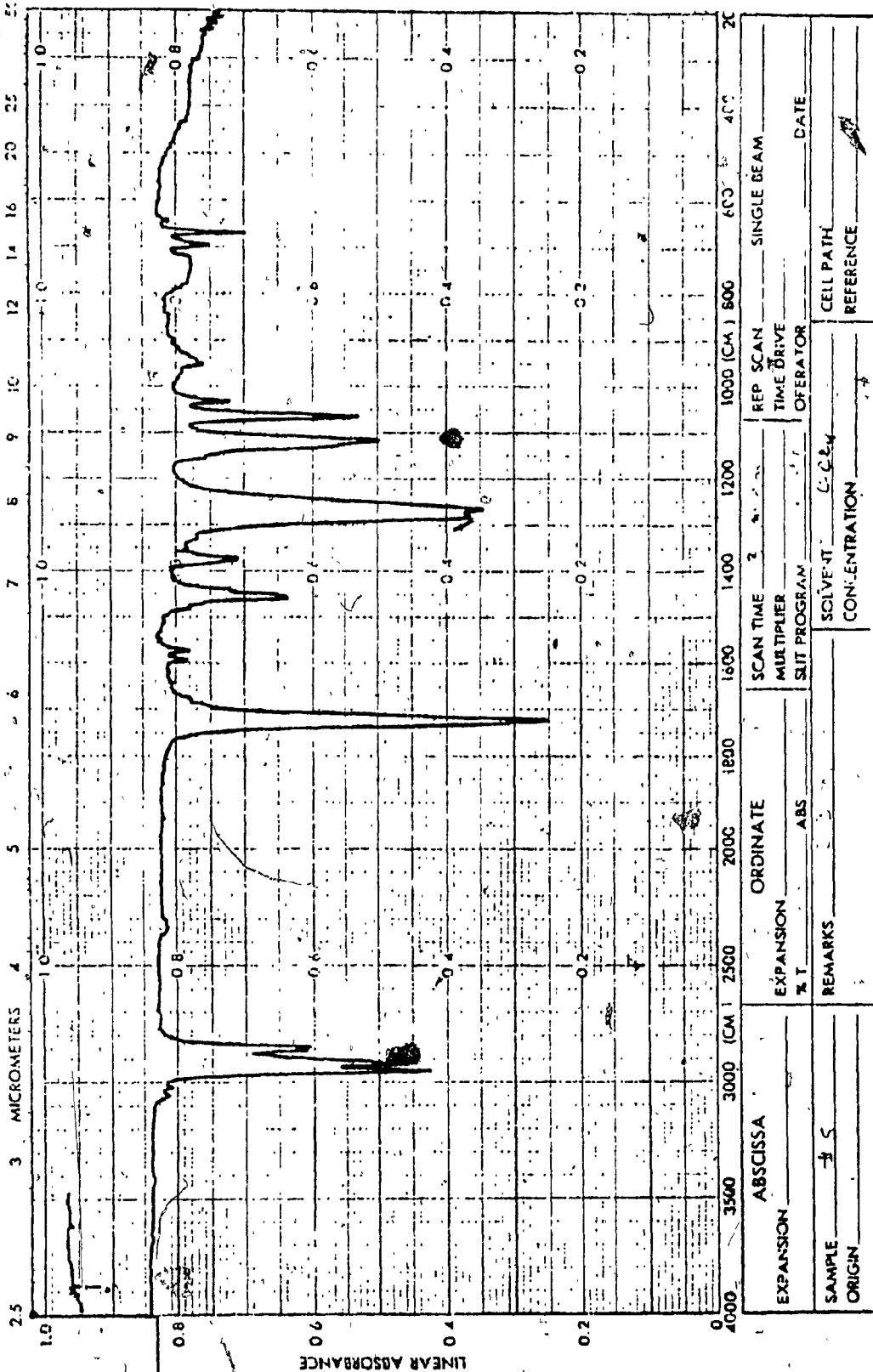
F I G U R E 13. Infrared Spectrum of # 5 compound, separated from benzene extract,

by column chromatography. Solvent: Carbon tetrachloride.
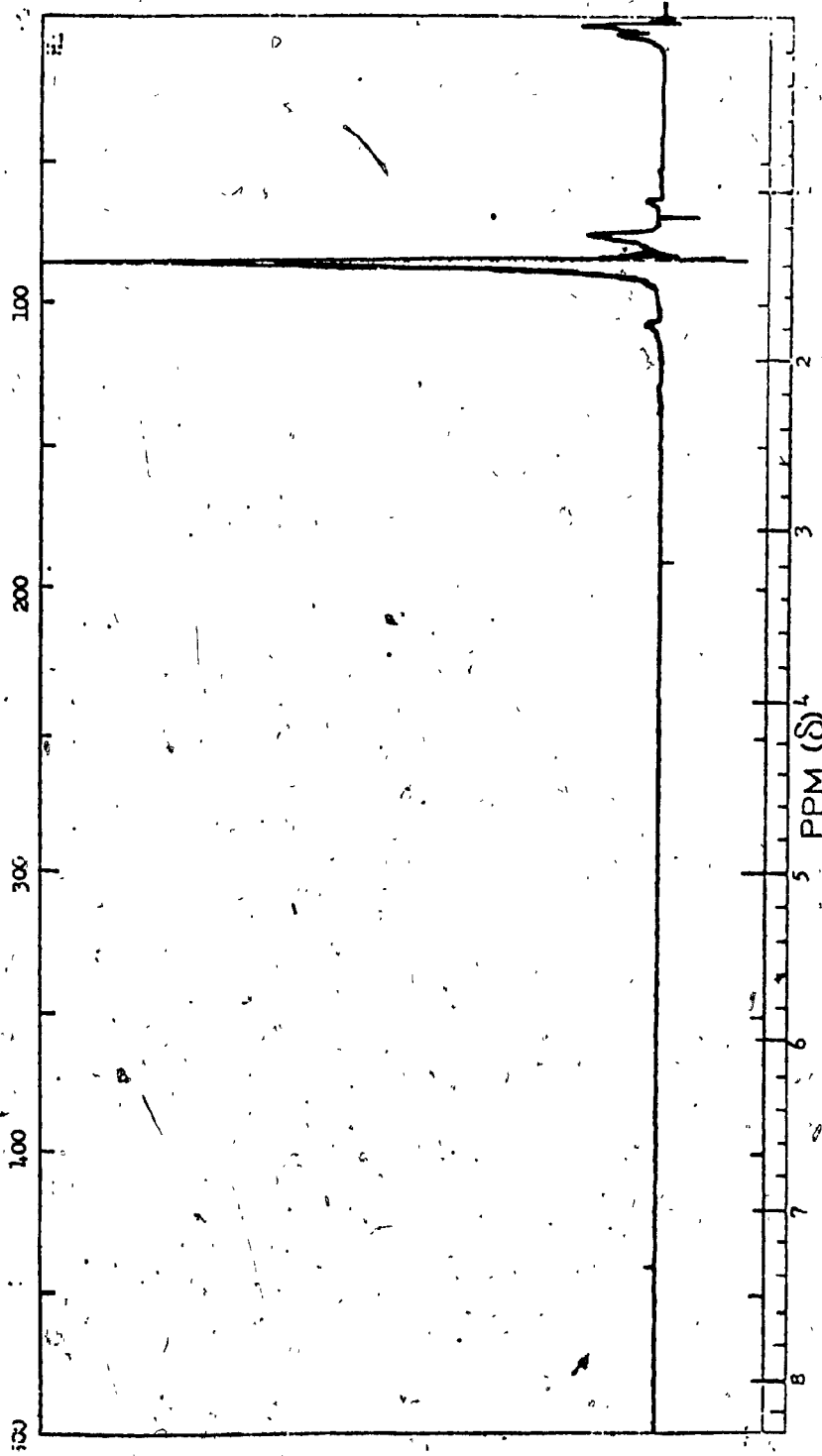
F I G U R E 14 : Nuclear Magnetic Resonance Spectrum of # 1 compound, separated from Hexane extract. Solvent: CCl_4 .

78
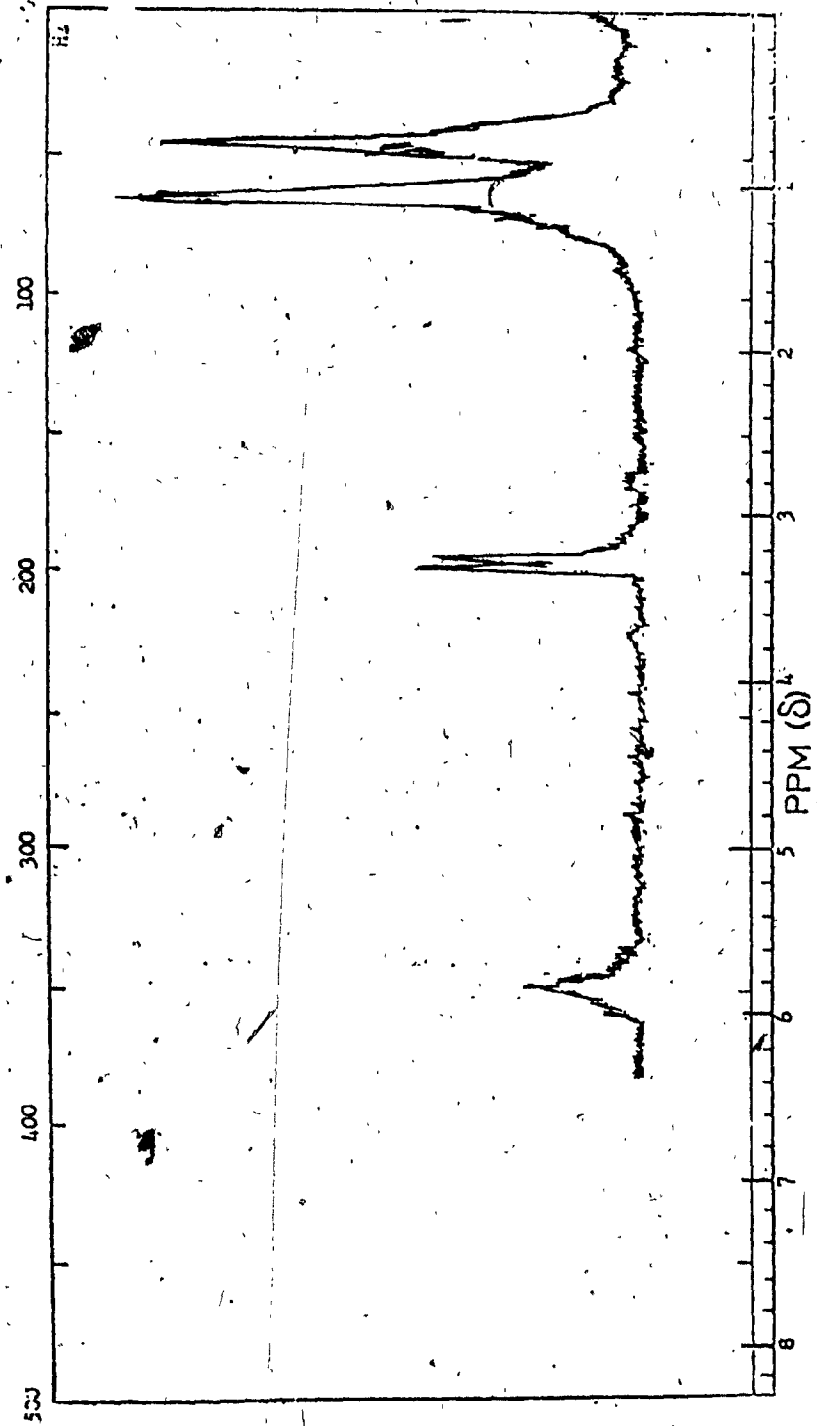
F I G U R E 15: Low field Nuclear Magnetic Resonance Spectrum of

5 compound, separated from Benzene extract.
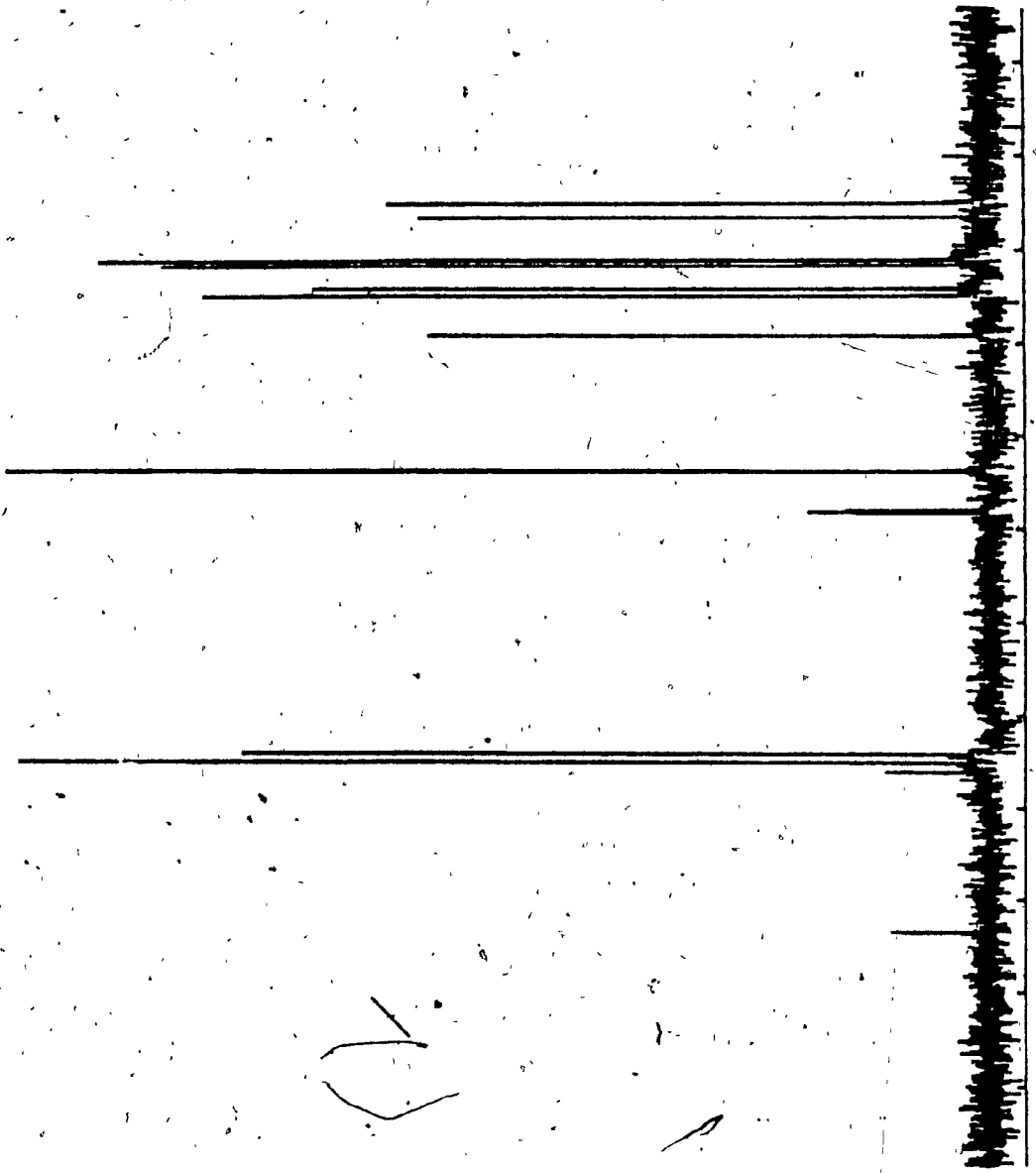
FIGURE 16 : Carbon-13 Spectrum of #5 compound, obtained from

Benzene extract by chromatography.
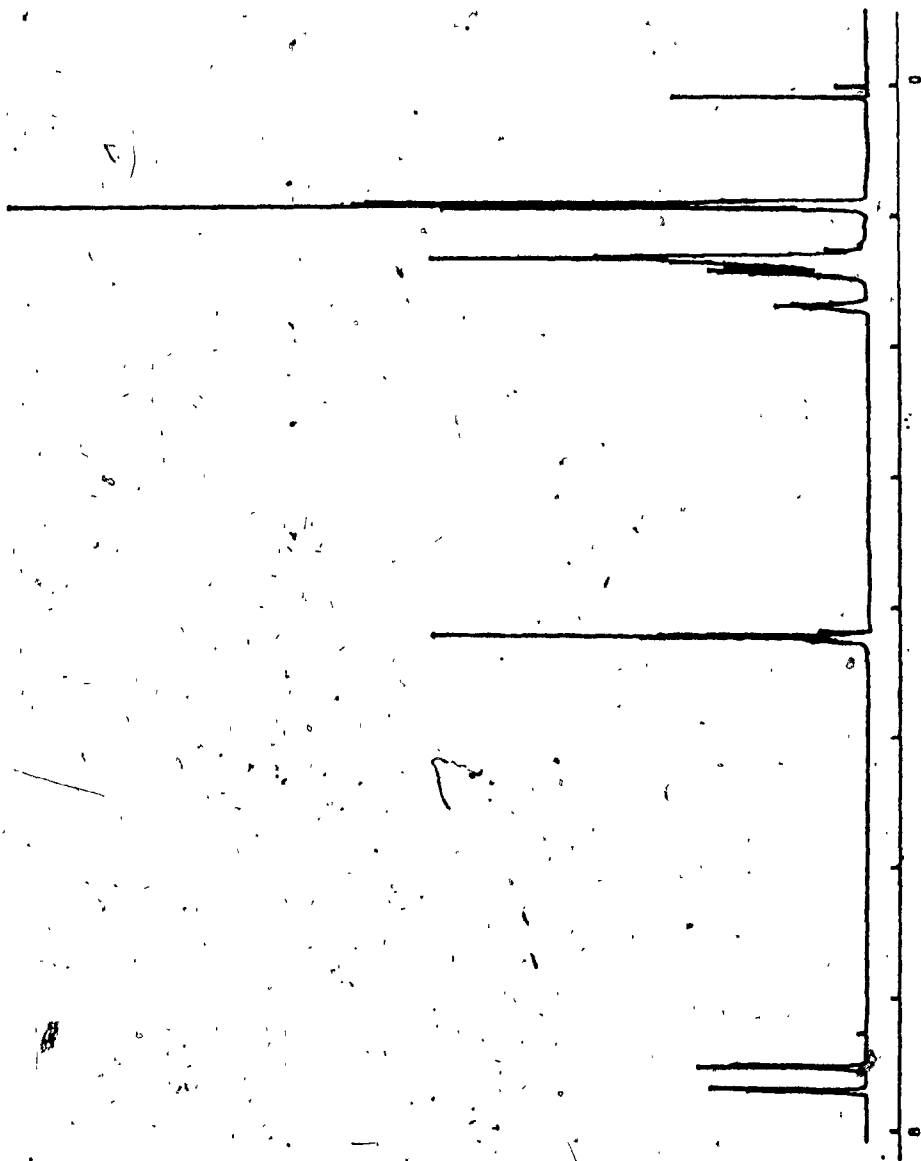
F I G U R E 17 : Proton Nuclear Magnetic Resonance Spectrum (400.00 MHz)

of # 5 compound, obtained from Benzene extract.
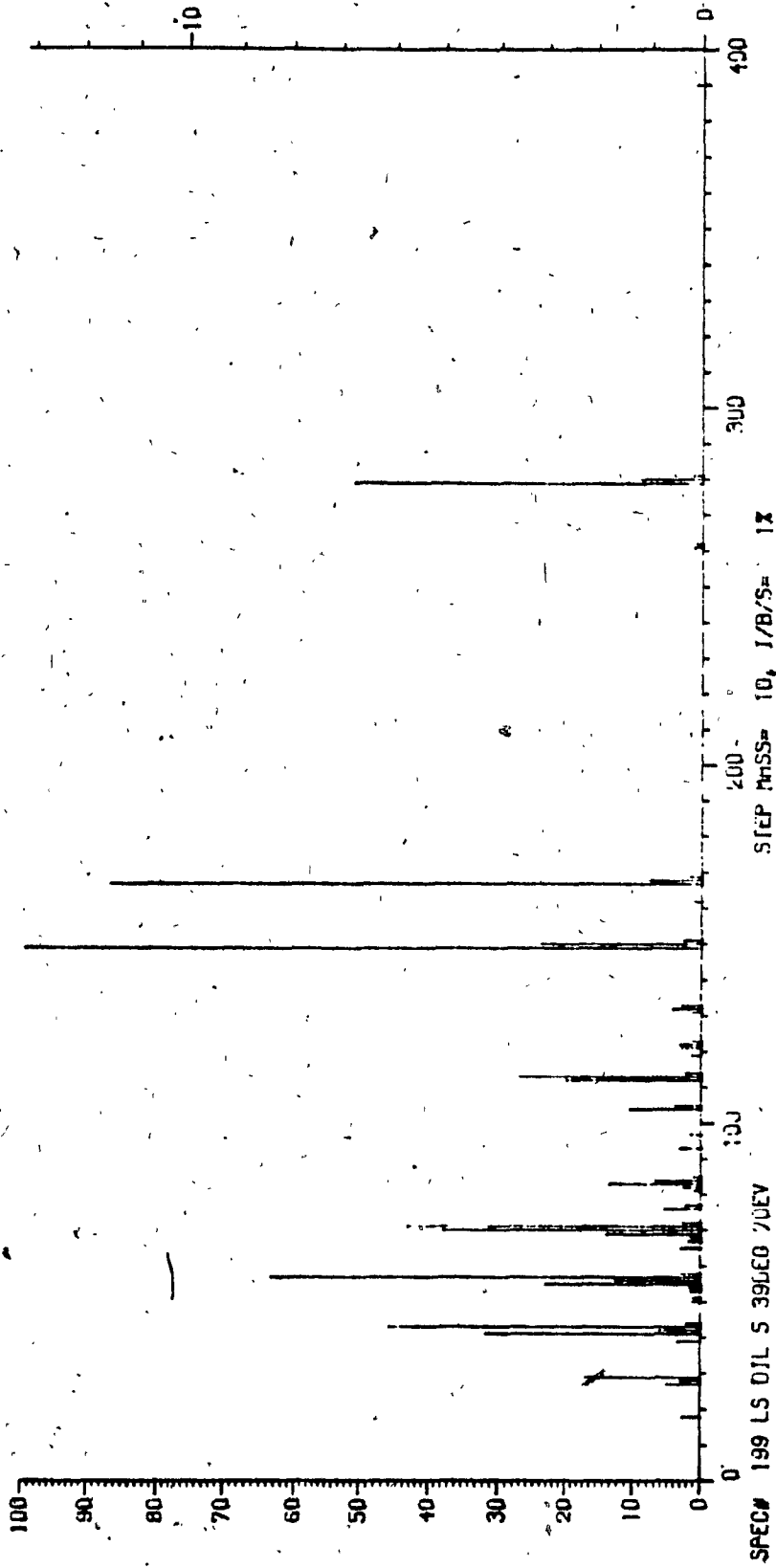
FIGURE 18. Mass Spectrum of # 5 compound, obtained from Benzene extract by chromatography.

FIGURE 19.

m/z: 390

$- C_8H_{17}$

m/z: 279

$- C_8H_{17}$

m/z: 167.

$- OH_2$

m/z: 149
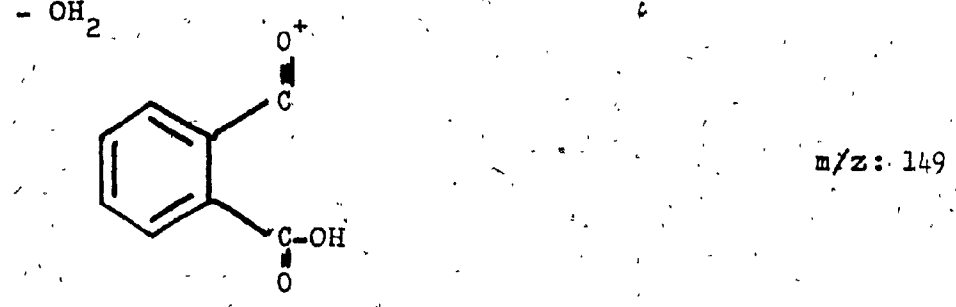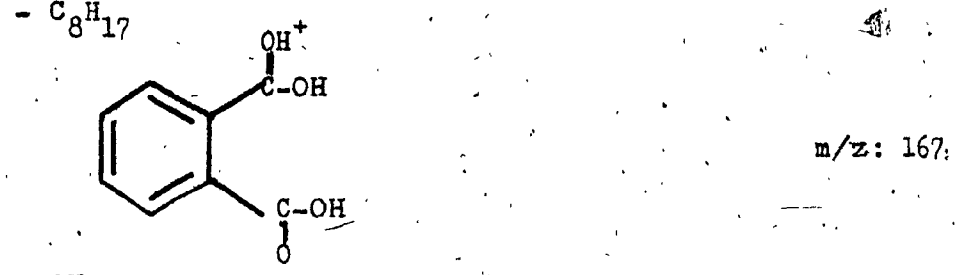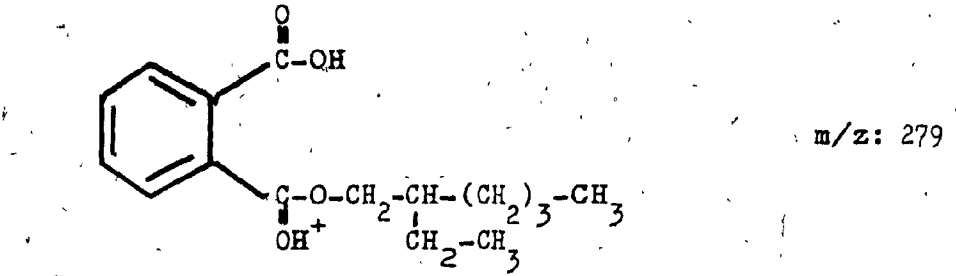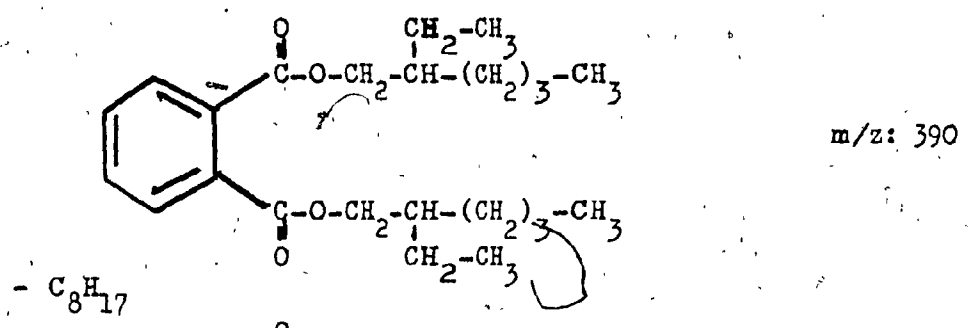
Probable degradation pathway and suggested structure for

5 compound, obtained from Benzene extract.
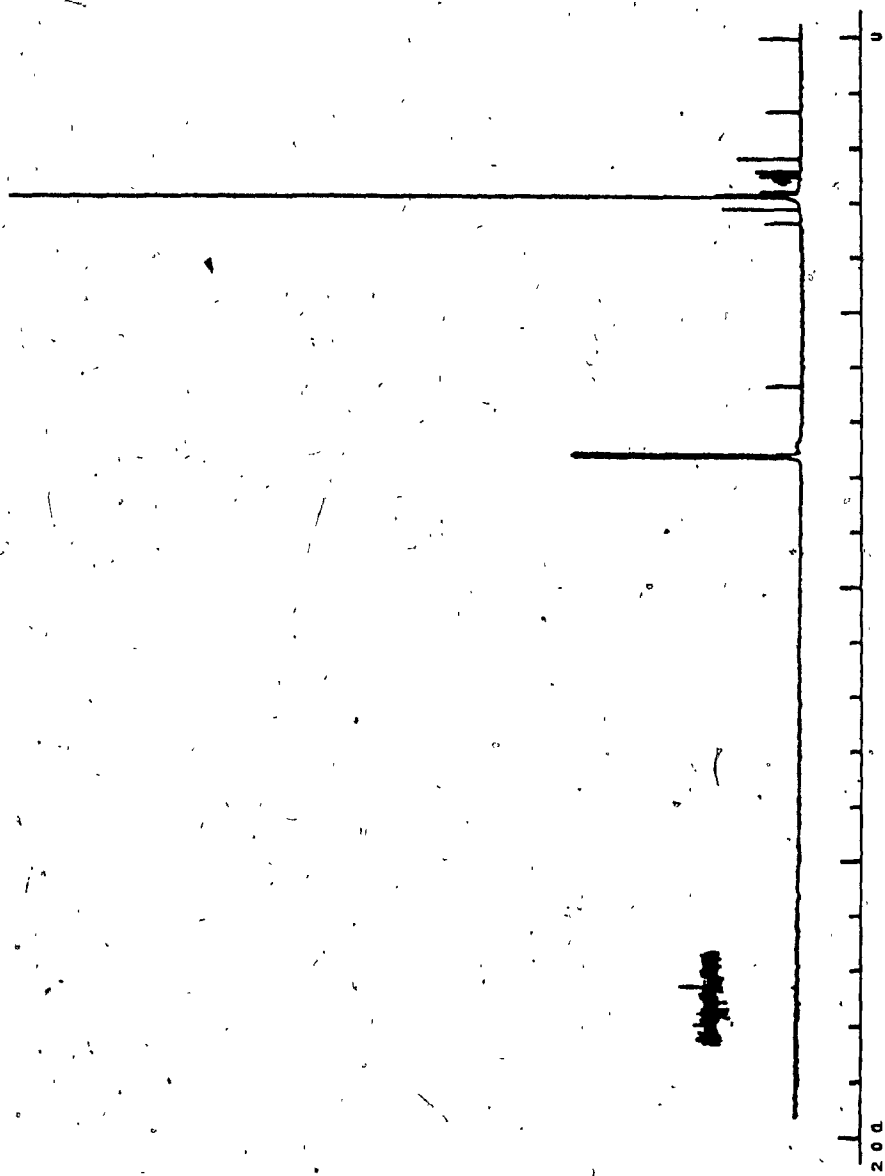
F I G U R E   20 :   Carbon-13 Spectrum of # 1 compound, obtained from Hexane extract.

200

84
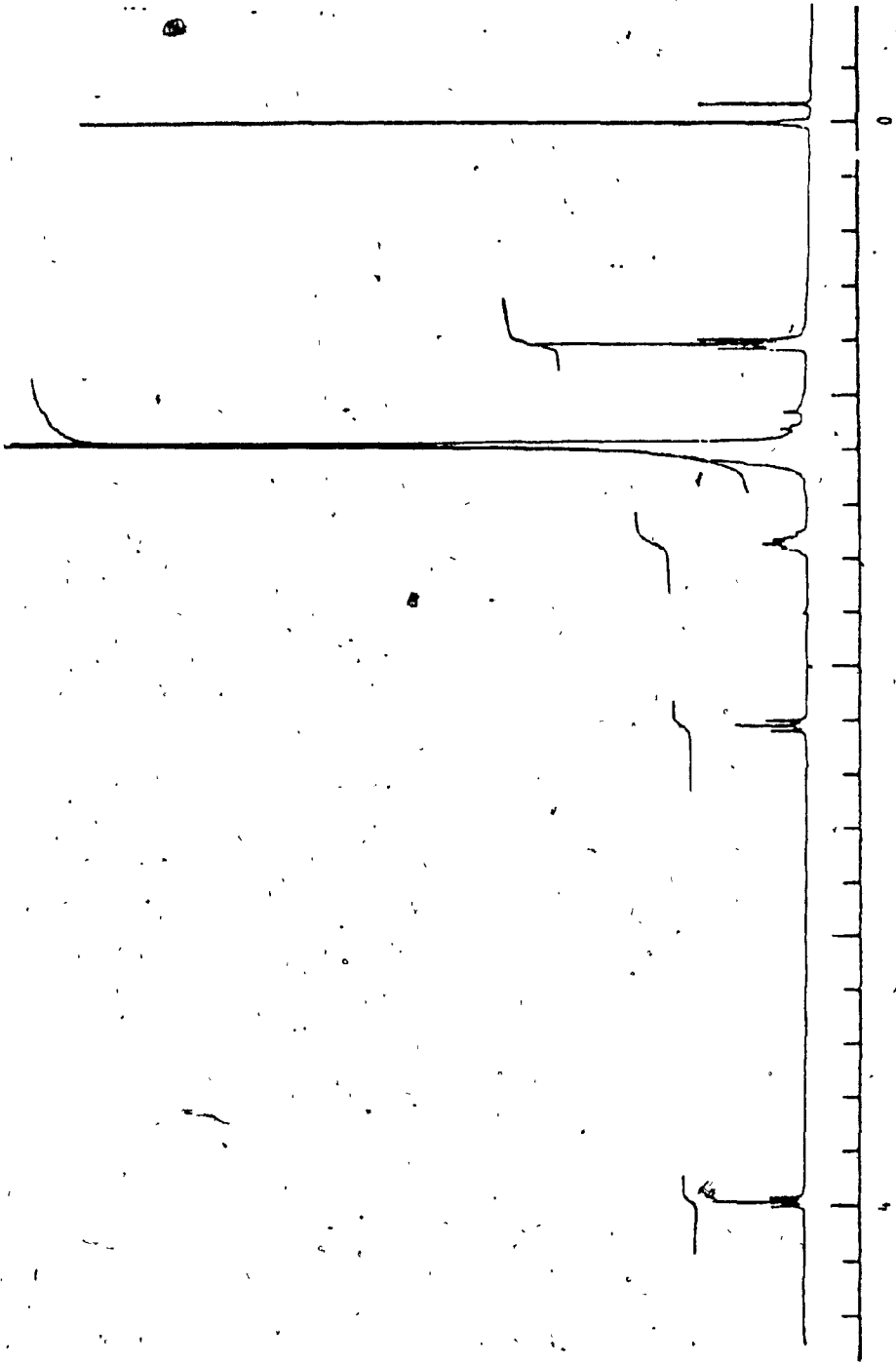
F I G U R E 21 : Proton Nuclear Magnetic Resohance Spectrum (400.00 MHz)

of # 1 compound, obtained from Hexane extract.
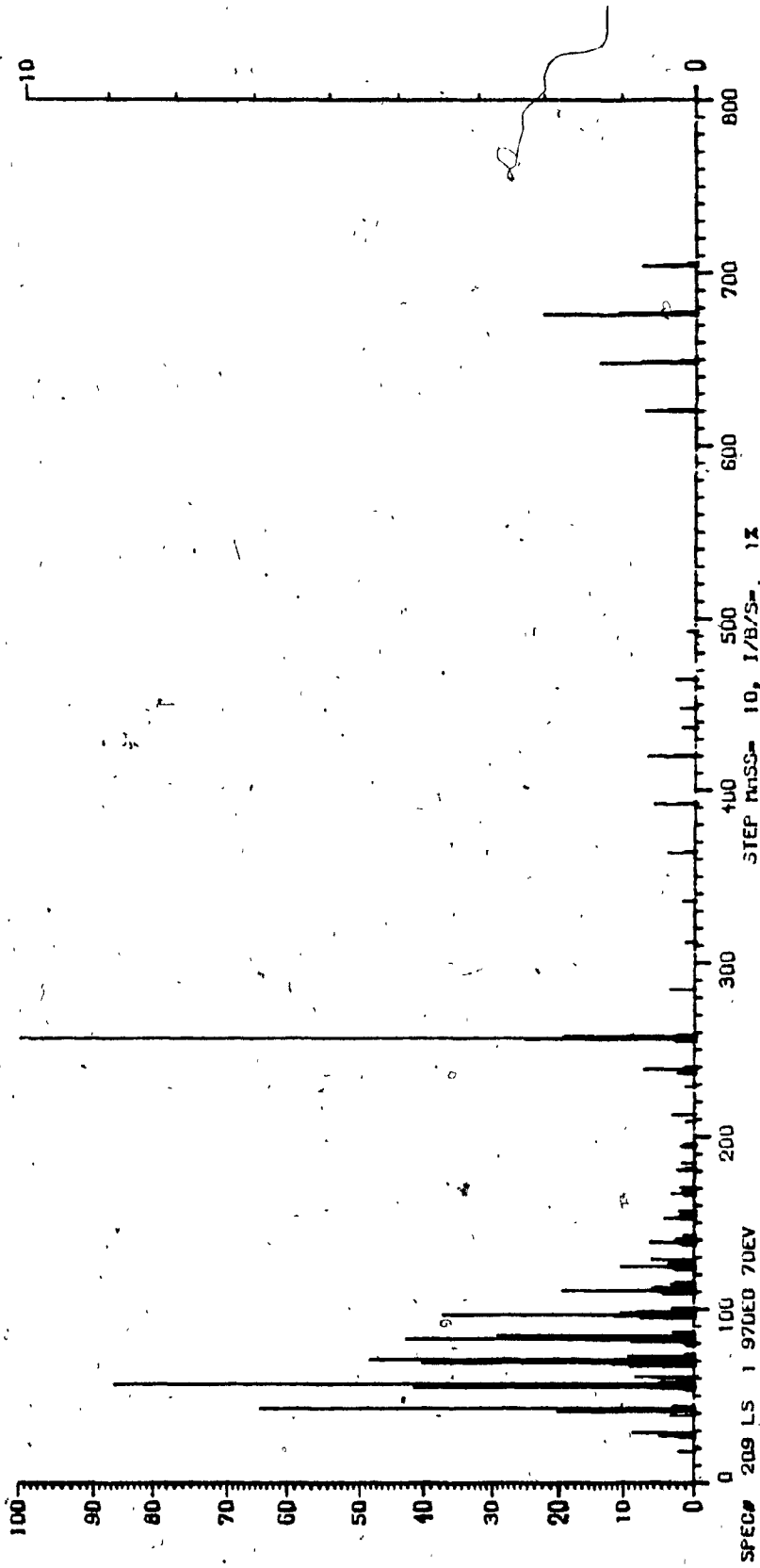
F I G U R E 22 . Mass Spectrum of # 1 compound, Obtained from Hexane extract.
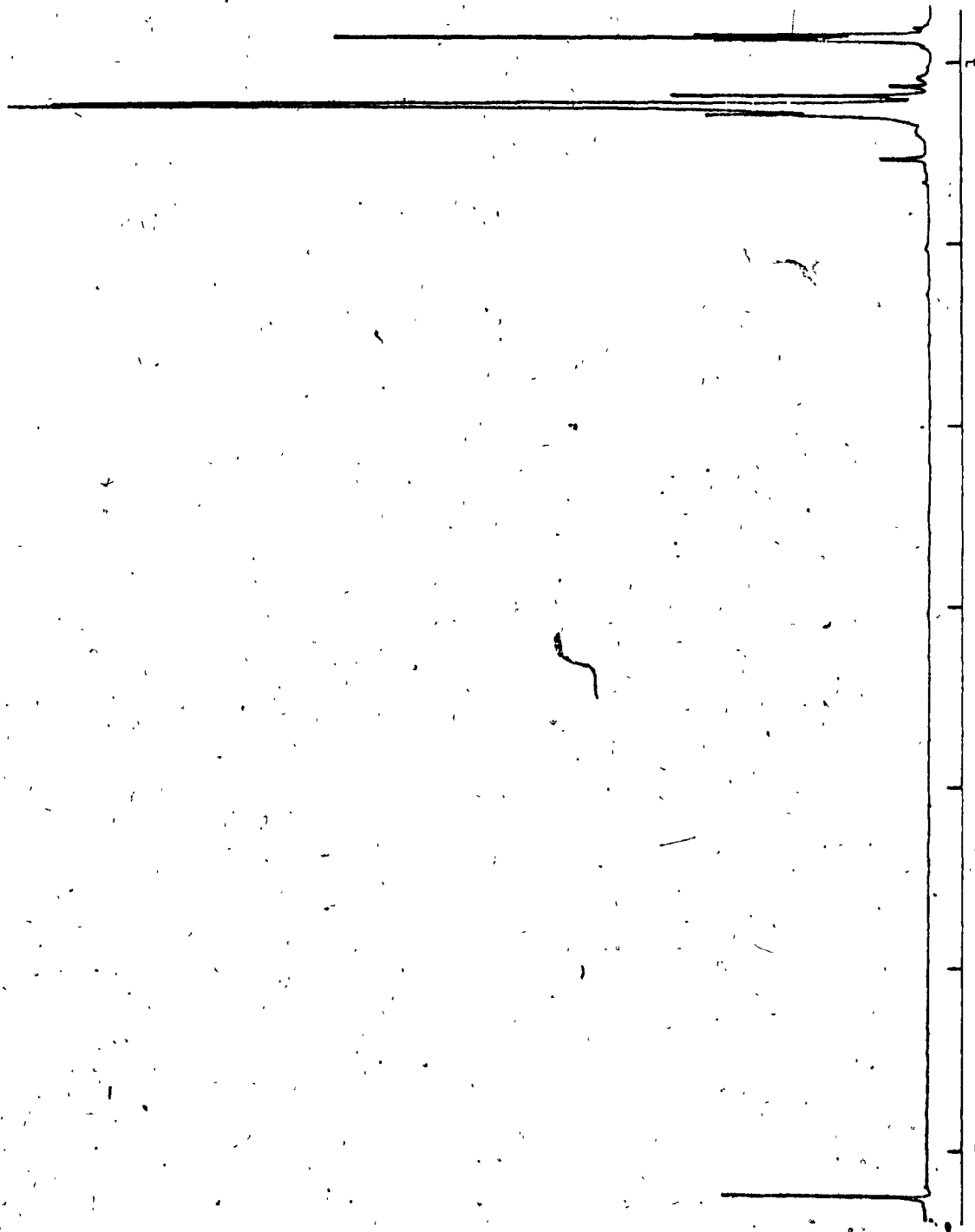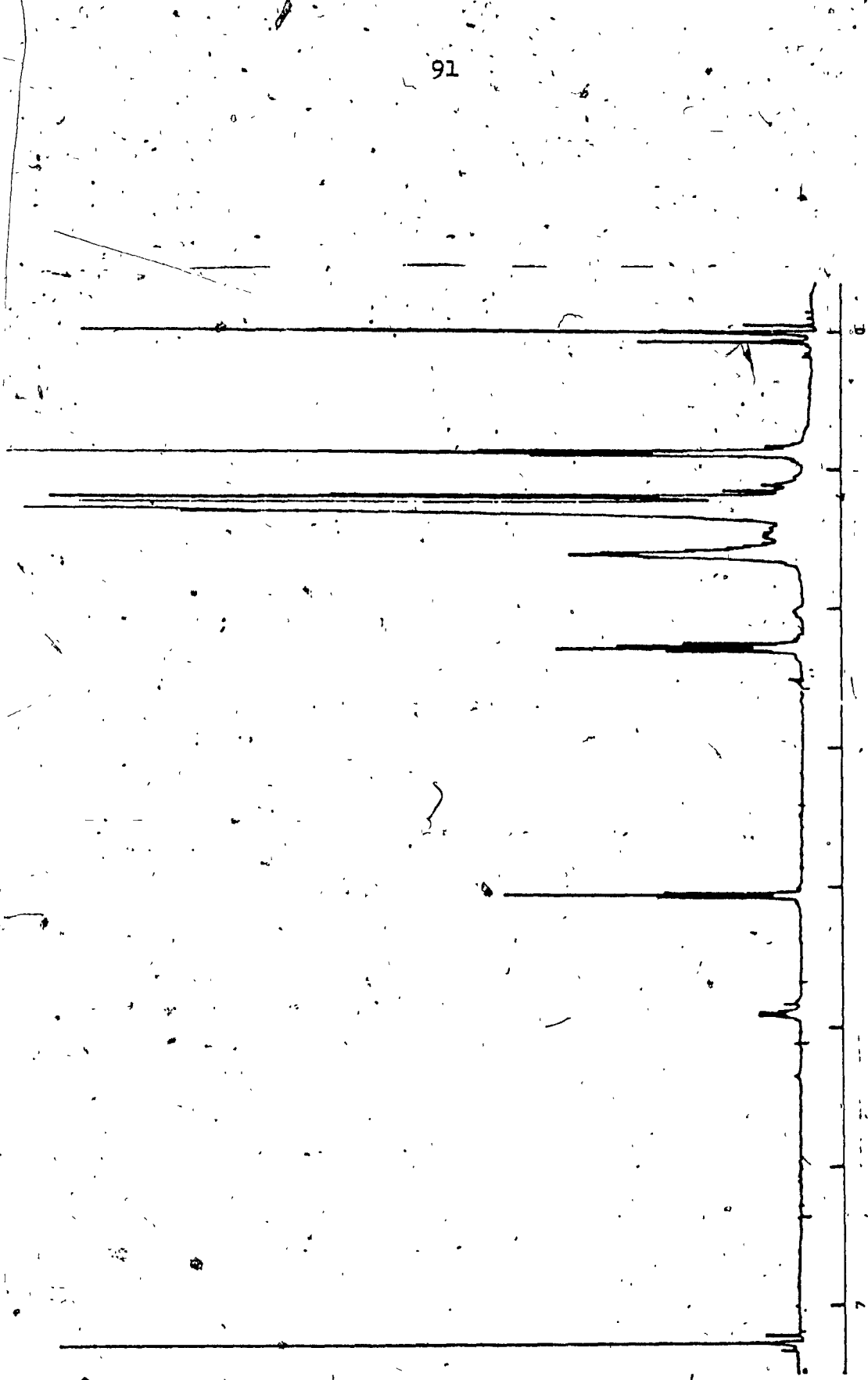
F I G U R E 23 : Proton Nuclear Magnetic Resonance Spectrum of

1/1 compound, isolated from Hexane extract.

87
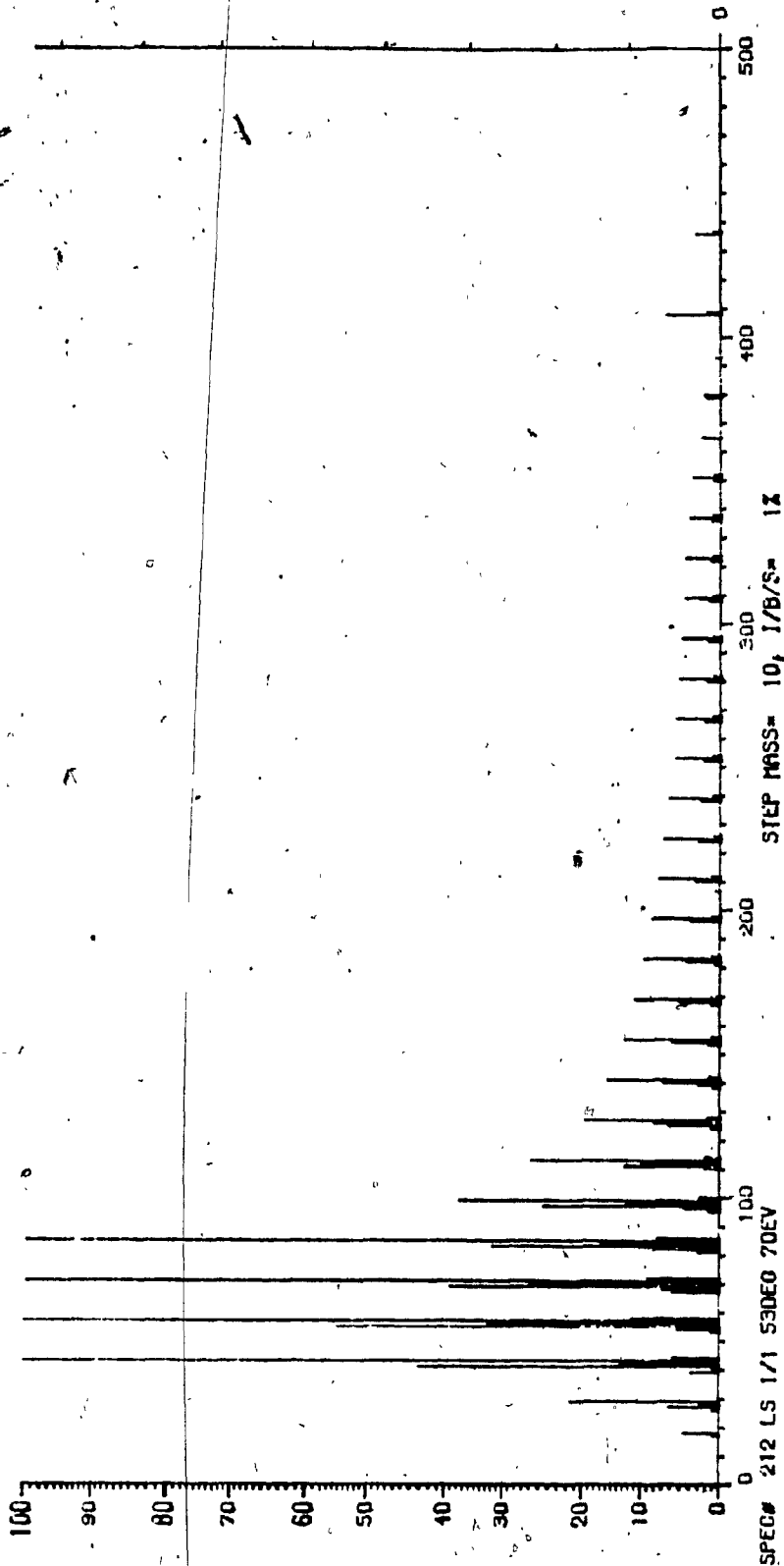
F I G U R E 24 . Mass Spectrum of # 1/1 compound, isolated from Hexane
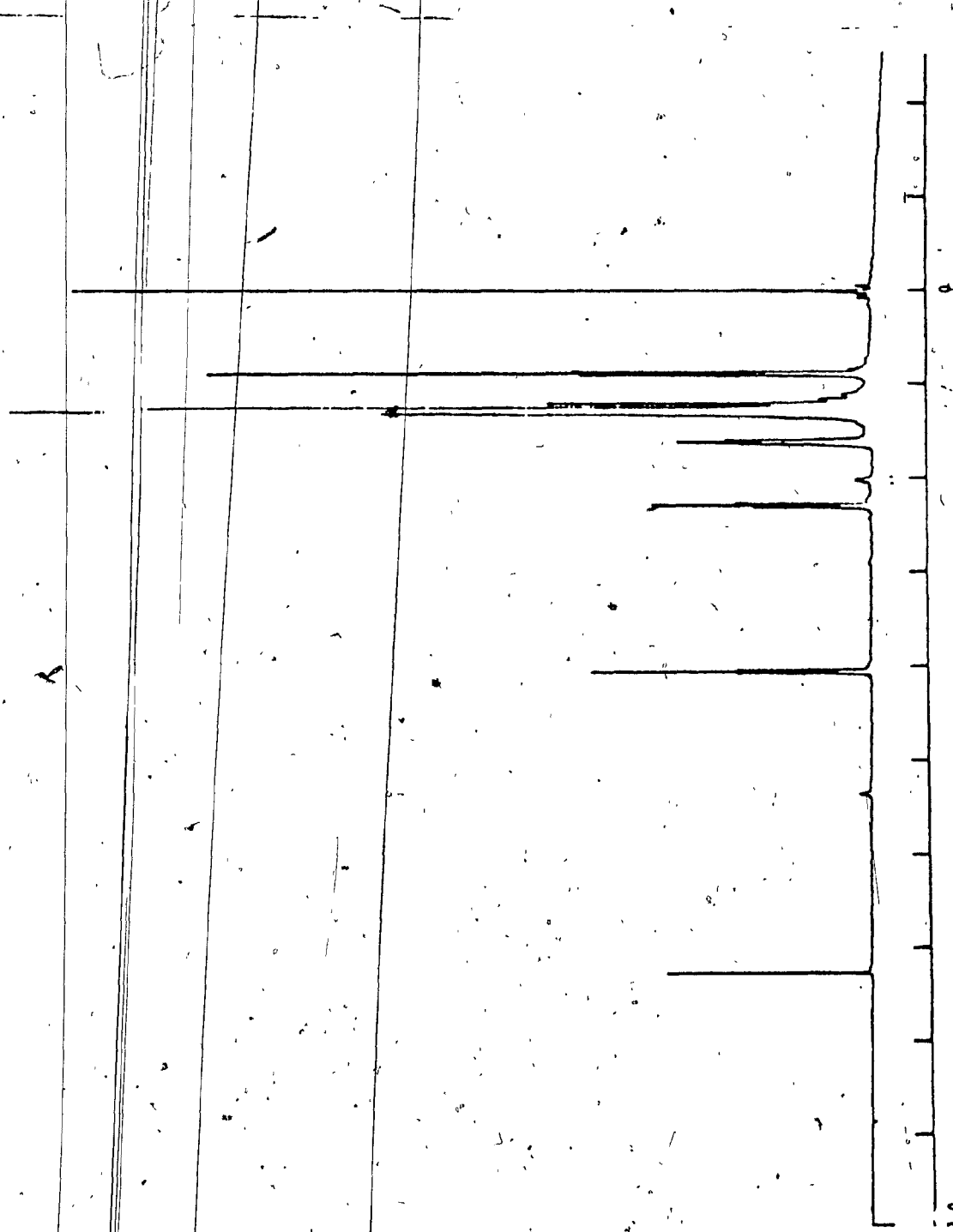
extract.

F I G U R E 25 : Proton Nuclear Magnetic Resonance Spectrum of
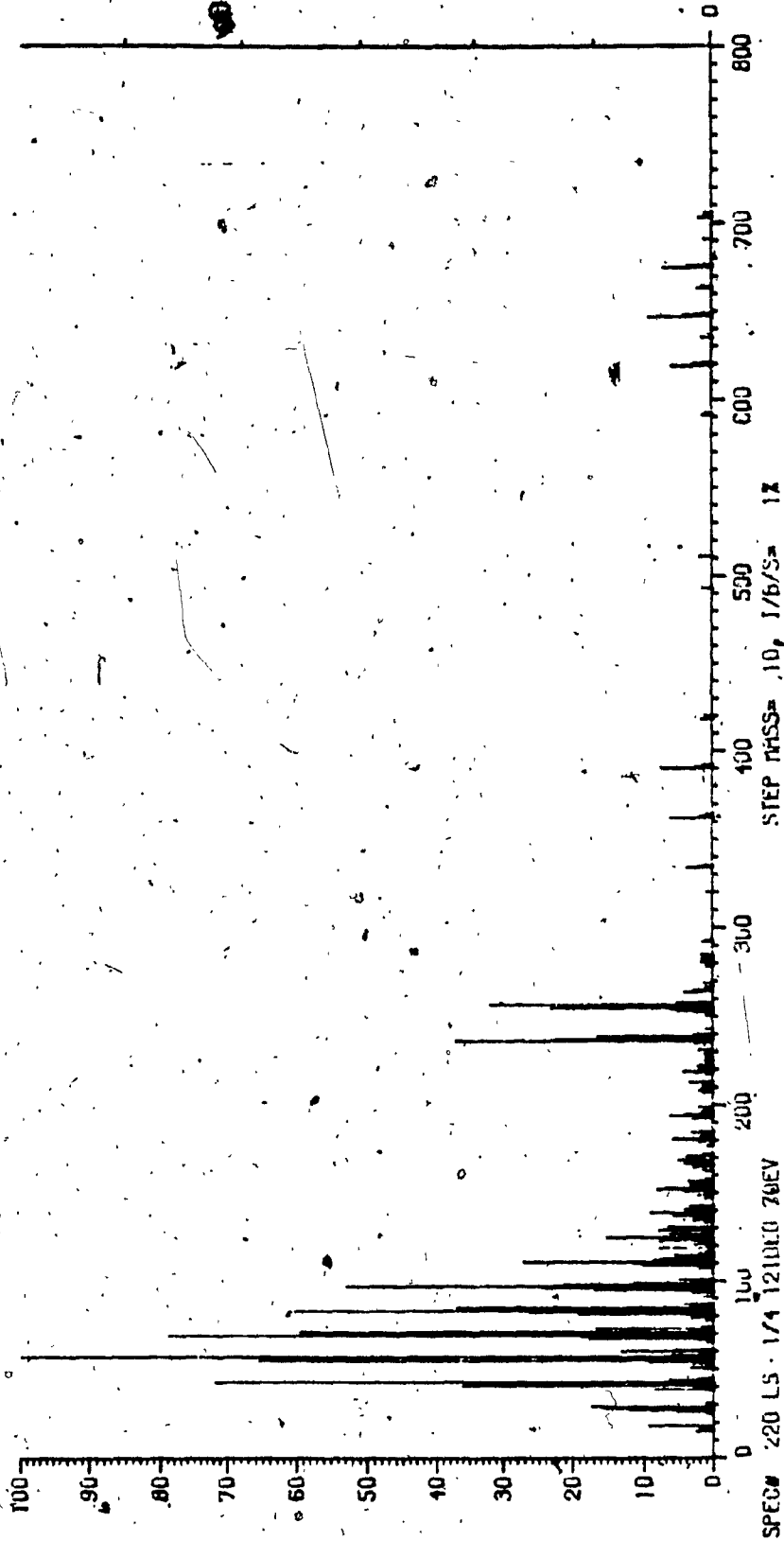
1/2 compound, isolated from Hexane extract.
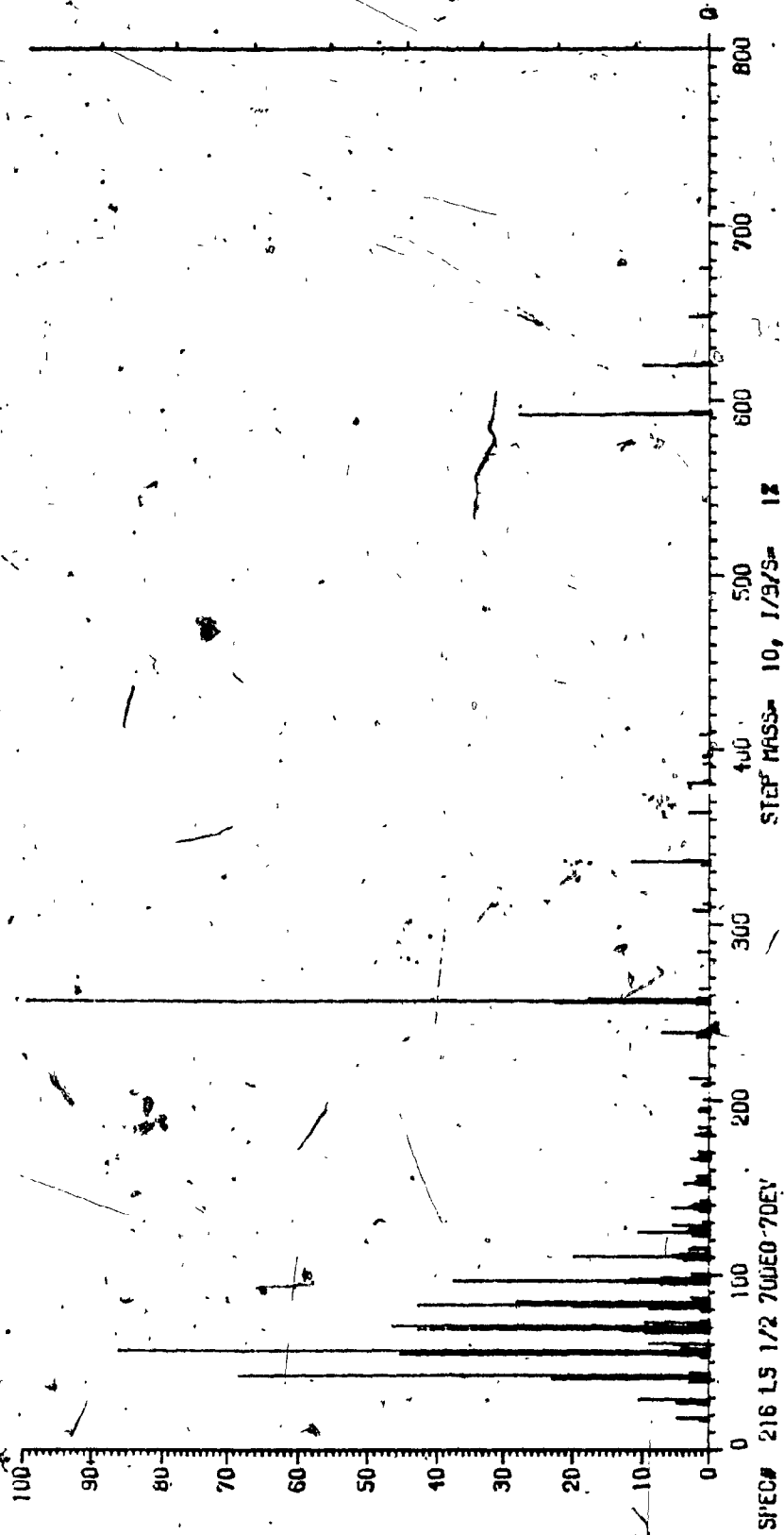
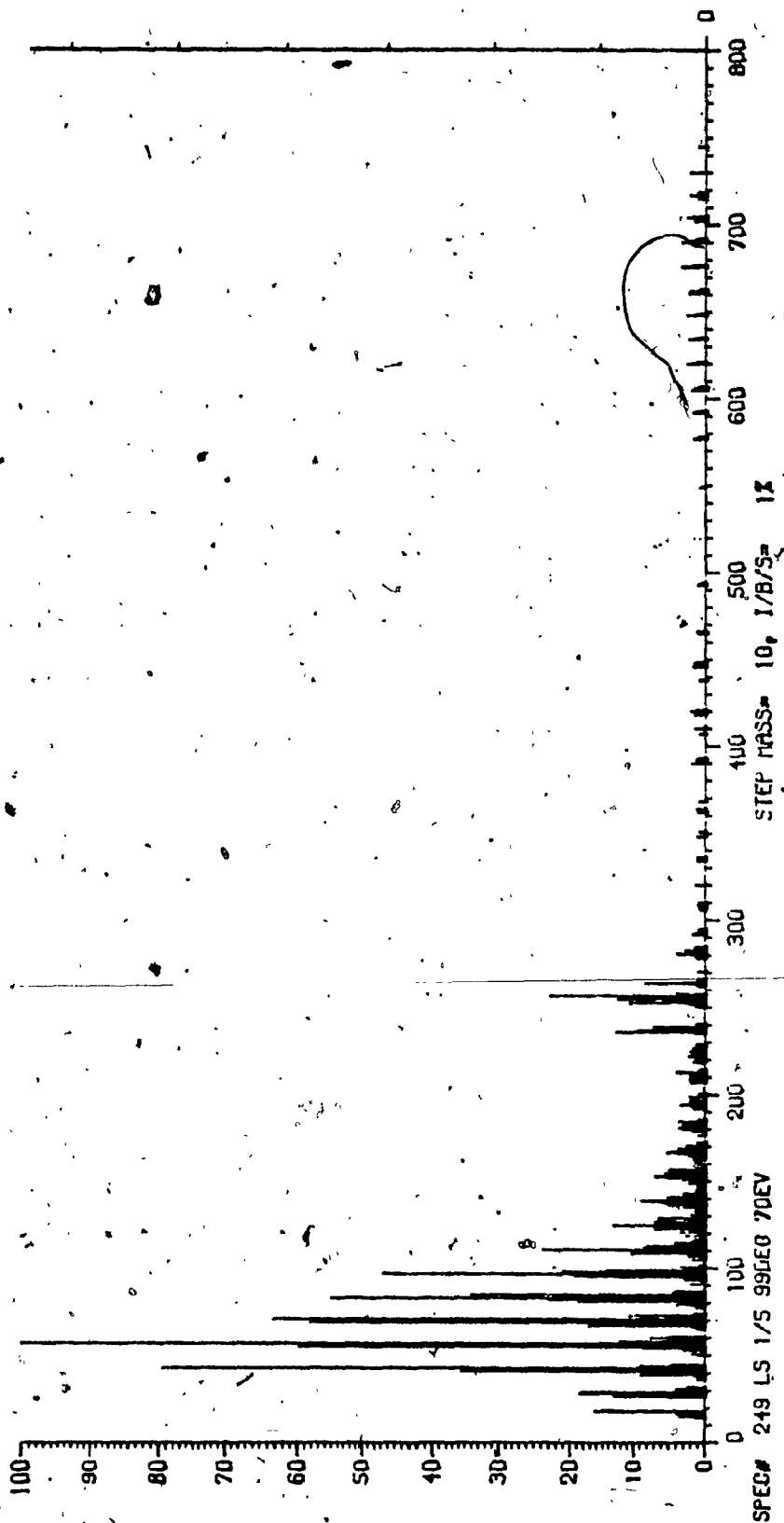F I G U R E 26 . Mass Spectrum of # 1/2 compound, isolated from Hexane
extract.
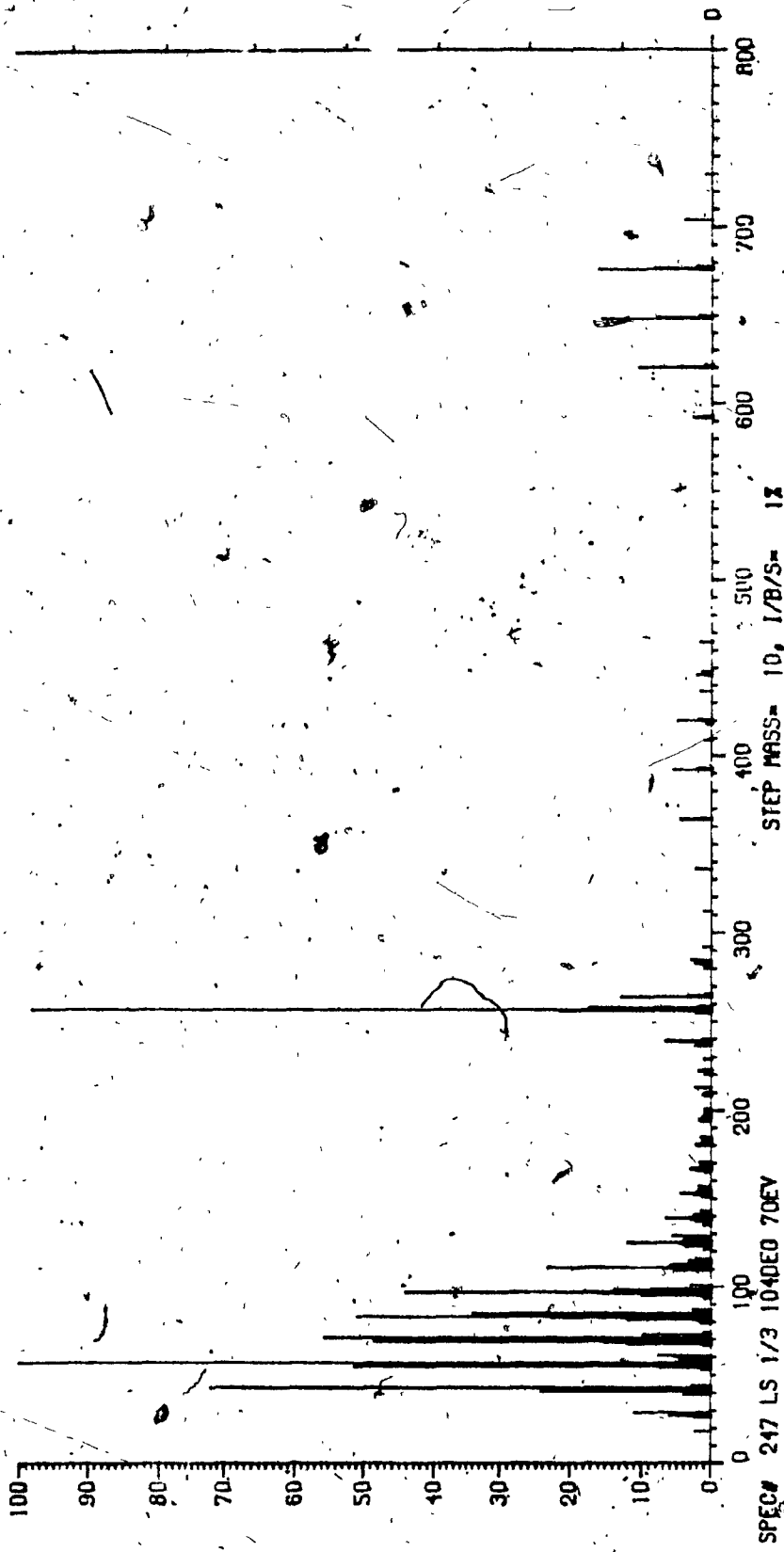
F I G U R E   27 .   Mass spectrum of # 1/3 compound, isolated from Hexane extract.

F I G U R E   28 : Proton Nuclear Magnetic Resonance Spectrum of

1/4 compound, isolated from Hexane extract.

F I G U R E 29. Mass Spectrum of # 1/4 compound, isolated from Hexane extract.

FIGURE 30. Mass Spectrum of # 1/5 compound, isolated from Hexane extract.
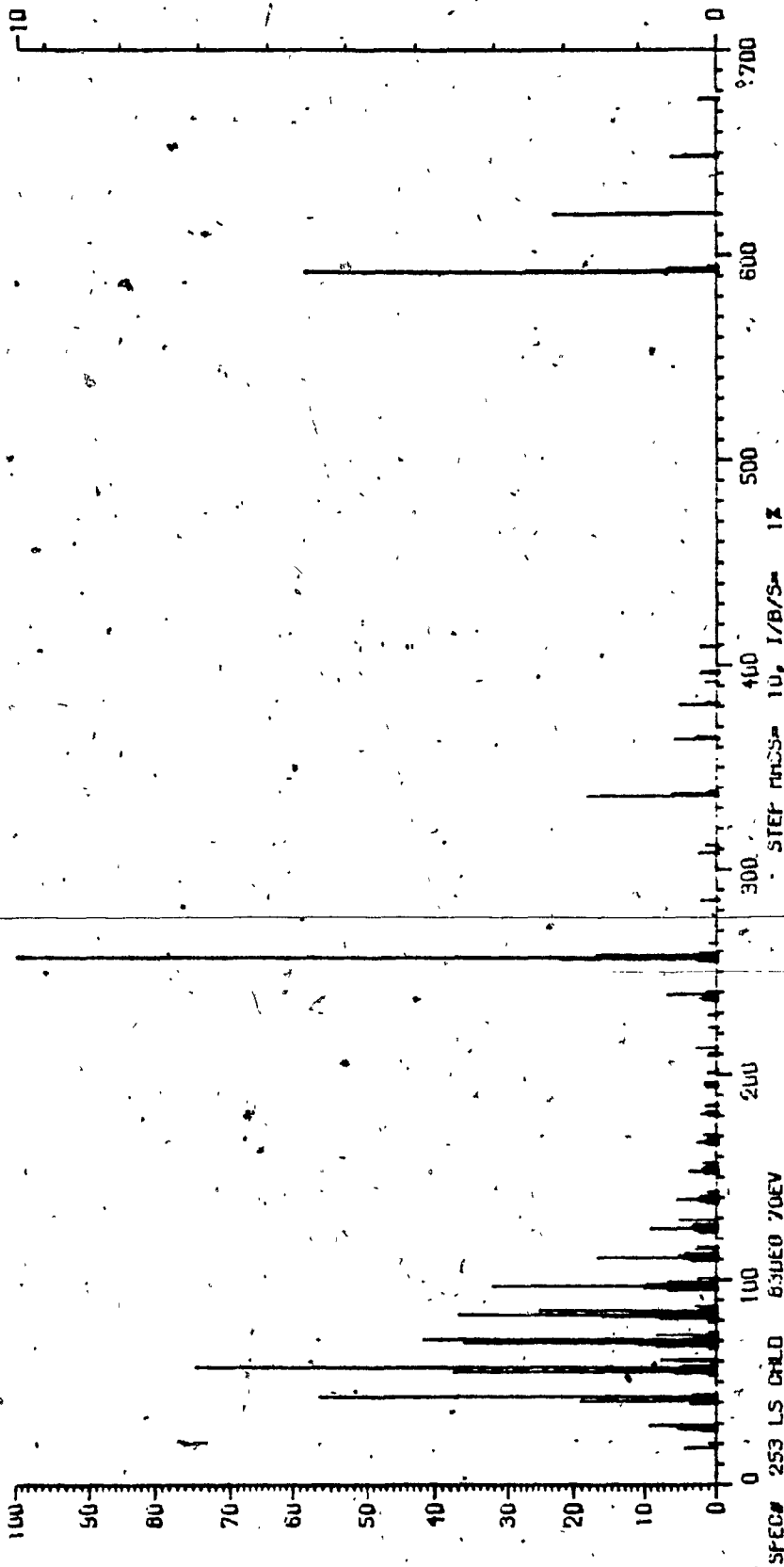
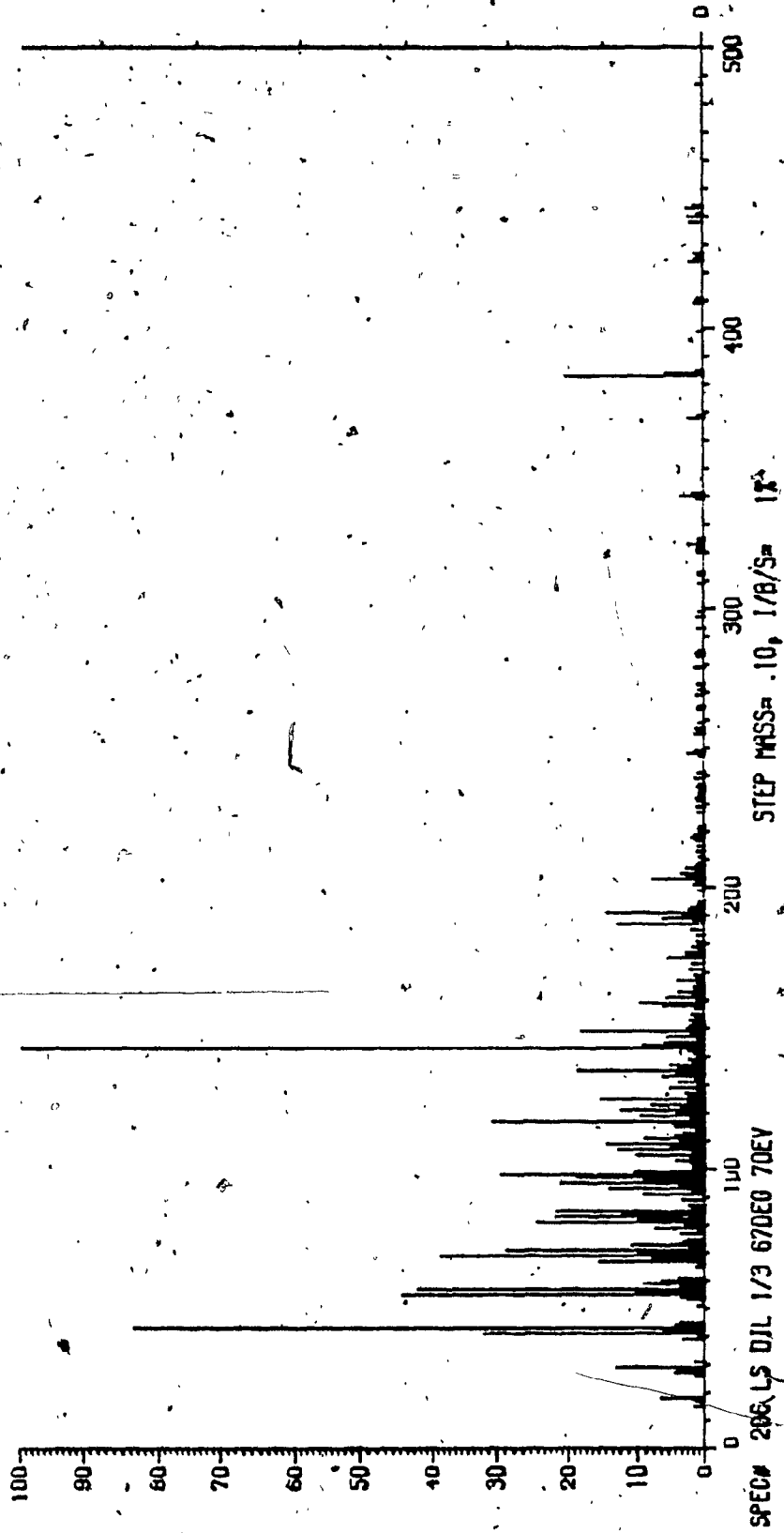F I G U R E   31 .   Mass Spectrum of # Chlo compound, isolated from Hexane
extract.

FIGURE 32. Mass Spectrum of # 33 oily compound, isolated by distillation.