A PHYTOCHEMICAL INVESTIGATION OF THE TOXIC PLANT *ISOCOMA WRIGHTII*

ISOLATION OF A SERIES OF BENZOFURANS AND STEROIDS

A THESIS

Presented to

The Faculty of the Division of Graduate Studies

by

John Robert Novak, Jr.

In Partial Fulfillment

of the Requirements for the Degree

Doctor of Philosophy in the

School of Chemistry

Georgia Institute of Technology

June, 1977

A PHYTOCHEMICAL INVESTIGATION OF THE TOXIC PLANT

ISOCOMA WRIGHTII

ISOLATION OF A SERIES OF BENZOFURANS AND STEROIDS

Approved:

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Date approved by Chairman: 5/25/77

TO MY PARENTS

ACKNOWLEDGMENTS

It is with pleasure that I express my gratitude to Professor Leon H. Zalkow for his invaluble guidance and counsel during the course of my graduate work and preparation of this dissertation. I also wish to thank Professor E. C. Ashby for his words of encouragement along the way and for serving as a member of my thesis committee. I also wish to thank Professors James A. Stanfield and Charles L. Liotta for serving as members of my reading committee.

I would like to especially thank my parents, to whom this work is dedicated. Without their moral and financial support, it would have been impossible.

I thank my wife, Jo-Ann, for her patience, understanding, encouragement and assistance in helping me towards my goals. I would also like to thank her for correcting my spelling and grammar and for typing this thesis. I would also like to thank her parents, Mr. and Mrs. Herbert A. Stout, for their help and encouragement.

Thanks are also rendered to the faculty and staff of the School of Chemistry at Georgia Tech for their help and advice during the course of this study.

I would also like to thank the National Cancer Institute for partial support in the form of a Research Assistantship (1976-1977) and to the School of Chemistry for partial support in the form of a Teaching Assistantship (1972-1976).

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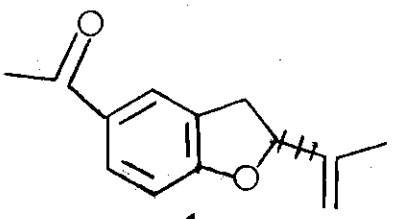
GLOSSARY OF ABBREVIATIONS

bm	broad multiplet (NMR)
bs	broad singlet (NMR)
bp	boiling point
C	Celsius
cc	cubic centimeter
cm^{-1}	wave number (IR)
Col	column
d	doublet (NMR)
ea	each
g	gram
GC	gas chromatography
hr	hour
Hz	Hertz (cycles per second)
IR	infrared
J	coupling constant (NMR)
kg	kilogram
l	liter
m	multiplet (NMR)
m/e	mass to charge ratio
mg	milligram
ml	milliliter
MP	melting point
nm	nanometers

NMR	nuclear magnetic resonance
ORD	optical rotatory dispersion
p.	page
ppm	parts per million
q	quartet (NMR)
R_t	retention time (GC)
s	singlet (NMR)
sec	seconds
t	triplet (NMR)
TMS	tetramethylsilane
UV	ultraviolet

SUMMARY

A reinvestigation of the toxic plant - Isocoma wrightii (rayless goldenrod)[23] has led to the isolation of the previously identified benzofurans - tremetone (1), dehydrotremetone (2), toxol (5), and 2,5 diacetylbenzofuran (6) and to the identification of a new benzofuran - toxyl angelate (23a).



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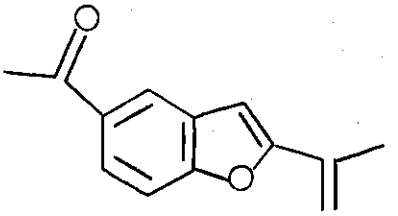

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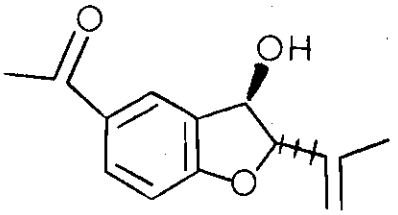

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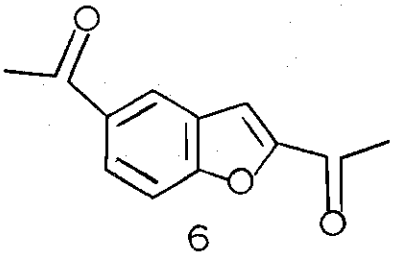

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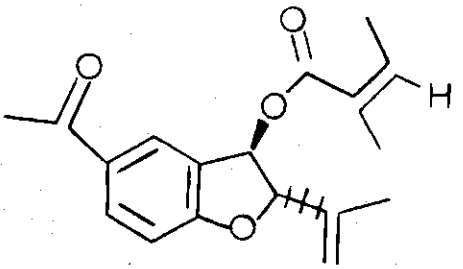
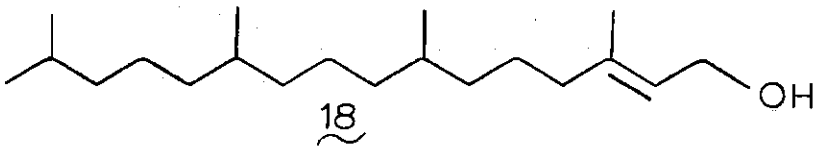


23a

Three compounds, previously unidentified as occurring in the plant, were isolated and identified - phytol (18), hentriacontane (19) and squalene (20).



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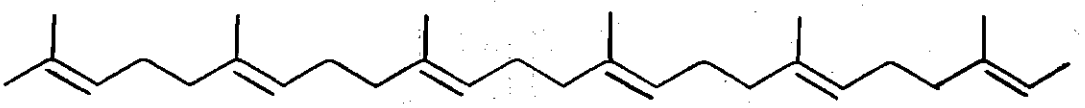
$CH_3-(CH_2)_{29}-CH_3$

19



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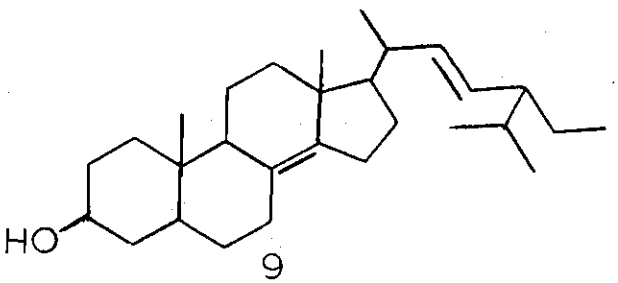
A series of three phytosterols were isolated and identified: stigmasta-8(14),22-dien-3β-ol (9), stigmasta-5,22-dien-3β-ol (21), and stigmasta-8(14)-en-3β-ol (22) - [(9) had been previously isolated from this plant].



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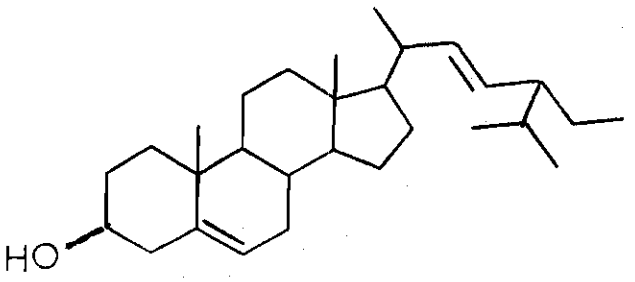

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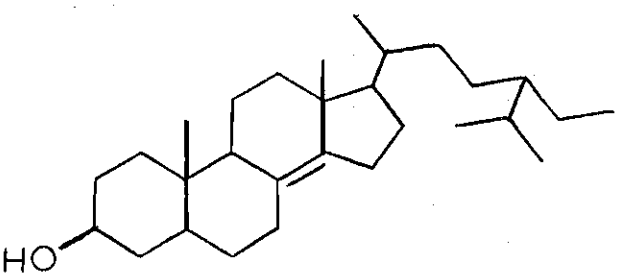


22

NMR (^{1}H and ^{13}C) and mass spectral studies were done on the benzofurans.

The chloroform extract of the plant (see Chart 2) was shown to exhibit anti-tumor activity. All of the benzofurans were sent to the National Institute of Health for testing, but none showed activity.

CHAPTER I

HISTORICAL INTRODUCTION

One of the hardships of the American pioneers was the mysterious malady which struck both humans and livestock alike. Known as "trembles" in cattle and "milksickness" in humans, the disease usually appeared in late August and disappeared in late October, and was one of the leading causes of death and disability in many parts of the American Midwest, Upper South and Southwest.[1] The disease was invariably fatal and occasionally reached epidemic proportions, sometimes wiping out whole communities.

In his Lincoln biography, Carl Sandburg describes this disease which caused the death of Lincoln's mother:

> . . . the "milksick," beginning with a whitish coat on the tongue, resulting, it supposed, from cows eating white snakeroot or other growths that poisoned their milk. . . . there came to Nancy Hanks Lincoln that white coating of the tongue; her vitals burned; the tongue turned brownish, her feet and hands grew cold and colder, her pulse slower and slower. Death came October 5, 1818. . . .[2]

The large number of deaths caused by "milksickness" in the nineteenth century was due in part to the fact that the etiology of the disease was not known. The medical reports on the "milksickness" were confused and conflicting. A number of theories evolved as to the cause of the

disease - one report was that it was due to poison ivy, others to mushrooms or Indian hemp.[3,4] An anonymous author of an 1811 article told as much about the malady as did most articles for half a century to come.[5] He held that "milksickness" was a true poisoning, that it was caused by poisoned milk and that the source of the poison was vegetation eaten by cows. All of which proved to be true.[6,7,8]

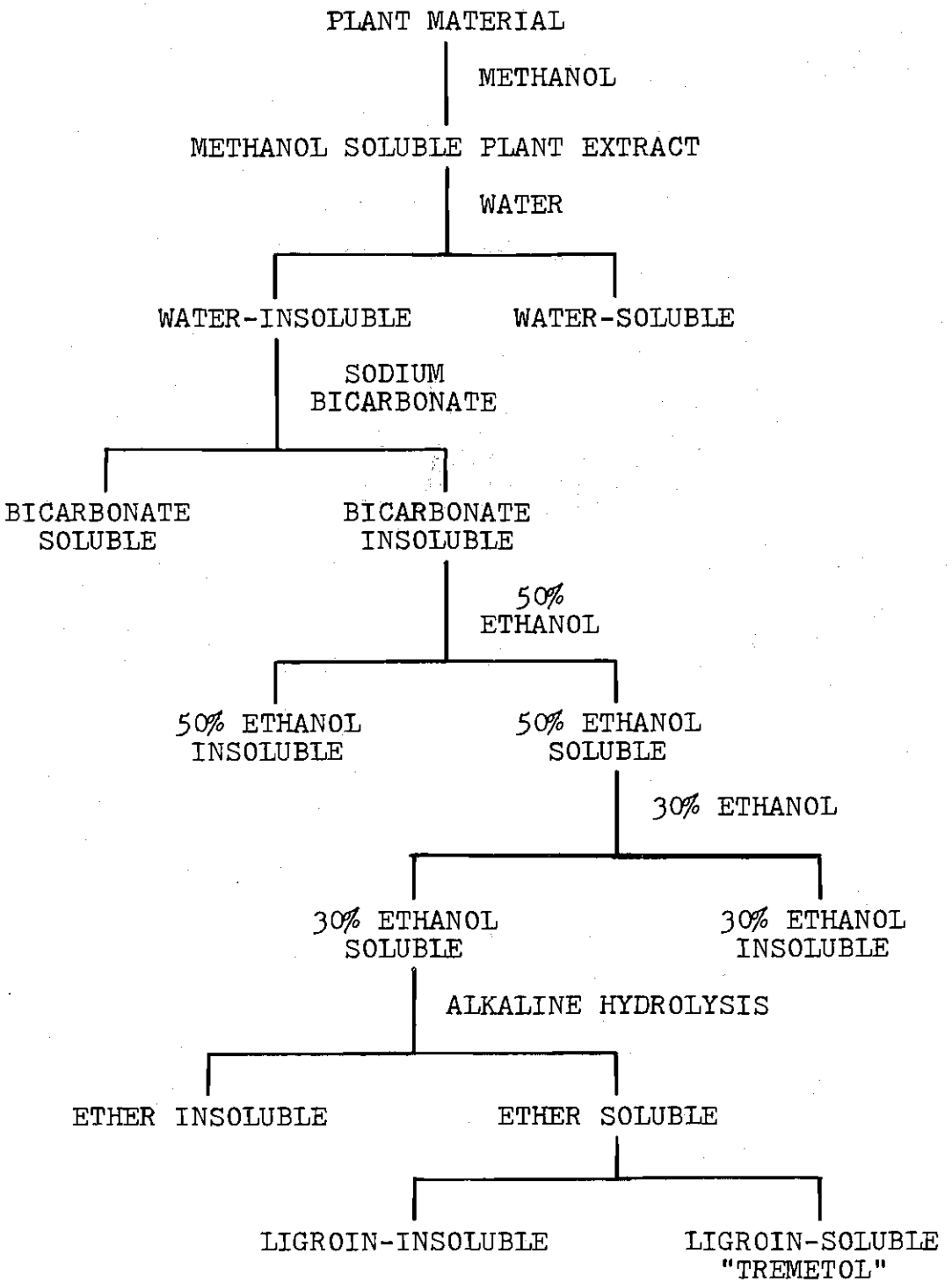
The disease was confined mainly to the backwoods and sparsely settled regions and was never prevalent in larger towns where it could come under scientific observation. Finally, in the 1920's, white snakeroot, Eupatorium rugosum, in the Midwest and Upper South, and rayless goldenrod, Isocoma wrightii, in the Southwest, were shown to be responsible for the disease.

After collecting and grinding some white snakeroot, Couch extracted it with ninty-five percent ethanol. The extract was evaporated to leave a dark green residue which was shown to be toxic to sheep and cattle. The water-soluble portion of the extract was not toxic. The toxin was, however, extractable into fifty percent hot ethanol and following that into thirty percent hot ethanol.

No further purification could be accomplished by solvent extraction; therefore the toxin was saponified. The ether extract of the saponified material contained

the toxic material with a considerable increase in purity, most of the weight having been removed as base-soluble fatty acids. The ether-soluble material yielded a non-toxic solid, mp 148-49°C, of the molecular formula $C_{18}H_{30}O$, as well as a yellow, viscous, toxic oil. All attempts to further purify the oil were unsuccessful and the material was considered to be pure. Couch named the oil, which he thought to be one compound, "tremetol." The isolation procedure is shown on Chart 1. Elemental analysis and molecular weight determinations of the toxic oil indicated the molecular formula to be $C_{16}H_{22}O_3$, $[\alpha]_D = -21.08°C$. Bromine titration showed the presence of two double bonds; no crystalline derivatives were obtained upon reaction with hydroxylamine or phenylhydrazine, and a negative Schiff's test indicated the probable absence of an aldehyde group. The toxin was stable to boiling water, but decomposed upon distillation, even at pressure less than one millimeter. Couch showed that "tremetol" showed a characteristic red color at the interface of a pet-ether solution of the toxin and concentrated sulfuric acid. From these data Couch asserted that the toxin was an aromatic alcohol with a side chain containing two double bonds.[9] Later studies by Couch showed that "tremetol" was also the toxic constituent of rayless goldenrod.[8] Although the plants have not

Chart 1. Couch's Separation Procedure



been considered closely related botanically, it is interesting that they produce the same toxin. Also noteworthy is that while white snakeroot loses its toxicity on drying, rayless goldenrod does so only slowly.[7,9] For unknown reasons Couch apparently stopped his work on "tremetol" and did not publish any further work after 1933.

The study of these plants lay dormant for about a decade until Dermer and his co-workers started a study on rayless goldenrod. Their work revealed that "tremetol" was not a pure substance, but rather a complex mixture. They attempted further to fractionate the oil using a Hickman molecular still. The oil was separated into various toxic fractions, but with the methods available to them at the time, they were unable to isolate a pure toxin.[10,11,12,13]

Further work on these poisonious plants apparently ceased during the next twenty year period and, fortunately, "milksickness" became less of a problem with time for two reasons. First, farmers were educated to recognize and eradicate the toxic plants, and secondly, milk was consumed on a local level less frequently and went into large dairy pools where toxin-containing milk became diluted.

No further work was reported in this area until the early 1960's when Bonner started his investigation of white snakeroot[14,15] and Zalkow iniated his study of rayless

goldenrod.[16] The similarities of compounds isolated from these plants is striking.

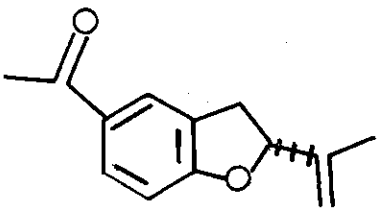
After extracting white snakeroot, Bonner successfully isolated an oil which showed the characteristic red color test of "tremetol." Partition chromatography of the oil on Celite yielded a partially crystalline sterol fraction and an oily ketone fraction. Adsorption chromatography of the sterol fraction on alumina yielded a sesquiterpene-like compound ($C_{15}H_{24}$, $[\alpha]_D$ = +44.7° ($CHCl_3$)), a sterol (mp 184.5-185.5°C, $[\alpha]_D$ = +57.2° ($CHCl_3$)), and a second sterol (mp 147-148°C, $[\alpha]_D$ = -32.8° ($CHCl_3$)).

The hydrocarbon showed the presence of two nonconjugated double bonds; sterol I was similar to α-amyrin; and sterol II was similar to β-sitosterol. However none of these compounds were toxic to goldfish and therefore were not investigated further.

The ketone fraction proved to be toxic to goldfish and gave the "tremetol" color reaction. This fraction yielded three compounds on adsorption chromatography. These compounds were tremetone ($C_{13}H_{14}O_2$, oil, $[\alpha]_D$ = -59.6° (EtOH)), dehydrotremetone ($C_{13}H_{12}O_2$, mp 87.5-88.5°C, $[\alpha]_D$ = 0.0° (EtOH)), and hydroxytremetone ($C_{13}H_{14}O_3$, mp 70-71°C, $[\alpha]_D$ = -50.7° (EtOH)). All three ketones showed a positive Couch color test for "tremetol." Bonner then established the structures of these compounds by degradation[14,15,17] and synthesis.[14,15,18,19,20] The parent

compound, tremetone, was shown to be 2-isopropenyl-2,3-dihydro-5-acetyl benzofuran (1), while dehydrotremetone is unsaturated between C-2 and C-3 (2) and hydroxytremetone has a hydroxyl group at C-6 (3). Hydroxytremetone is very similar in structure to euparin (4), isolated from a plant of the same family (Eupatorium purpurem).

Also during the early 1960's, Zalkow and co-workers reported the results of their study on rayless goldenrod.[16] Using both Couch's procedure (except substituting methanol for ethanol) and a modified procedure, they were able to isolate "tremetol" from the plant. Partition chromatography gave, in the first fraction, dehydrotremetone (2) and a second, more polar fraction. Adsorption chromatography of "tremetol" yielded dehydrotremetone and a more polar



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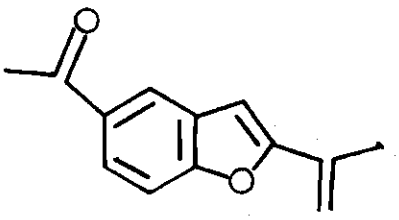

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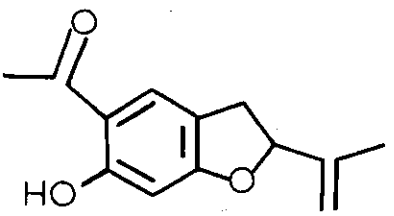

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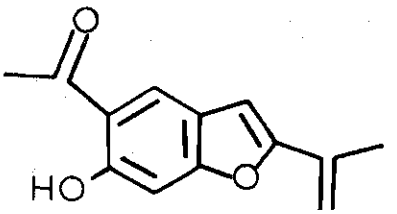

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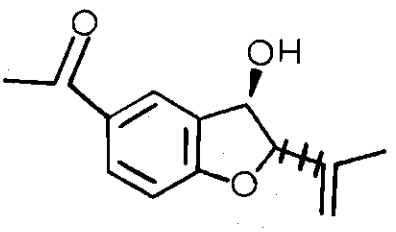
component, toxol ($C_{13}H_{14}O_3$, mp 52-53°C, $[\alpha]_D$ = -25.1°) was shown by IR and NMR to be an aromatic compound, containing one isolated double bond, a conjugated ketone, and a secondary hydroxyl group. A degradation study of toxol showed it to be another benzofuran (5).[23] Tremetone and another new benzofuran (6) have also been isolated from rayless goldenrod "tremetol."[24,25,26]

These benzofurans (1-3, 5, 6) are of particular interest; they have been isolated from these two plants which, while not considered closely related on morphological grounds, have been shown to be clinically similar in producing "milksickness." Simple benzofurans (1-6) are relatively rare in nature,[27] and undoubtedly are of importance from a chemotaxonomy point of view.

Since the investigations into white snakeroot and rayless goldenrod "tremetol" were conducted to find the toxic constituent, a brief summary of the toxicological and pharmacological studies of these plants follows. Using sheep as a screening animal, Couch was able to show that "tremetol" caused "trembles."[6,7,8,9] Couch also



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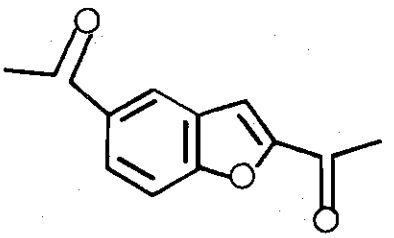

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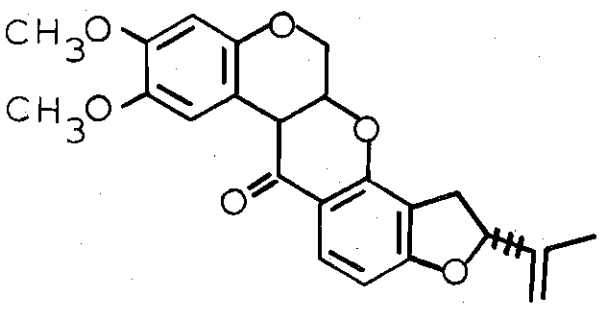
used guinea pigs, cats and rabbits for his studies, but found sheep to be best.[6] All of the animals that died of "trembles" showed large acetone concentrations in their urine and had the smell of acetone on their breath. Internal damage was confined mostly to the liver, with the kidneys, spleen and lungs being involved. The blood sugar level of the animals was depressed. Bonner studied the toxicity of tremetone on goldfish, mice, rabbits, sheep, chickens and insects.[20] Crude "tremetol" was found to cause the death of goldfish in 10 minutes (50 mg/l dose). Dehydrotremetone (30 mg/l) caused death in 20 minutes, tremetone (1) (30 mg/l) in 24 minutes, and hydroxytremetone (30 mg/l) in 21 minutes. Tremetone did not cause "trembles" in any of the test animals, but did prove toxic toward them. Zalkow found that a bacteriological screen was satisfactory for following the original chromatographic separation of rayless goldenrod "tremetol."[16] From a random selection of seventeen bacterial cultures, seven exhibited growth inhibition by the crude toxin. Bacillus cereus was chosen as an assay organism because of its hardiness and ease of cultivation; also the crude toxin was shown to be bacteriostatic for B. cereus. From this screen, he was able to judge that toxol was more toxic than dehydrotremetone. However, toxol is not present in white snakeroot, and Zalkow showed that in feeding experiments with sheep, that the

plant and "tremetol" were toxic but toxol (5) was not.[28] Therefore, although several biologically active benzofuran derivatives have been isolated from "tremetol," in no case have these proved to be responsible for toxicity to high animals.

It is interesting to note that toxol (5) and tremetone (1) have the same absolute configuration at C-2 as that of rotenone (7) at C-5[29] and presumably hydroxy-tremetone (3) has the same configuration. The common furan ring and absolute configuration at C-2 and C-5 in tremetone and rotenone respectively may be related to their insecticidal properties. Toxol (5) is the only benzofuran of this group which has a chiral center at C-3 and the final determination of the configuration at this center was only recently resolved.[30] Zalkow clearly showed that toxol (5) has the C-2, C-3 trans arrangement and this information taken together with its correlation with tremetone and rotenone lends to the 2S, 3R configuration as represented in 5.



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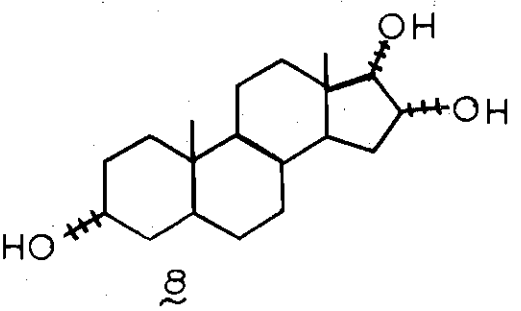
In addition to the above mentioned benzofurans, Zalkow also isolated two steroids, and was able to fully characterize them. From rayless goldenrod "tremetol," he was able to isolate 5-androstane-3,16,17-triol (8),[31] this was the first reported androstrane derivative to be isolated from a plant source. In addition, he isolated the steroid stigmasta-8(14),22-dien-3-ol (9),[32] a steroid important because of the biogenetic significance of the $\Delta^{8(14)}$ double bond.

Besides the more significant benzofurans and steroids, Zalkow's study of the extracts of Isocoma wrightii yielded the following terpenes: friedelin (10),[23] friedelan-3-ol (11),[33] (+)-limonene (12),[23] (-)-carvone (13),[23] (-)-borneol (14),[23] borneol acetate (15),[33] caryophyllene (16),[23] and caryophyllene oxide (17).[33] In addition, acidification of the alkaline solution remaining after saponification of the concentrated methanolic plant extract showed the presence of the following fatty acids: stearic, myristic, palmitic, lauric, octanoic and hexanoic.[23] Zalkow has also shown that the alcoholic plant extract of Iscoma wrightii contains a small, complex, alkaloid fraction. This alkaloid portion has not been characterized.

Both the investigation of Iscoma wrightii and Eupatorium rugosum has resulted in the isolation of several novel secondary plant metabolites, but none have been proven responsible for the toxicity of these plants. The



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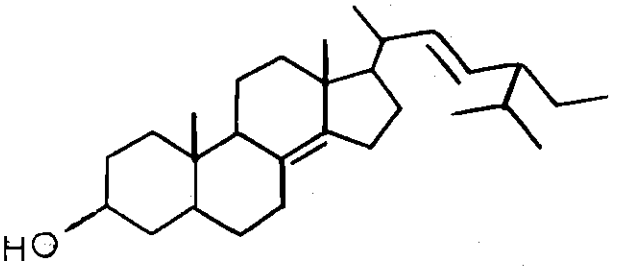

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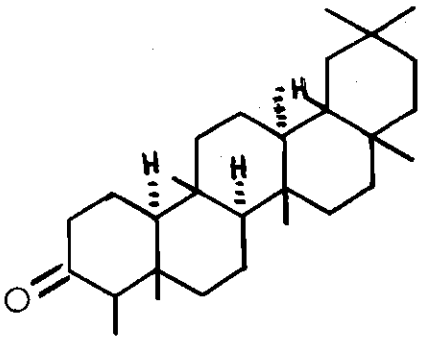

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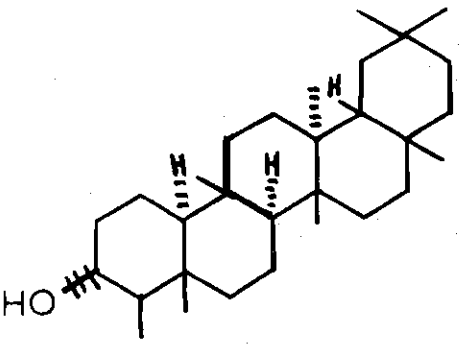

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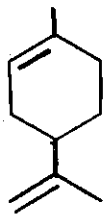

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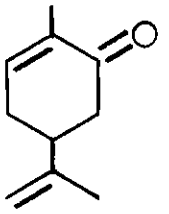

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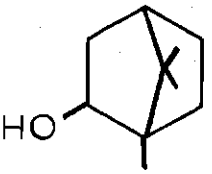

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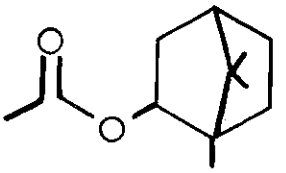

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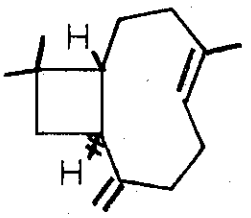

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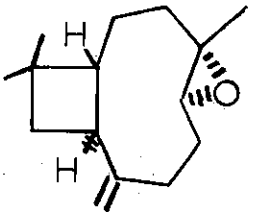


17

agent that causes "milksickness" and "trembles" has not yet been isolated.

Orginally, it was the purpose of this research to reinvestigate the plant Iscoma wrightii in order to find the toxic principle. However, it was found early in the investigation that the chloroform fraction of the ethanolic extract of the plant exhibited anti-tumor activity in P388 lymphacytic leukemia tumors. Therefore, a phytochemical investigation was initiated in order to find as many of the major secondary plant metabolites of the plant as possible and sent them to the National Institute of Health for anti-tumor testing. It is, of course, not inconceivable that the anti-tumor agent and the toxin are the same substance since toxicity has only been demonstrated on prolonged consumption of the plant by high animals, or prolonged consumption of affected milk by humans. In addition, both animals and humans removed from the source of contamination prior to fatal dosage, have been shown to recover.[6,7,8,9,34]

CHAPTER II

INSTRUMENTATION AND EQUIPMENT

Melting points are uncorrected and were determined on either a Thomas-Hoover capillary melting point apparatus or a Thomas-Kofler micro hot stage equipped with a microscope. Microanalyses were performed by Atlantic Microlabs, Atlanta, GA. Removal of solvents in vacuo was done using a Buchler Instruments rotary evaporator at water aspirator pressure. Infrared spectra were recorded using a Perkin-Elmer 237B spectrophtometer with solids in the form of potassium bromide pellets or in carbon tetrachloride or chloroform solution and liquids in the form of thin films between sodium chloride plates or in carbon tetrachloride or chloroform solution. The band at 1601 cm^{-1} of polystyrene film was used as a reference.

Nuclear magnetic resonance spectra were obtained using solutions containing tetramethylsilane as an internal standard. All 60 MHz ^{1}H-NMR spectra were obtained using Varian Associates A-60D or T-60A spectrometers. The 100 MHz ^{1}H-NMR and ^{13}C-NMR spectra were obtained using a JEOL PFT-100 Fourier transform spectrometer at 99.5 and 25.0 MHz, respectively. ^{13}C-NMR spectra are broad band proton decoupled unless specifically described as off

resonance proton decoupled spectra.

Ultraviolet spectra were obtained with either a Cary-14 or a Perkin-Elmer Model 202 spectrophotometer. Optical rotary dispersion curves were obtained using a JASCO ORD/UV-5 spectrophotometer. The mass spectra were obtained with an Hitachi Perkin-Elmer Model RMU-7L or a Varian Model M-66 mass spectrometer.

Gas chromatograph traces were obtained with either a Hewlett-Packard Model 402 gas chromatograph or an F and M Model 400 gas chromatograph; both gas chromatographs were equipped with flame ionization detectors. Preparative gas chromatography was performed on a Varian Aerograph Model 2740 gas chromatograph equipped with a 10:1 stream splitter and flame ionization detectors. The GC columns used are listed in Table 1.

Table 1. GC Columns

Column Number	Liquid Phase	Solid Support	Column Size	Column Material
I	3% SE-30	100/120 Gas Chrom Q	4' x 1/4"	glass
II	5% SE-30	60/80 Chromosorb W (acid-washed)	6' x 1/4"	glass
III	3% OV-17	100/120 Gas Chrom Q	6' x 1/4"	glass
IV	1.5% OV-101	100/120 H/P Gas Chrom G	5' x 1/8"	stainless steel
V	3% OV-17	60/80 Chromosorb W (acid-washed)	6' x 3/8"	glass

CHAPTER III

EXPERIMENTAL

Collection of Plant Material

The above-ground portion of Iscoma wrightii (rayless goldenrod) was collected near the Artesia, New Mexico area in October, 1968. The material was carefully dried in a low-humidity atmosphere then coarsely ground. The plant material was shipped in sealed, brown paper bags enclosed in cardboard containers.

Extraction Using NIH Procedure

Rayless goldenrod (5.05 kg) was placed in a large Soxhlet extractor along with 10-l of freshly distilled hexane (bp 66-67°C). The extraction continued until the hexane running through the plant material was colorless, about 24 hours. After removal of the solvent in vacuo, 84.3 g of hexane soluble extract was obtained.

The plant material was removed from the extractor and allowed to dry in a forced air draft overnight. The plant material was then placed back in the extractor with 10-l of freshly distilled 95% ethanol (bp 77-77.5°C). The extraction was continued until the solvent running through the plant material was clear and colorless. This

procedure took approximately four days. After removal of the solvent *in vacuo*, 363.4 g of ethanol soluble material was obtained.

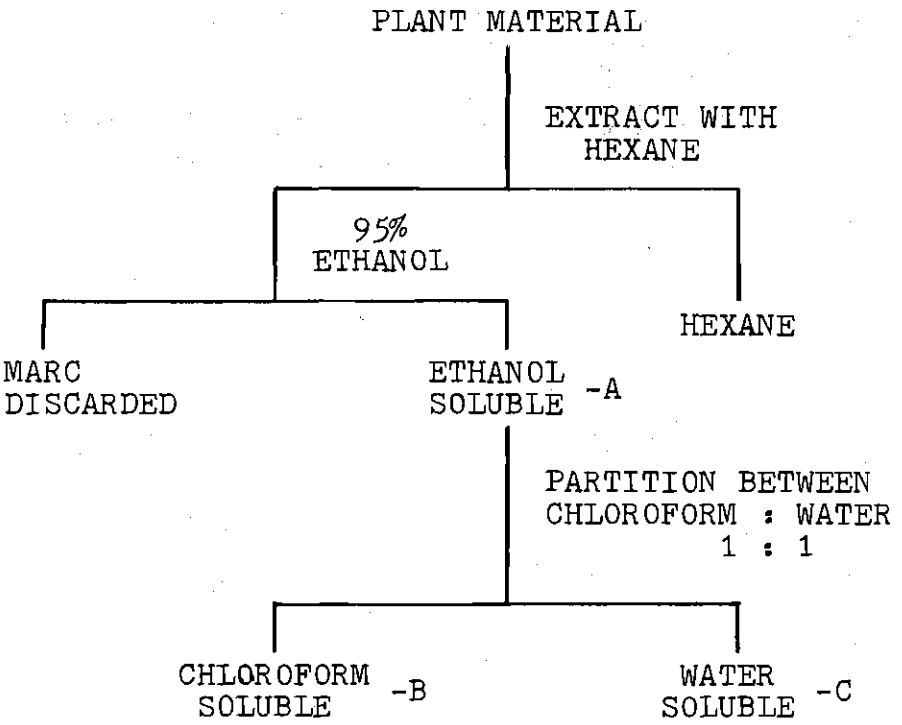
The ethanol soluble material (360 g) was dissolved in 4-l of ethanol-free chloroform and water (1:1) and placed into a large continuous chloroform extractor. The extractor was run continuously until the chloroform layer was colorless, about seven days. The chloroform and water layers were separated; after removal of the chloroform *in vacuo*, 140 g of chloroform soluble material was obtained. Removal of the water from the water layer was done by lyophilization, and 217 g of water soluble material was obtained.

Samples of the ethanol soluble material and of the chloroform soluble material were sent to the National Institute of Health for testing (see Chart 2 for complete procedure).

Attempted Separation of the Chloroform Soluble Material

Chloroform soluble plant material (1.1 g) was chromatographed on 100 g of alumina (Merck acid-washed, activity I), which was packed in benzene. The following elution scheme was followed:

Chart 2. NIH Procedure



A,B,C sent to NIH for testing.

Fraction	Solvent	Weight (g)
1(1-1)	Benzene	0.0205
2(.5-1)	1:4 Chloroform-Benzene	0.0129
3(.75-1)	1:4 Chloroform-Benzene	0.0159
4(.5-1)	1:1 Chloroform-Benzene	0.0141
5(.5-1)	Chloroform	0.0241
6(.75-1)	1:1 Chloroform-Ether	0.0412
7(.5-1)	Ether	0.0173
8(.75-1)	1:1 Ether-Ethanol	0.0717
9(1-1)	Ethanol	0.0000
		0.2177 g

The chromatography was followed by GC (Col II, 170°C); all of the fractions were complex mixtures, with most of the starting material staying on the column.

Partition of Chloroform Soluble Material Among Benzene, Ethanol and Water

A sample (79.9 g) of chloroform soluble plant extract was partitioned in the following solvent mixture: water, 250 ml, 95% ethanol, 750 ml and benzene, 3000 ml. The solution was stirred magnetically in a 6-l flask for one week. The mixture separated into two layers - a benzene rich layer and an ethanol-water rich layer. Evaporation of the solvents *in vacuo* yielded 53.3 g from the benzene rich layer and 13.7 g from the ethanol-water rich layer.

Attempted Separation of the Benzene Soluble Plant Material

The benzene soluble material (10.0 g) was charged to a chromatography column packed with 500 g of silica gel in 1:1 hexane-benzene. The column was eluted by the following scheme:

Fraction (250 ml ea)	Solvent	Weight (g)
1-6	1:1 Hexane-Benzene	2.16
7-23	Benzene	1.78
24-31	2% Ether in Benzene	1.28
32-48	10% Ether in Benzene	2.42
49-56	30% Ether in Benzene	0.72
57-68	Ethanol	0.32
		8.68 g

The chromatography was followed by GC (Col I, 170°C). It was noticed during this chromatography that not all of the peaks came off the GC at 170°C; the GC oven had to be raised to 260°C for all of the peaks to come off the GC column.

All of the fractions from this chromatography were complex showing many peaks at both 170°C and 260°C; some of the material still did not elute from the alumina column.

The benzene soluble fraction was chromatographed several times using different adsorbant - compound ratios but no pure compounds were obtained.

Base Washing of Benzene Soluble Material

Benzene soluble rayless goldenrod (10 g) dissolved in 75 ml of benzene was washed with 5% (ice-cold) sodium hydroxide until the base layer (aqueous layer) was clear (5 x 25 ml). The benzene layer was washed with water, brine and dried over magnesium sulfate. Evaporation of the solvent *in vacuo* yielded 1.26 g of non-acid, benzene soluble plant material. The aqueous layer was acidified with 6N hydrochloric acid and extracted (4 x 100 ml) with ether. Evaporation of the ether *in vacuo* yielded 8.53 g of acid plant material.

A GC study was done (Col II) in order to determine what known compounds were in the benzene, acid-free plant material. At 170°C toxol, tremetone and 2,5 diacetylbenzofuran were identified by retention time and mixed injection (see Appendix 1 p. 89 for GC traces). At 260°C, the GC traces showed at least seven peaks (see Appendix 1, p. 92 for GC trace).

Steam-Distillation of Base-Washed Benzene Soluble Plant Material

Base-washed, benzene-soluble rayless goldenrod extract (.95 g) was placed into a 250 ml round-bottomed flask with 175 ml of distilled water. This was attached to a continuous steam-distillation ether extraction apparatus.[68]

The extractor was run continuously for three days. The residue was recovered from the water by extraction with benzene (4 x 100 ml). After drying with magnesium sulfate, the solvent was evaporated *in vacuo* to yield 0.83 g of non-steam volatile residue. The steam distillate was recovered by distilling the ether through a 12-inch Vigreux column - 0.12 g of steam-volatile oil was obtained.

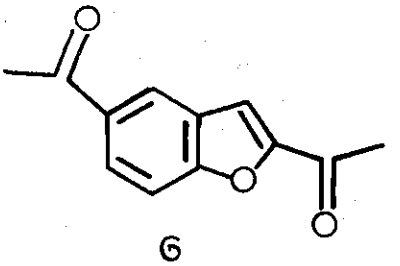
A GC study (C01 II) showed that the high molecular weight material and 2,5 diacetylbenzofuran were in the non-steam volatile residue and that tremetone, toxol and almost all the low boiling components were in the steam distillate (see Appendix 1 p. 93 for GC traces).

Isolation of 2,5 Diacetylbenzofuran

Preliminary micro-chromatographies of the non-steam volatile residue showed 2,5 diacetylbenzofuran could be separated from the high molecular weight materials by adsorbing the mixture on a silica gel column and eluting with 10% ether in benzene solution. A portion (4.71 g) of the residue was chromatographed on 200 g of silica gel packed in hexane. Fifty-eight column volumes (250 ml) were collected using the following elution scheme:

Fractions	Solvent	Weight (g)
1-6	1:1 Hexane-Benzene	0.11
7-23	Benzene	0.64
24-31	2% Ether in Benzene	0.41
32-40	10% Ether in Benzene	0.32
41-48	10% Ether in Benzene	0.57
49-56	30% Ether in Benzene	0.82
57-58	1:1 Ether-Ethanol	1.60
		4.50 g

Fraction 36 yielded 0.046 g of pure 2,5 diacetyl-benzofuran (6) which had the following spectral and physical properties:



6

GC: R_t = 6.6 min (Col II, 170°C)

MP: 139-140°C lit.[26] mp = 139-140°C

IR: ν_{CHCl_3} (cm^{-1}): 1680, 1630, 1570, 1365, 1305, 1280, 1160

^{1}H-NMR (100 MHz, δ, $CDCl_3$): 2.63 (3H, s, ketone methyl), 2.68 (3H, s, ketone methyl), 7.57 (1H, s, aromatic proton), 7.62 (1H, d, J = 9.0 Hz, aromatic proton), 8.13 (1H, dd, J = 1.7, 9.0 Hz, aromatic proton), 8.35 (1H, d, J = 1.7 Hz, aromatic proton).

^{13}C-NMR (δ, $CDCl_3$): 195.4 (s[35], ketone carbon), 186.7 (s, ketone carbon), 156.7 (s, aromatic carbon), 127.5 (d, aromatic carbon), 132.8 (s, aromatic

carbon), 126.4 (s, aromatic carbon), 124.3 (d, aromatic carbon), 111.8 (d, aromatic carbon), 152.8 (s, aromatic carbon), 113.1 (d, aromatic carbon), 26.5 (q, methyl carbon), and 26.3 (q, methyl carbon).

Mass Spectrum: M^{+}_{found} = 202 (41%), 187 (100%) see Appendix 2, p. 115.

Fraction 46 yielded 0.026 g of impure toxol (5) no other pure compounds were obtained in this chromatography. The isolation procedure for obtaining 2,5 diacetylbenzofuran is shown in Chart 3.

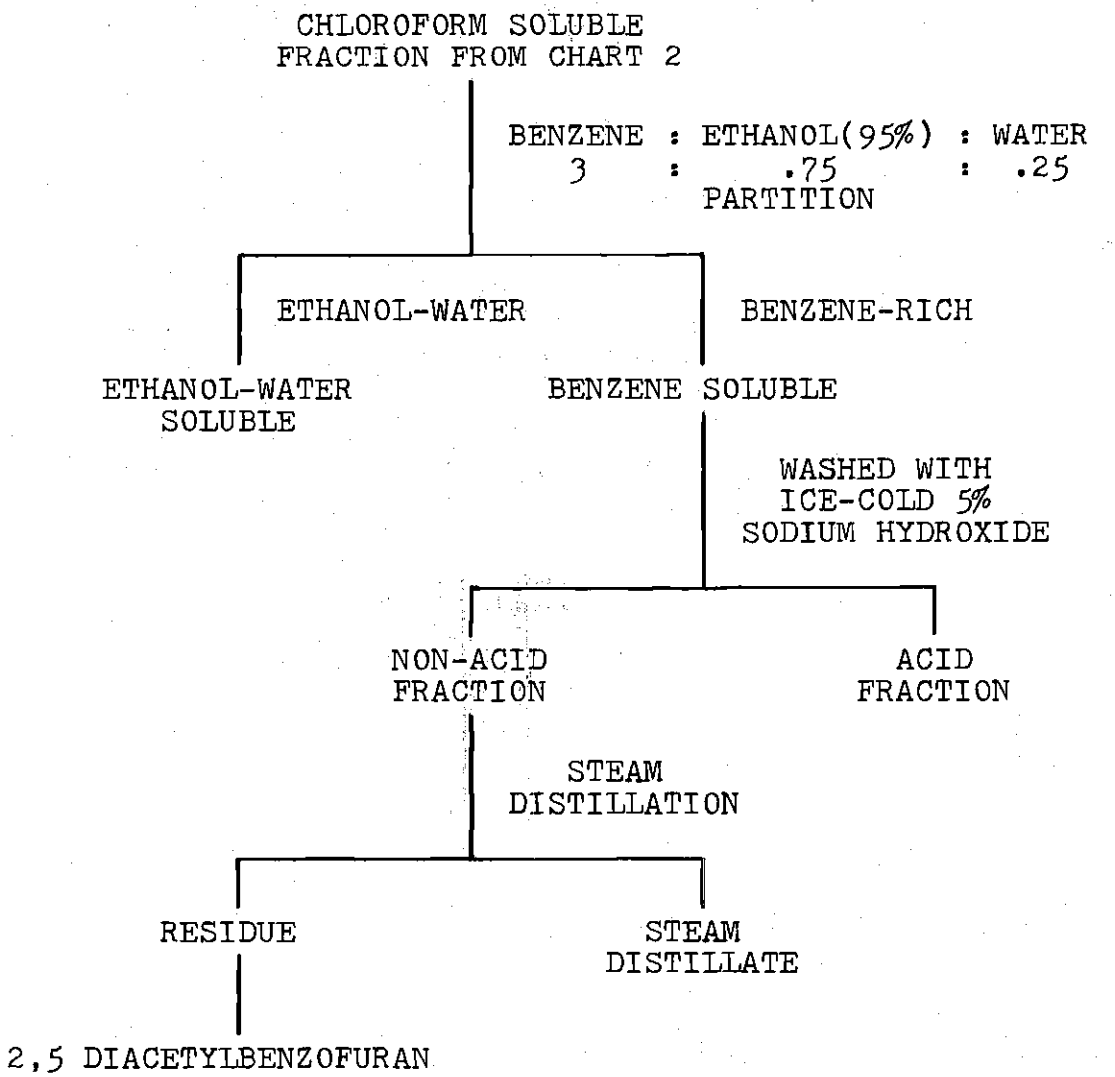
Modification of Burke's Procedure:[23]

Isolation of the "Red Jelly"

Rayless goldenrod (2.3 kg) was placed in a large Soxhlet extractor and extracted exhaustively with 10-l of freshly distilled hexane. The plant material was removed from the extractor and air-dried overnight in a fume hood. Evaporation of the hexane *in vacuo* yielded 26.8 g of hexane soluble material. The plant material was packed back into the extractor and extracted exhaustively with 10-l of distilled 95% ethanol. The ethanol was removed by rotary evaporator to yield 192.8 g of a dark green viscous gum.

The ethanol soluble material (192.8 g) was dissolved in 1.5-l of 50% aqueous methanol containing

Chart 3. Isolation of 2,5 Diacetylbenzofuran

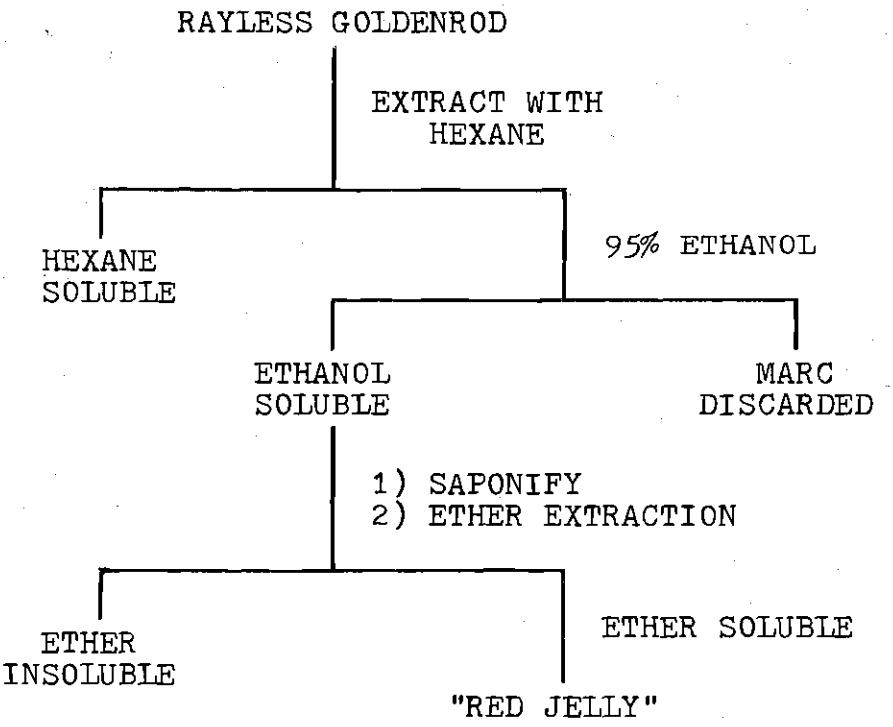


105.0 g of potassium hydroxide (reagent grade). This solution was placed into a three liter round-bottomed flask equipped with a heating mantle and a reflux condenser. The solution was refluxed for forty-eight hours. The methanol was removed by distillation and the resulting solution was diluted with one liter of water. This solution was continuously extracted with ether for forty-eight hours. The ether was dried over magnesium sulfate and evaporated *in vacuo* to yield 7.4 g of a red jelly. The procedure is outlined in Chart 4.

Separation of the "Red Jelly"

The "red jelly" (4.53 g) was dissolved in 50 ml of distilled methanol containing 4.00 g of Girard's T Reagent (see figure 1, p. 31) and 1.0 ml of glacial acetic acid. This solution was heated at reflux for one hour. The solution was then cooled in an ice bath and poured into 50 ml of a 10% sodium carbonate solution. This solution was extracted with ether (3 x 25 ml). The ether layer was separated and dried over magnesium sulfate. The ether layer was evaporated *in vacuo* to yield 3.6 g of the non-ketone fraction. The aqueous layer was acidified with 6N hydrochloric acid to a pH of 2. This solution was then extracted with ether (3 x 25 ml). After drying the ether layer over magnesium sulfate, evaporation

Chart 4. Isolation of "Red Jelly"



yielded 0.88 g of the ketone fraction of the "red jelly." This procedure is outlined in Chart 5.

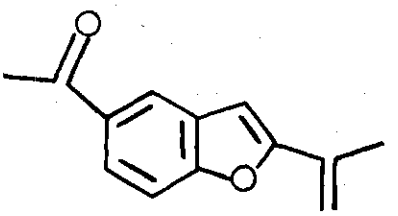
Separation of the Ketone Fraction

GC analysis (Col II, 170°C) of the ketone fraction showed three major components:
R_t = 5.3 min (17.3%), R_t = 6.3 min (13.2%), and R_t = 8.6 min (69.5%).

The ketone fraction (0.88 g) was chromatographed on 100 g of silica gel packed in 4:1 hexane:benzene. The column was eluted using the following scheme:

Fractions	Solvent	Weight (g)
1-30	Benzene	0.10
31-39	3% Ether in Benzene	0.02
40-41	10% Ether in Benzene	0.04
42-44	10% Ether in Benzene	0.01
45-75	10% Ether in Benzene	0.40
76-90	30% Ether in Benzene	0.14
		0.71 g

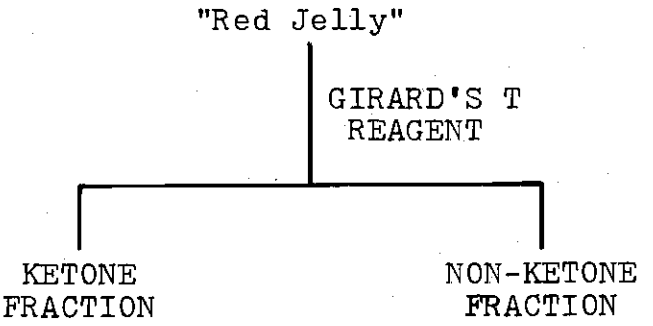
Fraction 41 yielded 43.2 mg of dehydrotremetone (2) which had the following spectral and physical properties:

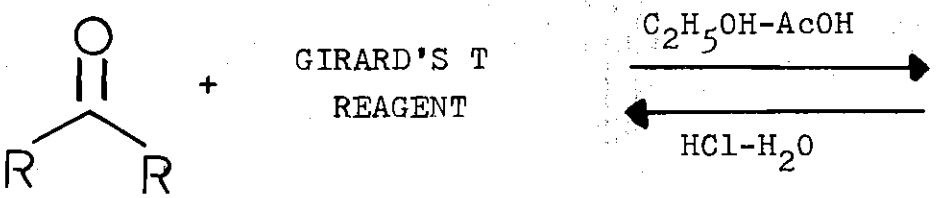


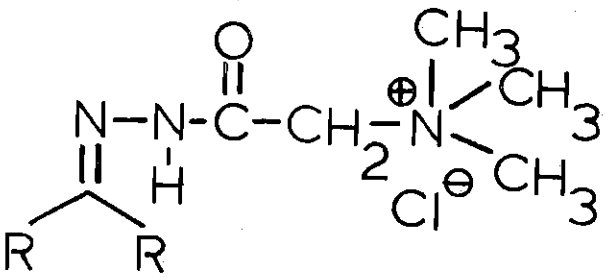
2

GC: R_t = 5.3 min (Col II, 170°C)
MP: 84-85°C (from hexane)
IR: $\nu_{KBr}(cm^{-1})$ 2955, 2925, 1675, 1595, 1565, 1370, 1310,

Chart 5. Separation of "Red Jelly"







Water-Soluble Derivative

Figure 1. Girard's T Derivative Formation

1275, 1245, 1165, 925, 840, 820

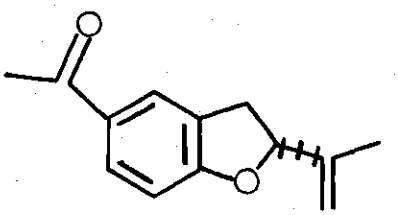
^{1}H-NMR (100 MHz, δ, $CDCl_3$): 1.80 (3H, s, olefinic methyl), 2.22 (3H, s, ketonic methyl), 5.23 (1H, bs, olefinic proton), 5.83 (1H, bs, olefinic proton), 6.67 (1H, s, aromatic proton), 7.43 (1H, d, J = 8.7 Hz, aromatic proton), 7.93 (1H, dd, J = 2.0, 8.7 Hz, aromatic proton), 8.17 (1H, d, J = 2.0 Hz, aromatic proton).

^{13}C-NMR (δ, $CDCl_3$): 196.3 (s, ketone carbon), 157.5 (s, aromatic carbon), 156.4 (s, aromatic carbon), 131.9 (s, olefin carbon), 128.4 (s, aromatic carbon), 121.4 (d, aromatic carbon), 113.6 (t, olefin carbon), 102.5 (d, aromatic carbon), 26.4 (q, methyl ketone carbon), and 19.0 (q, methyl olefin carbon).

UV (95% ethanol): 254 (36,900), 282 (18,260), 285 (16,700), 294 (14,000), 310 (4,660).

Mass Spectrum: M^{+}_{found} = 200 (52%), 185 (100%) see Appendix 2, p. 113.

Fraction 43 and 44 yielded ten mg of tremetone (1) which had the following physical and spectral properties:



1

GC: R_t = 6.3 min (Col II, 170°C)

MP: 38-39°C

IR: ν_{CCl_4} (cm^{-1}): 1672, 1605, 1265, 1230, 905

^{1}H-NMR (100 MHz, δ, $CDCl_3$): 1.76 (3H, bs, olefinic methyl protons), 2.53 (3H, s, ketonic methyl protons), 3.08 (1H, m), 3.36 (1H, m), 5.09 (1H, bs, olefinic proton), 5.18 (1H, bs, olefinic proton), 5.27 (1H, t, J = 9.0 Hz, aromatic proton), 6.81 (1H, d, J = 9.0 Hz, aromatic proton), 7.84 (2H, m, aromatic protons).

^{13}C-NMR (δ, $CDCl_3$): 195.3 (ketone carbon), 163.3 (aromatic carbon), 142.6 (olefinic carbon), 130.3 (aromatic carbon), 129.9 (aromatic carbon), 126.9 (aromatic carbon), 124.9 (aromatic carbon), 111.9 (olefinic carbon), 108.3 (aromatic carbon), 86.5 (benzofuran methine carbon), 33.9 (benzofuran methylene carbon), 26.1 (ketone methyl), and 17.0 (olefinic methyl).

ORD (c = 0.87, abs. ethanol): $[\emptyset]_{589}$ = -76.6($[\alpha]^{20}_{589}$ = -37.9, $[\emptyset]_{335\ trough}$ = -871.7

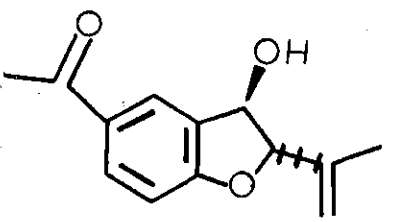
UV (abs. ethanol): 226 (8630), 279 (8100), 286 (7,900).

Analysis: ($C_{13}H_{14}O_2$) calculated: C 77.20, H 6.98

found C 77.15, H 7.01

Mass Spectrum: M^+_{found} = 202 (91%), 43 (100%) see Appendix 2, p. 112.

Fractions 76-90 yielded 140.0 mg of pure toxol (5) which had the following spectral and



5

physical properties:

GC: R_t = 8.6 min (Col II, 170°C)

IR: ν_{CHCl_3} (cm^{-1}): 3450, 1670, 1660, 1610

^{1}H-NMR (100 MHz, δ, $CDCl_3$): 1.73 (3H, s, olefinic methyl protons), 2.51 (3H, s, ketonic methyl protons), 3.87 (1H, bm, hydroxyl proton), 4.93 (1H, bs, olefinic proton), 5.08 (1H, bs, olefinic proton), 4.97 (1H, m), 5.15 (1H, m), 6.88 (1H, d, J = 8.5 Hz, aromatic proton), 7.88 (1H, dd, J = 2.0, 8.5 Hz, aromatic proton), 8.02 (1H, d, J = 2.0 Hz, aromatic proton).

^{13}C-NMR (δ, $CDCl_3$): 196.3 (s, ketone carbon), 163.5 (s, aromatic carbon), 140.5 (s, olefinic carbon), 131.7 (d, aromatic carbon), 130.1 (s, aromatic carbon), 128.5 (s, aromatic carbon), 126.3 (d, aromatic carbon), 112.2 (t, olefinic carbon), 109.4 (d, aromatic carbon), 94.3 (d, benzofuran methine carbon), 75.3 (d, benzofuran hydroxyl carbon), 26.3 (q, ketone methyl carbon), 17.4 (q, olefinic methyl carbon).

ORD (c = 4.80, abs. ethanol): $[\phi]_{589}$ = -25.1 ($[\alpha]^{20}_{589}$ = -11.5), $[\phi]_{550}$ = -31.9, $[\phi]_{500}$ = -45.4, $[\phi]_{450}$ = -68.3, $[\phi]_{400}$ = -122.7

Mass Spectrum: M^+_{found} = 218 (28%), 43 (100%) see Appendix 2, p. 114.

An outline of the isolation procedure used to obtain toxol (5), dehydrotremetone (2), and tremetone (1), is shown

in Chart 6.

Separation of Non-Ketone Fraction

A GC study (Col I, program temp: 150°C, hold for eight min, then programmed at 20°C/min to 245°C and held at 245°C) showed that the non-ketone mixture was complex having low and high boiling components (see Appendix 1, p. 96 for the GC trace).

The non-ketone fraction (33.9 g) was chromatographed on 380 g of alumina (acid-washed, Merck, activity I) packed in hexane. The column was eluted using the following scheme:

Fractions	Solvent	Weight (g)
1A	Hexane	2.01
2A	Hexane	0.16
3A	Benzene	1.20
4A	Benzene	4.08
5A	Benzene	1.14
6A	Chloroform	4.91
7A	Chloroform	3.25
8A	1:1 Chloroform-Methanol	9.03
9A	Methanol	3.27
10A	Methanol	1.26
11A	Methanol	1.31
		31.90 g
		93% recovery

The chromatography was followed by GC (Col I, program temp: same as above). The GC traces are in

Chart 6. Separation of Ketone Fraction

KETONE FRACTION

CHROMATOGRAPHY ON 100g OF SILICA GEL

10% ETHER IN BENZENE (100 ml)	DEHYDROTREMETONE
10% ETHER IN BENZENE (300 ml)	TREMETONE
30% ETHER IN BENZENE	TOXOL

Appendix 1, p. 97 . An outline of the chromatography as shown in Chart 7.

Isolation of Phytol

GC trace #4A (Appendix 1, p. 99) showed that there was mostly one component in this fraction. Fraction 4A (4.08 g) was chromatographed on 200 g of alumina (Activity I) packed in benzene. The following elution scheme was used:

Fraction	Solvent	Weight (g)
1B	Benzene	0.03
2B	Benzene	0.53
3B	Benzene	0.44
4B	Benzene	0.45
5B	Benzene	0.70
6B	Benzene	0.69
7B	Benzene	0.34
8B	Benzene	0.27
9B	Chloroform	0.15
10B	Chloroform	0.18
11B	Chloroform	0.00
		3.78 g

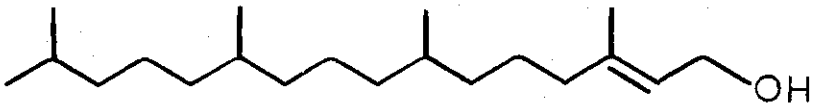
The chromatography was followed by GC (Col I, program temp: same as above). Fractions 8B and 9B were combined to yield 0.42 g of a compound that had the same spectral properties as phytol (18)[36]:

Chart 7. Separation of Non-Ketone Fraction

NON-KETONE FRACTION

CHROMATOGRAPHY ON 380g ALUMINA (ACTIVITY I)

HEXANE (500 ml)	1A
HEXANE (500 ml)	2A
BENZENE (250 ml)	3A
BENZENE (500 ml)	4A
BENZENE (250 ml)	5A
CHLOROFORM (500 ml)	6A
CHLOROFORM (500 ml)	7A
CHLOROFORM 1:1 METHANOL (500 ml)	8A
METHANOL (100 ml)	9A
METHANOL (500 ml)	10A
METHANOL (2000 ml)	11A



18

GC: R_t = 11.5 min (Col I, Program temp: same as above).

IR: $\nu_{CHCl_3}(cm^{-1})$: 3400, 2935, 1650, 1460, 1380, 980

^{1}H-NMR (60 MHz, δ, $CDCl_3$): 0.83 (6H, d, J = 4.0 Hz), 0.84 (6H, s), 1.30 (18H, bs), 1.65 (3H, s), 2.00 (4H, m), 4.19 (2H, bd), 5.43 (1H, m)

Mass Spectrum: M^+_{found} = 296 (1%), 71 (100%) see Appendix 2, p. 117.

The isolation procedure used to obtain phytol (18) is shown on Chart 8.

Isolation of Hentriacontane

GC trace #1A (Appendix 1, p. 97) showed that there were three major components in this fraction - R_t = 15.6 min, 16.5 min, and 19.0 min. Fraction 1A (1.89 g) was chromatographed on 200 g of alumina (activity I) packed in hexane. The following elution scheme was used:

Fraction	Solvent	Weight (g)
1C	Hexane	0.88
2C	Hexane	0.00
3C	Hexane	0.10
4C	Hexane	0.00
5C	Hexane	0.00

Chart 8. Isolation of Phytol

FRACTION 4A (CHART 7)

CHROMATOGRAPHY ON 200g ALUMINA (ACTIVITY I)

- BENZENE (100 ml) — 1B
- BENZENE (200 ml) — 2B
- BENZENE (400 ml) — 3B
- BENZENE (400 ml) — 4B
- BENZENE (400 ml) — 5B
- BENZENE (400 ml) — 6B
- BENZENE (400 ml) — 7B
- BENZENE (400 ml) — 8B
- CHLOROFORM (400 ml) — 9B

PHYTOL (8B, 9B)

- CHLOROFORM (400 ml) — 10B
- CHLOROFORM (400 ml) — 11B

6C	Hexane	0.00
7C	Hexane	0.13
8C	Hexane	0.00
9C	Hexane	0.00
10C	1:1 Hexane-Benzene	0.33
11C	Benzene	0.06
12C	Benzene	0.00
13C	Chloroform	0.07
14C	Chloroform	0.04
		1.16 g
		85% recovery

The chromatography was followed by GC (Col I, program temp: same as above). The components with an R_t of 16.5 and 19.0 min came off in the first fraction, #1C. The component with the R_t of 15.6 min came off in the third fraction, #3C but was contaminated with the first two components.

Fraction #1C, (0.782 g), was chromatographed on 200 g of alumina (activity I) packed in hexane. The following elution scheme was used:

Fraction	Solvent	Weight (g)
1F	Hexane	0.0000
2F	Hexane	0.0000
3F	Hexane	0.0000
4F	Hexane	0.0000
5F	Hexane	0.0144
6F	Hexane	0.4491
7F	Hexane	0.2911

8F	Hexane	0.0194
9F	Hexane	0.0060
10F	Hexane	0.0000
		0.7800 g

The chromatography was followed by GC (Col IV, program temp: 160°C, then program at 8°C/min to 285°C and held at 285°C). Fraction 7F was composed mostly of the component with the longest R_t - on this column (Col IV) the R_t = 15.6 min. Fraction 7F (0.2911 g) was chromatographed on 150 g of alumina (activity I) packed in hexane. The following elution scheme was used:

Fraction	Solvent	Weight (g)
1H	Hexane	0.0000
2H	Hexane	0.0000
3H	Hexane	0.0000
4H	Hexane	0.0000
5H	Hexane	0.0082
6H	Hexane	0.1024
7H	Hexane	0.0598
8H	Hexane	0.0286
9H	Hexane	0.0135
10H	Hexane	0.0140
11H	Hexane	0.0164
12H	Hexane	0.0142
13H	Hexane	0.0101
14H	Hexane	0.0048
15H	Hexane	0.0000
		0.2720 g
		93% recovery

The chromatography was followed by GC (Col IV, same program as before). Fraction 10H (0.0140 g) contained the high R_t component; it was 95% pure (by GC). The component had the same spectral and physical properties as hentriacontane (19)[37]:

$$CH_3-(CH_2)_{29}-CH_3$$

19

GC: R_t = 5.4 min (Col III, 290°C)

MP: 66-68°C lit.[39] = 67°C

IR: ν_{CCl_4} (cm^{-1}): 2950, 2930, 2850, 1460, 1255, 1095, 1015

^{1}H-NMR: (60 MHz, δ, $CDCl_3$) 1.28 (s)

Mass Spectrum: M^+_{found} = 436 (4%), 57 (100%) see Appendix 2, p. 118.

Mass spectral and gas chromatographic studies were done on fraction 10H in order to show that it was indeed hentriacontane (19). Mass spectra were obtained for triacontane (C_{30}) and dotriacontane (C_{32}). These can be compared in Appendix 2, p. 119. These are identical except for their respective molecular ions. GC traces were obtained for triacontane and for dotriacontane, R_t = 4.4 and 6.5 min respectively (Col IV, 290°C). A mixed injection of fraction 10H and the C_{30} and C_{32}

hydrocarbons show that the retention time of fraction 10H to be inbetween the C_{30} and C_{32} hydrocarbons. The GC traces are shown in Appendix 1, p. 108. The isolation procedure used to obtain hentriacontane (19) is shown on Chart 9.

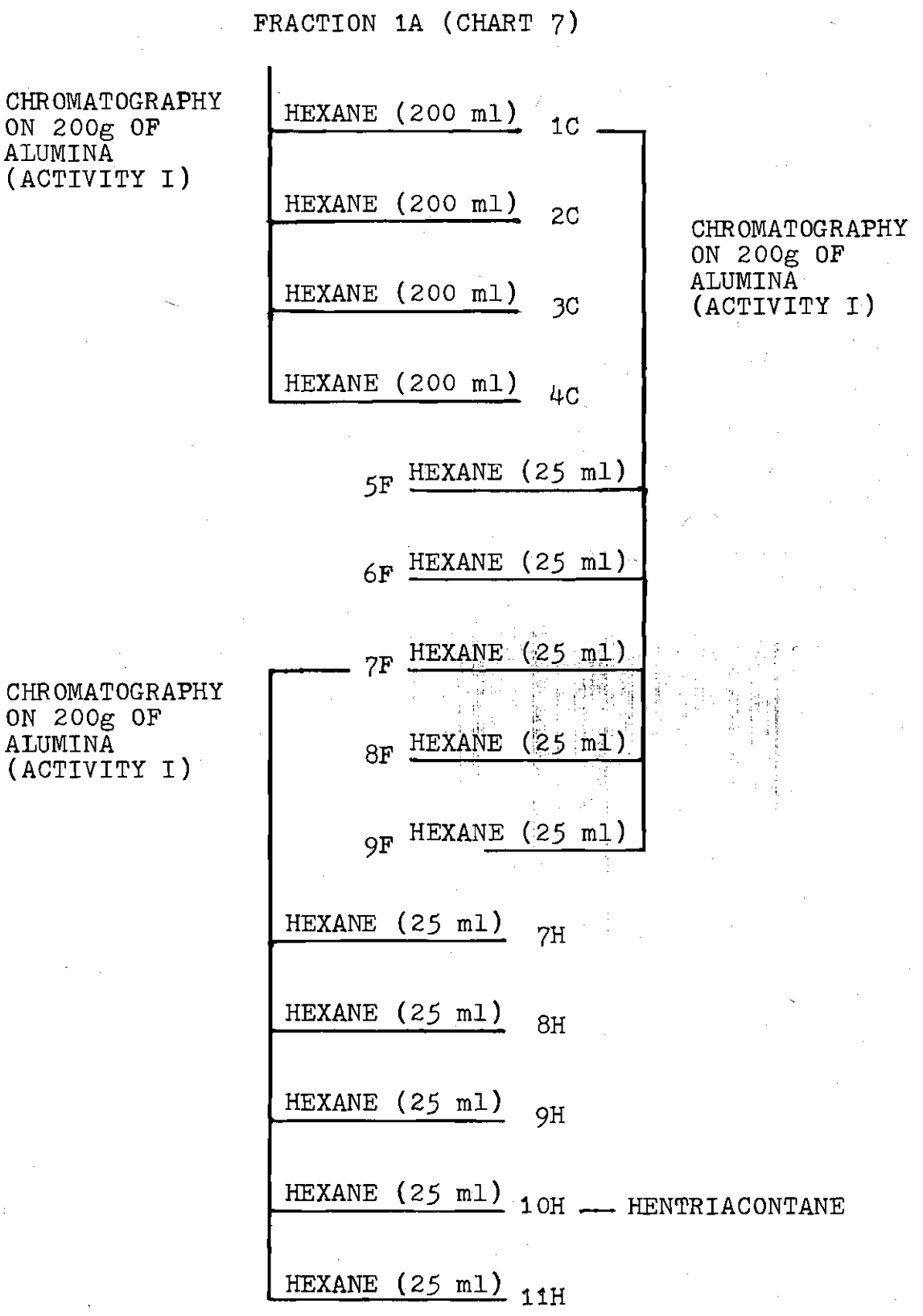
Isolation of Squalene

GC trace #3C, Appendix 1, p. 107, showed mostly one component (Col I, program temp: start at 210°C, held for 2 min, then 20°C/min to 245°C and held), R_t = 5.5 min. Fraction #3C (156.7 mg) was chromatographed on 16.0 g of alumina (activity I) packed in hexane. The following elution scheme was used:

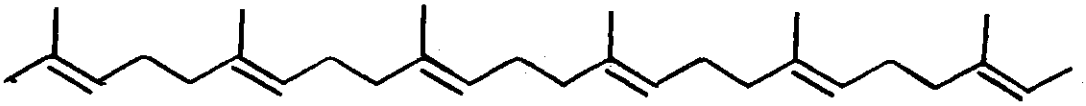
Fraction	Solvent	Weight (g)
1E	Hexane	0.0494
2E	Hexane	0.0417
3E	Hexane	0.0476
4E	Hexane	0.0000
5E	Hexane	0.0000
6E	Hexane	0.0000
7E	Hexane	0.0000
		0.1387
		89% recovery

The chromatography was followed by GC (Col I, program temp: same as above). Fraction 3C was composed of 95% of the component with the R_t = 5.5 min. The component

Chart 9. Isolation of Hentriacontane



had the same spectral properties as squalene (20)[38]:



20

GC: R_t = 5.5 min (Col I, program temp: same as above)

^{1}H-NMR: (60 MHz, δ, $CDCl_3$) 1.60 (24H, s, methyls), 1.98 (20H, bs, methylenes), 5.10 (6H, bm, olefin protons).

Mass Spectrum: M^+_{found} = 410 (2%), 81 (100%) see Appendix 2, p. 121.

The isolation procedure used to obtain squalene (20) is shown on Chart 10.

Isolation of the Sterol Fraction

GC trace #7A (Appendix 1, p. 102) showed that fraction 7A consisted mostly of three components of R_t = 19.7, 20.4, and 21.4 min respectively. Fraction 7A (3.25 g) was chromatographed on 300 g of alumina (activity I) packed in benzene. Thirty-eight fractions of 300 ml each were taken using benzene with increasing amounts of chloroform to 100% chloroform as an eluent. GC analysis (Col I, 285°C) of the fractions showed that they could be combined as follows:

Chart 10. Isolation of Squalene

FRACTION 3C (CHART 9)

CHROMATOGRAPHY ON 16.0g ALUMINA (ACTIVITY I)

- HEXANE (10 ml) — 1E
- HEXANE (10 ml) — 2E
- HEXANE (10 ml) — 3E — SQUALENE
- HEXANE (10 ml) — 4E
- HEXANE (10 ml) — 5E
- HEXANE (10 ml) — 6E
- HEXANE (10 ml) — 7E

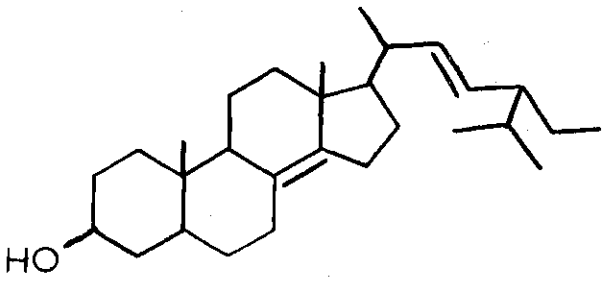
Fractions	Solvent	Weight (g)
1D comb (1-12)	Benzene	0.16
2D comb (13-18)	2:1 Benzene-Chloroform	0.22
3D comb (19-21)	1:1 Benzene-Chloroform	0.64
4D comb (22-26)	Chloroform	1.16
5D comb (27-38)	Chloroform	0.86
		3.04 g
		94% recovery

Fraction 4D comb contained the three high molecular weight components. All three components eluted from the column together. Fraction 4D comb (1.16 g) was chromatographed on 100 g of alumina (activity III) packed in hexane. Fifty 25 ml fractions were taken, using benzene as the eluent. GC analysis (Col IV, 285°C) showed that the fractions could be combined as follows:

Fractions	Solvent	Weight (g)
1G comb (1-25)	Benzene	0.4944
2G comb (26-29)	Benzene	0.1902
3G comb (30-32)	Benzene	0.0900
4G comb (33-50)	Benzene	0.1770
		0.9516 g
		82% recovery

Fraction 3G comb was shown by GC analysis to be composed mostly of the component with the middle R_t - 20.4 min (Col I, 285°C), 90% pure by GC. The spectral and physical properties of fraction 3G comb

were shown to be identical with those of stigmasta-8(14), 22-dien-3β-ol (9)[32]:



9

GC: 20.4 min (Col I, 285°C), 6.3 min (Col IV, 285°C)

MP: 164-165°C lit.[32] = 165-166°C

IR: ν_{CCl_4} (cm^{-1}) 3600, 2950, 2865, 1460, 1375, 1145, 975

^{1}H-NMR (100 MHz, δ, $CDCl_3$): 0.55 (3H, s), 0.80 (3H, s), 0.78-1.80 (39H, overlapping multiplets), 3.62 (1H, bm), 5.09 (2H, m).

Mass Spectrum: M^+_{found} = 412 (3%), 43 (100%) see Appendix 2, p. 122.

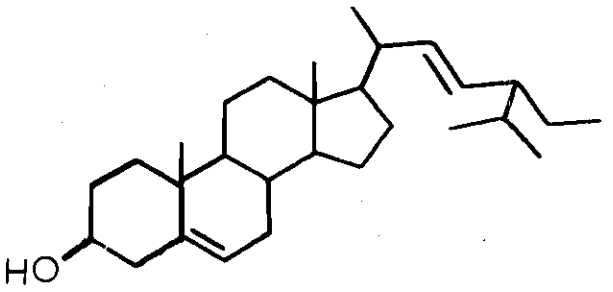
The other two high molecular weight components did not separate on this column. Fraction 1G comb contained all three components. GC analysis of this fraction on another column, Col V at 290°C, showed that the mixture had actually four components, R_t = 14.0, 16.3, 18.4 and 20.1 min respectively, (see Appendix 1, p. 110 for the GC trace). The component with the R_t = 16.3 min was shown to be 2 by R_t and mixed injection.

Chromatography on silica gel and on silica gel

impregnated with silver nitrate failed to separate the mixture.

The mixture was finally separated by preparative gas chromatography (Col V, 290°C).

The component with the R_t of 14.0 min had the same spectral and physical properties as an authentic sample of stigmasta-5,22-dien-3β-ol (21)[40]:



21

GC: R_t = 14.0 min (Col V, 290°C), authentic sample has same R_t.

MP: 168-170°C; MP of authentic sample = 170°C. Mixed MP showed no depression.

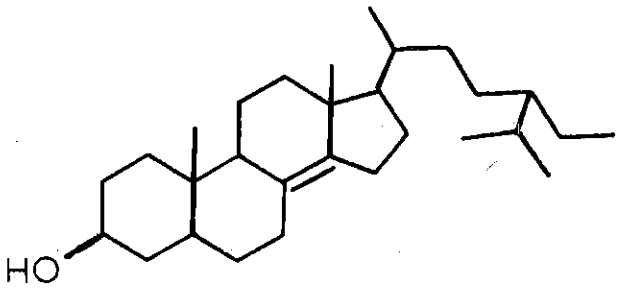
IR: ν_{CHCl_3} (cm^{-1}) 3600, 2965, 2875, 1460, 1380, 1030, 974

^{1}H-NMR: (100 MHz, δ, $CDCl_3$) 0.70 (3H, s), 0.77-0.88 (9H, overlapping multiplets), 1.01 (3H, s), 1.03 (3H, d, J = 10 Hz), 1.1-2.0 (26H, bm), 3.52 (1H, bm), 5.09 (2H, t, J = 6 Hz), 5.35 (1H, bd, J = 6 Hz).

Mass Spectrum: M^+_{found} = 412 (17%), 55 (100%) see Appendix 2, p. 123 for the spectrum of the

natural material and the authentic sample.

The component with the R_t = 18.4 min had the same spectral properties and MP as stigmasta-8(14)-en-3β-ol (22)[41]:



22

GC: R_t = 18.4 min (Col V, 290°C)

MP: 109-111°C lit. MP = 108-111°C.

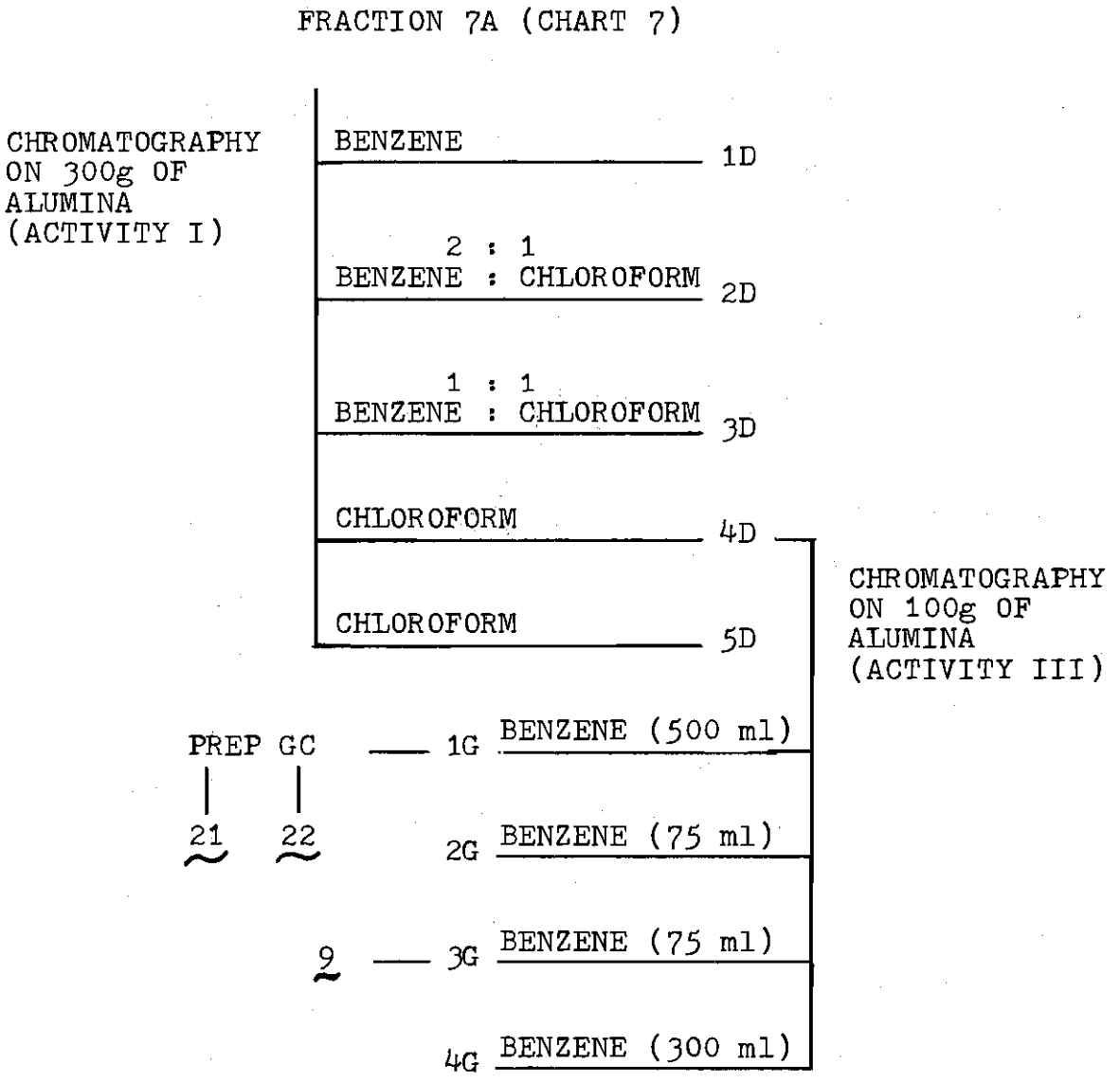
IR: ν_{CCl_4} (cm^{-1}) 3600, 2945, 2855, 1455, 1375, 1035

^{1}H-NMR: (100 MHz, δ, $CDCl_3$) 0.54 (3H, s), 0.80 (3H, s), 0.78-1.60 (41H, overlapping multiplets), 3.60 (1H, bm).

Mass Spectrum: M^+_{found} = 414 (100%), 255 (45%) see Appendix 2, p. 125.

The component with the R_t = 20.1 min could not be obtained in enough quantity so that spectral measurements could be obtained. The isolation procedure used in obtaining the sterols is shown on Chart 11.

Chart 11. Isolation of the Three Steroids



CHAPTER IV

DISCUSSION OF RESULTS

As stated in the Historical Introduction, the purpose of this research was to find as many of the major secondary plant metabolites of Isocoma wrightii as possible with the hope of finding the agent responsible for the anti-tumar activity of the chloroform fraction of the ethanolic extract of the plant.

Extraction of Plant Material and the Isolation of a Series of Benzofurans

The plant material used in this study was the above-ground portions of Isocoma wrightii (rayless goldenrod) collected near the Artesia, New Mexico area in October, 1968. This material was dried in a low-humidity atmosphere and then coarsely ground.

The plant material was extracted using a procedure recommended by the National Institute of Health. This procedure is shown on Chart 2, p. 20. The rayless goldenrod was placed in large Soxhlet extractors and extracted first with hexane to defat the plant. The plant material was then removed from the extractors and dried overnight in a fume hood. The material was then exhaustively extracted

with 95% ethanol. Two Soxhlet extractors were usually used, holding a total of approximately five kilograms of plant material and a total of 20 liters of each solvent. Evaporation of the hexane gave 80-85 g of hexane soluble plant extract. Evaporation of the ethanol yielded 350-365 g of ethanol soluble plant extract. The extraction procedure usually took about one week.

The ethanol soluble material was then dissolved in a total of four liters of ethanol-free chloroform and water (1:1) and placed into a large continuous chloroform extractor. The extractor was run until the chloroform layer was colorless; this usually took about one week. The two layers were then separated. About 140 g of chloroform soluble extract were obtained, after removal of the solvent. This was the extract that exhibited the anti-tumor activity. The results of the anti-tumor testing will be discussed in a later section. Removal of the water by lyophilization, yielded about 220 g of water soluble plant extract.

Separation of the chloroform extract was attempted using column chromatography on alumina; however, only complex mixtures were obtained with most of the chloroform extract staying on the column even after elution with ethanol. It was decided that further solvent partitioning was needed. The chloroform plant extract (79.9 g) was

dissolved in a mixture of benzene (3000 ml), water (250 ml), and 95%ethanol (750 ml). This mixture, stirred in a six liter flask for one week, separated on standing into an upper benzene-rich layer and a lower ethanol-water layer. After separation, evaporation of the solvents yielded about 53 g of benzene soluble material and 14 g of ethanol-water soluble material.

The benzene soluble plant extract was chromatographed on silica gel but again only complex mixtures resulted with some material still not eluting from the column. Separation of the benzene soluble plant material was attempted several times using different adsorbant (silica gel) - compound ratios but no separation was achieved.

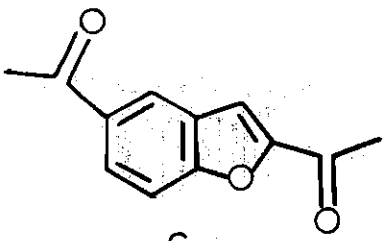
The benzene soluble plant material (10 g) was dissolved in 75 ml of benzene and washed with 5% sodium hydroxide solution (ice-cold) in order to remove any acid material present in the extract. The benzene layer was then washed with water, with brine and then dried over magnesium sulfate. Evaporation of the benzene yielded 1.3 g of non-acid, benzene soluble plant material. The aqueous layer was acidified with 6N hydrochloric acid and extracted with ether. Evaporation of the ether layer yielded 8.5 g of acid material. Therefore, approximately 85% of the benzene soluble plant material dissolved in the sodium hydroxide solution. This large acid fraction

has been observed in both Isocoma wrightii and Eupatorium rugosum.[42a] A gas chromatography study of the acid-free, benzene soluble extract (Col II, 170°C) showed that toxol (5), tremetone (1), and 2,5 diactylbenzofuran (6) were present in this extract. This was shown by GC R_t comparison and mixed injection of extract and known samples. At 260°C on the same column, the GC traces showed at least seven peaks. See Appendix 1, p. 92.

It was hoped that if the benzene soluble extract was steam distilled, the benzofurans would be freed of the higher molecular weight compounds. Therefore, the benzene soluble extract (0.95 g) was placed into a 250 ml round-bottomed flask with 175 ml of distilled water. This flask was attached to a continuous steam distillation-ether extraction apparatus. The extractor was run for three days. The residue was recovered from the water by extraction with benzene. After drying with magnesium sulfate, evaporation of the solvent yielded 0.83 g of non-steam volatile residue. The steam distillate was recovered by distilling the ether through a Vigreux column - 0.12 g of steam-volatile oil was obtained. GC analysis (Col II, 170°C and 260°C) showed that the high molecular weight material and 2,5 diacetylbenzofuran were in the non-steam volatile residue and that toxol, tremetone and almost all of the lower boiling components were in the steam distillate.

The GC traces are in Appendix 1, p. 95.

Column chromatography on silica gel of the non-volatile steam distillation residue gave a crystalline solid, MP = 139-140°C. It had the same MP and IR spectrum as 2,5 diacetylbenzofuran (6), as reported by Ramming.[26] It had the same R_t on GC (Col II, 170°C) as an authentic sample - alone and on admixture. A sample of 6 was sent to NIH for anti-tumor testing. The procedure used to



6

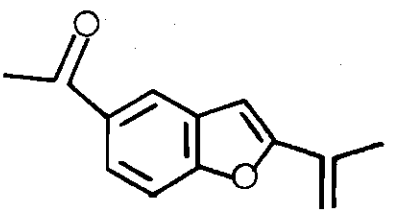
obtain 6 is shown on Chart 3. No other pure compounds were isolated. The steam volatile portion of the benzene extract was not investigated at this time, since it was a complex mixture and could be obtained only in small quantities.

Since we wanted to send samples of toxol (5), tremetone (1) and dehydrotremetone (2) to NIH for testing, it was decided to abandon the NIH procedure at this point and go on with a modification of Burke's procedure for obtaining 2 and 5.[23]

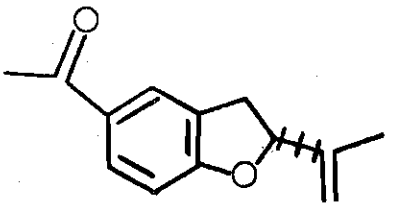
Rayless goldenrod was extracted as described above to yield the hexane soluble fraction and the ethanol

soluble fraction. The ethanol soluble fraction (192.8 g) was dissolved in 1.5-l of 50% aqueous methanol containing 105.0 g of potassium hydroxide. This solution was heated at reflux for forty-eight hours. The methanol was then removed by distillation and the resulting solution was diluted with one liter of water. This solution was continuously extracted with ether for forty-eight hours. The ether solution was dried over magnesium sulfate and evaporated to give 7.4 g of a red jelly-like material. This "red jelly" was separated into a ketone and a non-ketone fraction using Girard's T reagent, see Figure 1, p. 31. This separation procedure is outlined on Charts 4 and 5.

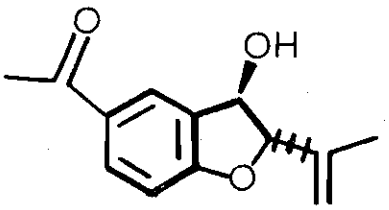
The ketone fraction was chromatographed on silica gel. Elution with 10% ether in benzene yielded first a crystalline solid (A) and an oily liquid (B). Elution with 30% ether in benzene yielded another oily liquid (C). The solid (A) had a MP of 84-85°C. It had the same MP and IR spectrum as reported for dehydrotremetone (2).[17] Oil (B) had the same IR spectrum as tremetone (1).[24] The second oil (C) had the same IR spectrum as toxol (5).[23]



2



1



5

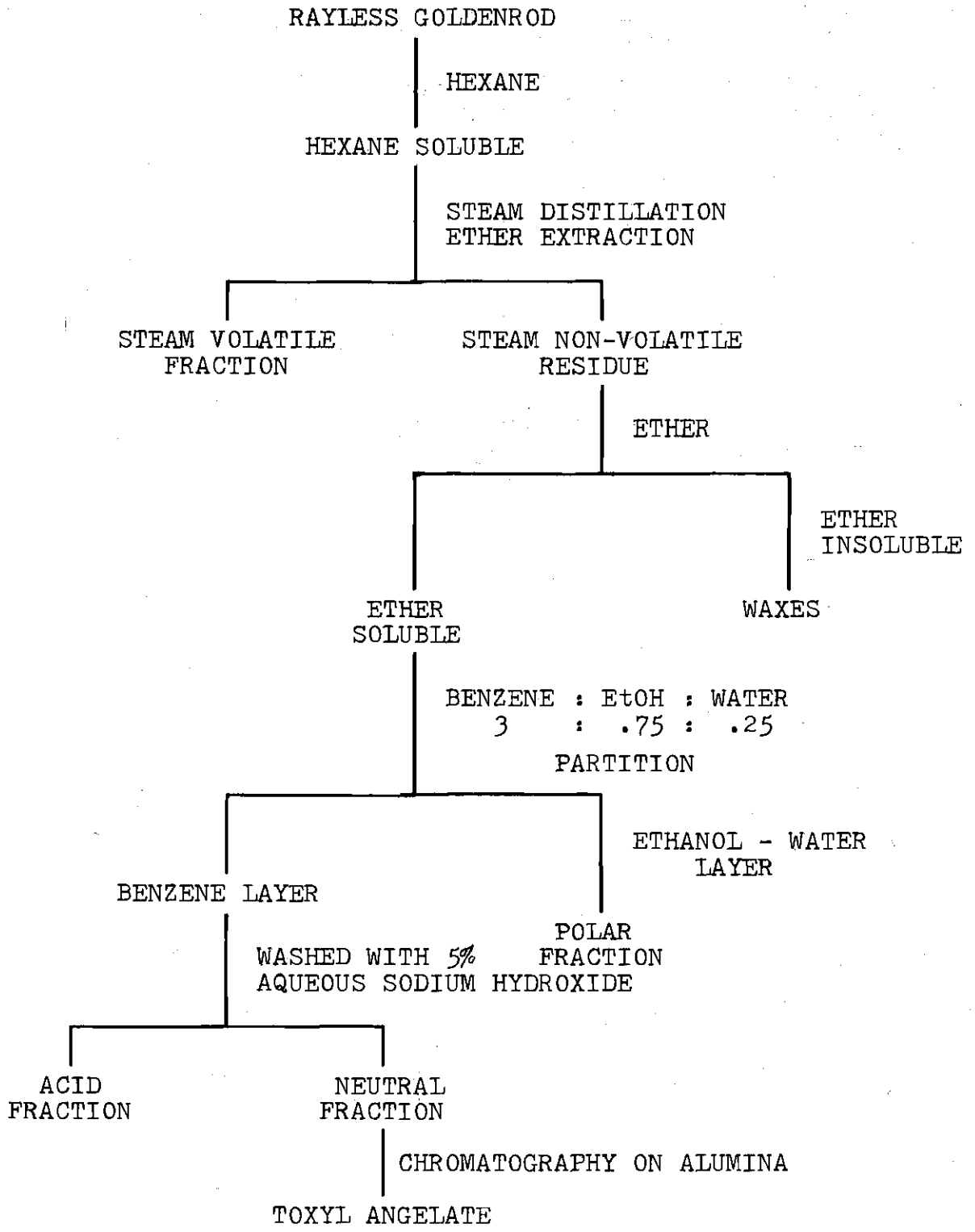
Both tremetone (1) and toxol (5) had the same retention time as authentic samples, both alone and on admixture. An authentic sample of dehydrotremetone (2) was not available. ^{1}H-NMR spectra (100 MHz) and mass spectra were obtained on all of the benzofurans. In addition, enough of the benzofurans was isolated so that ^{13}C-NMR spectra were also obtained. These spectra will be discussed later. Samples of the three benzofurans were sent to NIH for anti-tumor testing. An outline of the isolation procedure used to obtain toxol (5), dehydrotremetone (2) and tremetone (1) is shown in Chart 6.

Recently in our laboratories another new benzofuran was isolated, 23.[42b] The isolation procedure used to obtain this compound is shown in Chart 12.

The structure of 23 was elucidated as follows:

1) Analysis indicated the molecular formula to be $C_{18}H_{20}O_4$.
2) It showed a negative Cotton effect in its ORD curve [like toxol (5)], and had an optical rotation of $[\alpha]_D^{25} = -114.7$ (c = 4.62) determined by polarimeter and $[\alpha]_{589}^{30.5} = -105.6$ (c = 2.31) calculated from ORD curve.

Chart 12. Isolation of Toxyl Angelate



3) The infrared spectrum (film) showed two strong carbonyl bands at 1680 cm^{-1} (conjugated C=O) and 1715 cm^{-1} (conjugated C=O),as well as bands at 1645 cm^{-1} (conjugated C=C), 1600 cm^{-1} (C=C aromatic) and very strong bands in the 1150-1300 cm^{-1} region (α,β-unsaturated esters).

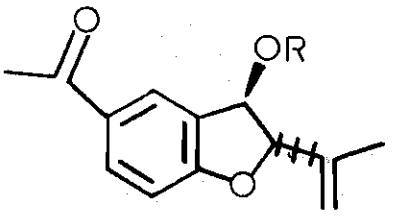
4) The 60 MHz ^{1}H-NMR spectra (CCl_4) showed the presence of a methyl ketone [δ 2.47 (3H)] and a methyl on a double bond [δ 1.78 (3H)] - both of these absorptions are indicative of benzofurans of the type isolated so far in the plant. The NMR spectra also indicated two more methyls on a double bond [δ 7.93-8.05 (2H, m) and δ 6.89 (1H, d)]. The NMR spectra also showed a doublet centered at [δ 5.0 (3H)] and a multiplet centered at [δ 6.1 (2H)].

5) The mass spectra gave a molecular ion, M^+_{found} = 300 (3%) and other fragments at 217 (5%), 201 (22%), 200 (100%), 185 (80%), 157 (19%), 145 (4%), 128 (10%), 115 (5%), 100 (11%), 91 (4%), 85 (5%), 83 (90%), 77 (6%), 63 (5%), 55 (53%), 51 (5%), 43 (58%), 41 (8%), 39 (10%), 29 (8%), and 27 (5%). This fragmentation pattern will be discussed in the NMR-mass spectrum section.

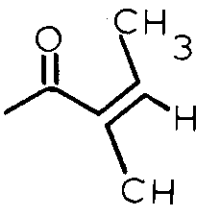
6) Hydrolysis of a sample of 23 yielded toxol (5) whose retention time (Col II, 170°C) was identical with that of an authentic sample - alone and on admixture. Its ^{1}H-NMR and IR spectra were also identical with

those of toxol.

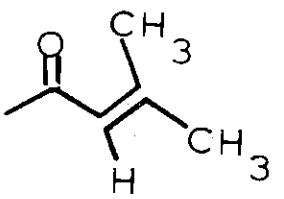
The spectral, chemical and analytical data presented so far indicated that the compound (23) could be one of three possible structures:



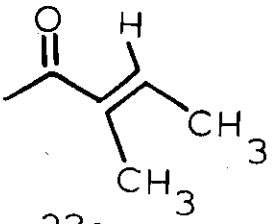
23

R= 

23a

R= 

23b

R= 

23c

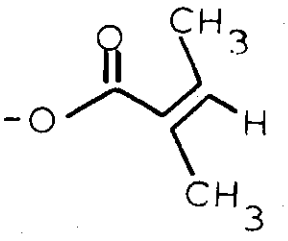
Confirmation of the structure of the compound as 23a was done from its 100 MHz ^{1}H-NMR, ^{13}C-NMR, and mass spectra. These will be discussed in the next section. Structure 23a has been named toxyl angelate.

NMR and Mass Spectral Studies of the Benzofurans

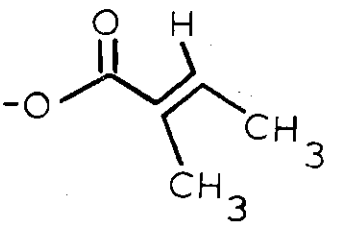
As mentioned in the previous section of the discussion, a 100 MHz ^{1}H-NMR spectrum was taken on each of the benzofurans isolated - dehydrotremetone (2), tremetone (1), toxol (5), 2,5 diacetylbenzofuran (6) and the new benzofuran - toxyl angelate (23a). These data are presented in Table 2.

First, the structure elucidation of toxyl angelate (23a) based on its 100 MHz ^{1}H-NMR spectrum will be presented; then a comparison of all the ^{1}H-NMR spectra of the isolated benzofurans from Isocoma wrightii will be discussed.

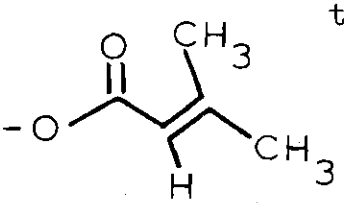
As shown before, the spectral, chemical and physical data showed that the new benzofuran (23) was an ester with the alcohol group being toxol (5). The acid portion of the benzofuran could be one of three possibilities:



angelate

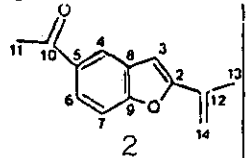
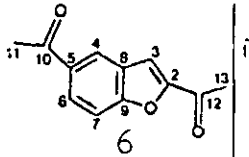
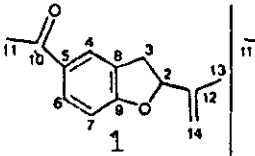
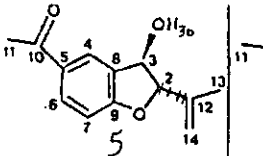
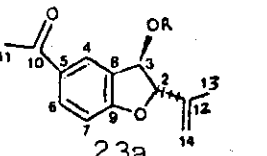


tigiate



or senecioate

Table 2. ^{1}H-NMR Data of the Isolated Benzofurans[a]

	 2	 6	 1	 5	 23a
2	--	--	5.27 (t , 9.0)	5.15 (m)	5.00 (d , 2.5)
3	6.67 (s)	7.57 (s)	a) 3.08 (m) b) 3.36 (m)	a) 4.97 (m) b) 3.87 (bm)	6.17 (d, 2.5)
4	8.17 (d , 2.0)	8.35 (d , 1.7)	7.84 (m)	8.02 (d, 2.0)	8.04 (d , 2.0)
5	--	--	--	--	--
6	7.93(dd, 2.0, 8.7)	8.13(dd, 1.7, 9.0)	7.84 (m)	7.88(dd, 2.0, 8.5)	7.93(dd, 2.0, 8.5)
7	7.43 (d , 8.7)	7.62 (d , 9.0)	6.81 (d , 9.0)	6.88 (d , 8.5)	6.89 (d , 8.5)
8	--	--	--	--	--
9	--	--	--	--	--
10	--	--	--	--	--
11	2.22 (s)	2.68 (s)	2.53 (s)	2.51 (s)	2.47 (s)
12	--	--	--	--	--
13	1.80 (s)	2.63 (s)	1.76 (bs)	1.73 (s)	1.71 (s)
14	a) 5.23 (bs) b) 5.83 (bs)	--	a) 5.09 (bs) b) 5.18 (bs)	a) 4.93 (bs) b) 5.08 (bs)	a) 4.90 (d , 1.0) b) 5.03 (d , 1.0)
					R = -C-C(CH$_3$)=CH 1.92(dq,7.3,1.5) CH$_3$ 6.06(qq,7.3,1.5) 1.77(dq, 1.5,1.5).

[a]Spectra were run at 99.95 MHz in $CDCl_3$ solution using a JEOL PFT-100 instrument in FT mode; chemical shift values are expressed in δ values (PPM) relative to TMS.

The 100 MHz ^{1}H-NMR spectrum of 23 showed that the two unassigned methyl resonances were: δ 1.77 (3H, dq, J = 1.5, 1.5 Hz) and δ 1.92 (3H, dq, J = 1.5, 7.3 Hz). The olefinic proton of the α,β-unsaturated ester resonated at δ 6.06 (1H, qq, J = 1.5, 7.3 Hz). These data are typical of an angeloyl group.[43] This assignment can also be confirmed by comparison with three similar benzofurans which have been recently isolated by Bohlmann and Grenz.[44] Their data are from 270 MHz ^{1}H-NMR spectra. These three compounds - B, K, and I are shown in Table 3.

The methyl resonances of the angeloyl group of B are: δ 1.82 (3H, dq, J = 1, 1 Hz) and δ 1.96 (3H, dq, J = 1, 7 Hz). The methyl resonances of the angeloyl group of K are: δ 1.98 (3H, dq, J = 1, 1 Hz) and δ 1.94 (3H, dq, J = 1, 7 Hz). The methyl resonances of the angeloyl group of I are: δ 1.89 (3H, dq) and δ 2.00 (3H, dq). These are in very good agreement with the methyl resonances of the new benzofuran (23). The olefinic proton of the angeloyl group of B, K and I are δ 6.05 (1H, qq, J = 1, 7 Hz), δ 6.02 (1H, qq, J = 1, 7 Hz) and δ 6.16 (1H, qq) respectively. These data are also in good agreement with the olefinic proton resonance of the new benzofuran - toxyl angelate (23a).

Examination of Table 2 shows that all of the isolated benzofurans exhibit similar ^{1}H-NMR spectra. These data agree well with the data in Table 3. Table 3 represents

Table 3 ^{1}H-NMR Data of Similar Natural Benzofurans

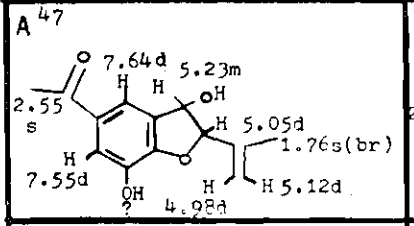
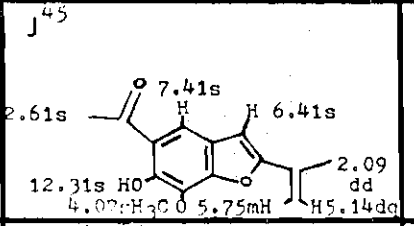
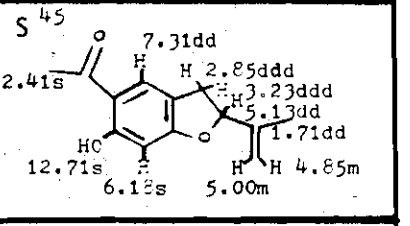
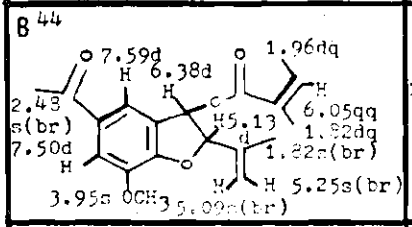
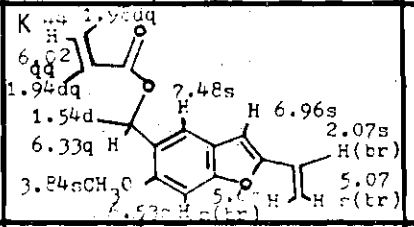
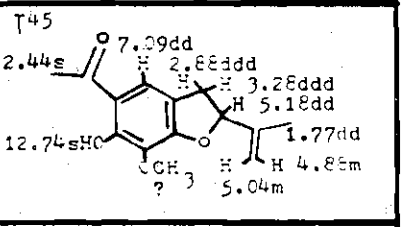
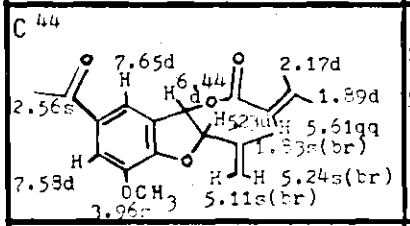
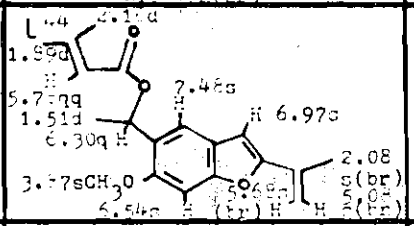
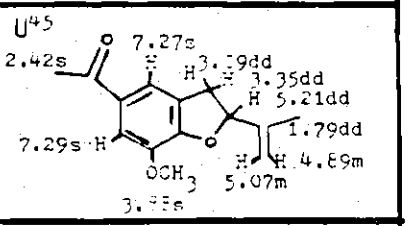
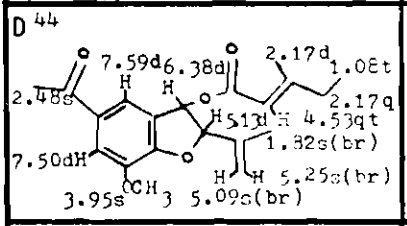
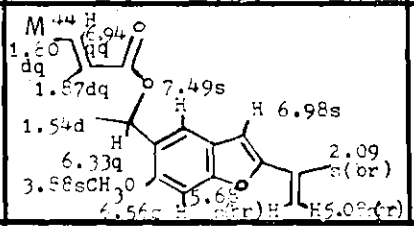
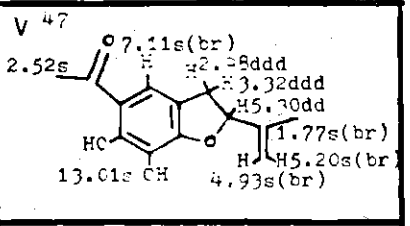
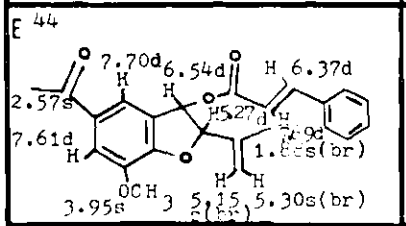
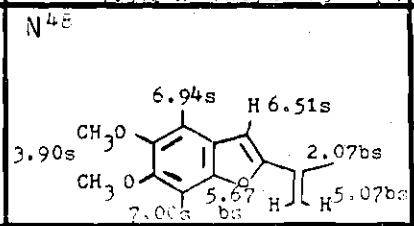
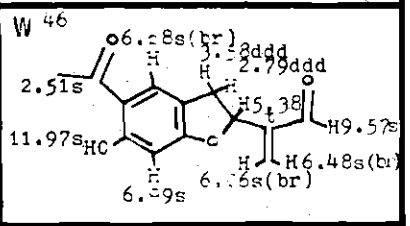
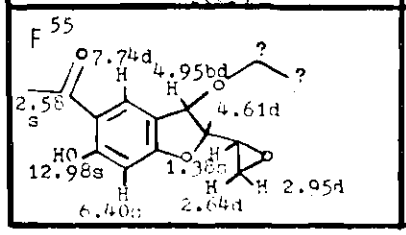
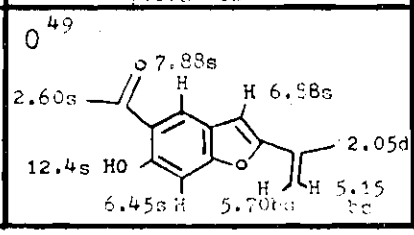
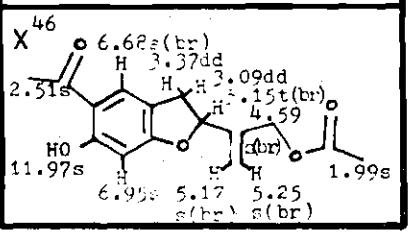
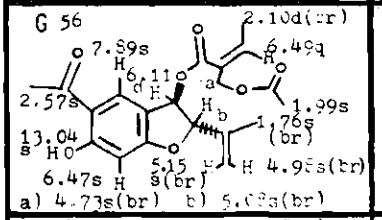
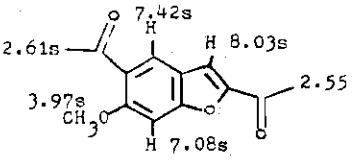
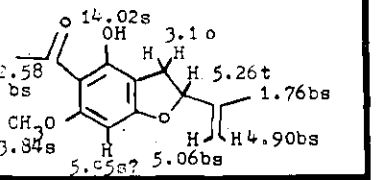
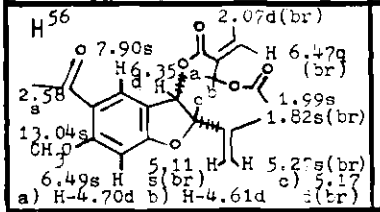
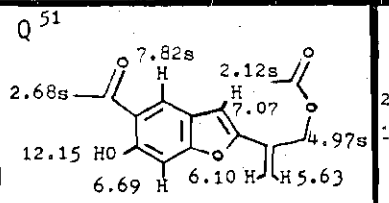
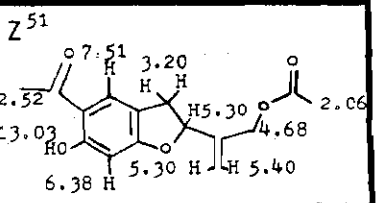
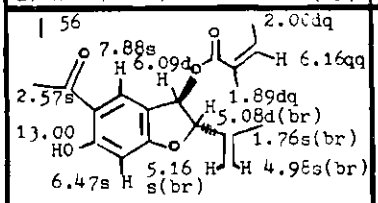
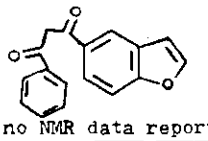
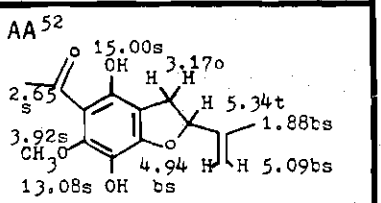
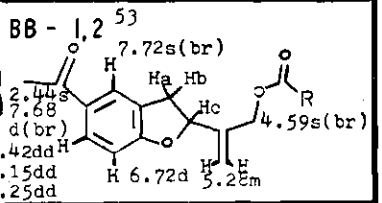
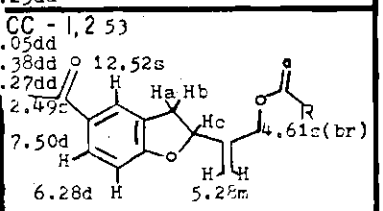
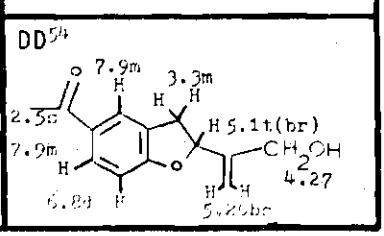
Toxol, Toxyl Angelate - Like	Dehydrotremetone, 2, 5, Di-Acetyl Benzofuran - Like	Tremetone - Like
A [47] 	J [45] 	S [45] 
B [44] 	K [44] 	T [45] 
C [44] 	L [44] 	U [45] 
D [44] 	M [44] 	V [47] 
E [44] 	N [48] 	W [46] 
F [55] 	O [49] 	X [46] 

Table 3 Continued

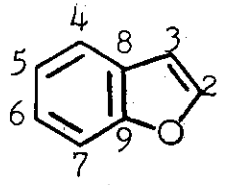
Toxol, Toxyl Angelate -- Like	Dehydrotremetone, 2,5- Di-Acetyl Benzofuran - Like	Tremetone - Like
G [56]  a) 4.73s(br) b) 5.08s(br)	P [49] 	Y [50] 
H [56]  a) H-4.70d b) H-4.61d	Q [51] 	Z [51] 
I [56] 	R [57]  no NMR data reported	AA [52] 
		BB - 1,2 [53]  1) R=CH(CH$_3$)$_2$ a) 3.42dd b) 3.15dd c) 5.25dd 2) R=CHCH$_2$CH$_3$ (CH$_3$) a) 3.05dd b) 3.38dd c) 5.27dd
		CC - 1,2 [53] 
		DD [54] 

the ^{1}H-NMR data on all of the natural benzofurans which have been isolated from plants.

As had been mentioned before, enough of the benzofurans were isolated so that ^{13}C-NMR spectra could be obtained. Since no other ^{13}C-NMR spectra have been reported on naturally occurring benzofurans, the interpretation of the spectra was based on off-resonance decoupling data and on a series of model compounds shown in Table 4.

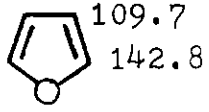
The ^{13}C-NMR spectral data for the isolated benzofurans of Isocoma wrightii are listed in Table 5. Examination of the first two columns of Table 5 - compounds 2 and 6, one can see that for the aromatic benzofurans the assignment of resonances to carbon atoms agrees well across the table. In compound 2 carbons 4 and 6 were not uniquely assigned to individual resonances because of the closeness of their absorption and the lack of a suitable model compound assignment. Carbons 2 and 9 were not uniquely assigned for the same reasons; however, in 6, carbons 2 and 9 can be assigned because of the known assignment in model compound 27. For the next three columns, the dihydrobenzofurans also agree well across the columns. Again carbons 4 and 6 were not uniquely assigned in any of these compounds. The off-resonance decoupling experiment was not done for 1 because of the small amount of material isolated from the plant - its assignments being based on

Table 4. ^{13}C-NMR Model Compounds

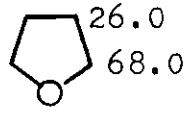


24 ref. 58

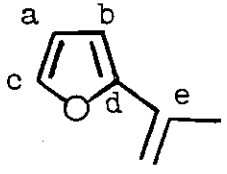
2) 144.7
3) 111.4
4) 124.2*
5) 121.2*
6) 122.8*
7) 106.5
8) 127.6
9) 155.1



25 ref. 58

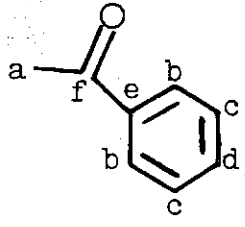


26 ref. 58



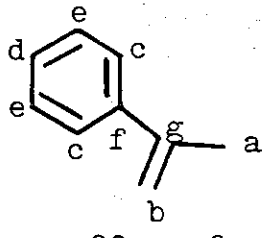
27 ref. 59

a) 112.9
b) 121.6
c) 148.7
d) 153.8
e) 178.2



28 ref. 58

a) 26.3
b) 128.3*
c) 128.6*
d) 133.0
e) 137.3
f) 197.4



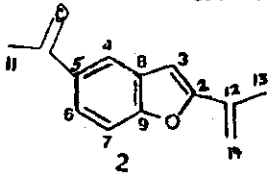
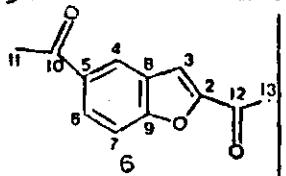
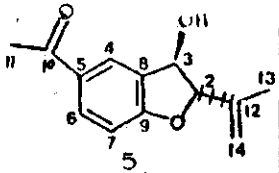
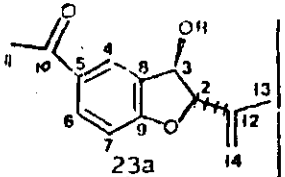
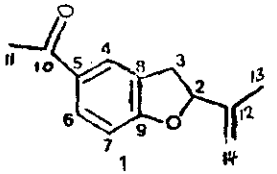
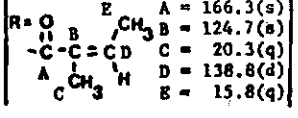
29 ref. 59

a) 21.8
b) 112.3
c) 125.6
d) 127.4
e) 128.2
f) 141.4
g) 143.4

* Tentative assignment

All chemical shift values are expressed in δ values (ppm) relative to TMS.

Table 5. ^{13}C-NMR Data of the Isolated Benzofurans[a]

	 2	 6	 5	 23a	 1
2	156.4 (s) [c]	152.8 (s)	94.3 (d)	90.9 (d)	86.5
3	110.2 (d)	113.1 (d)	75.3 (d)	76.3 (d)	33.9
4	124.5 (d) [b]	127.5 (d) [b]	131.7 (d) [b]	132.5 (d) [b]	130.3 [b]
5	131.6 (s)	126.4 (s)	128.5 (s)	126.6 (s)	126.9
6	121.4 (d) [b]	124.3 (d) [b]	126.3 (d) [b]	127.6 (d) [b]	124.8 [b]
7	102.5 (d)	111.8 (d)	109.4 (d)	109.5 (d)	108.3
8	128.4 (s)	132.8 (s)	130.1 (s)	130.8 (s)	129.9
9	157.5 (s) [c]	156.7 (s)	163.5 (s)	163.9 (s)	163.3
10	196.3 (s)	195.4 (s)	196.3 (s)	194.8 (s)	195.3
11	26.4 (q)	26.5 (q) [c]	26.2 (q)	26.1 (q)	26.1
12	131.9 (s)	186.7 (s)	140.5 (s)	139.6 (s)	142.6
13	19.0 (q)	26.3 (q) [c]	17.4 (q)	17.6 (q)	17.0
14	113.6 (t)	--	112.2 (t)	113.1 (t)	111.9
				 A = 166.3(s) B = 124.7(s) C = 20.3(q) D = 138.8(d) E = 15.8(q)	

[a]Spectra were run at 25 MHz in $CDCl_3$ solution using a JEOL PFT-100 instrument in FT mode; chemical shift values are expressed in δ values (PPM) relative to TMS. b,c Assignments may be reversed.

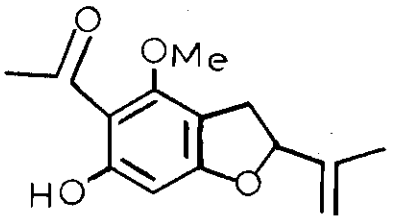
the other two dihydrobenzofurans (5) and (23a) and on the model compounds.

Further confirmation of the structure of toxyl angelate (23a) was obtained from comparison of the angelate side chain carbons with those of reported angelate side chain coumarins.[60] The comparison is shown below:

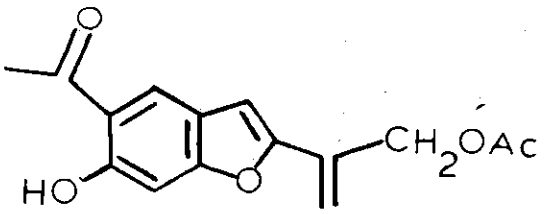
	C-15	C-16	C-17	C-18	C-19
toxyl angelate	166.3	124.7	138.8	20.3	15.8
reported values for angelate coumarins	166.3	126.5	139.4	20.0	15.5

Mass spectra were run on each of the benzofurans isolated from the plant. These are presented in Appendix 2. The typical fragmentation pattern of benzofurans has been discussed in the literature.[61,62,63] These spectra are characterized by an intensive molecular ion, this being due to the stability of the aromatic heterocyclic ion formed without ring fission. It has been noted, however, that for dihydrobenzofurans other fragmentation processes can occur; these frequently involving ring fission and subsequent recyclization. The typical fragmentation pattern for 2-isopropenyl-5-acetyl benzofurans is formation of an intense molecular ion with the base peak of the spectrum being either $[M^+ - CH_3]$ or m/e 43 due to the acetyl ion $[CH_3-C\equiv O^+]$. This type of pattern can be seen from the examples given in Table 6. The fragmentation pattern for

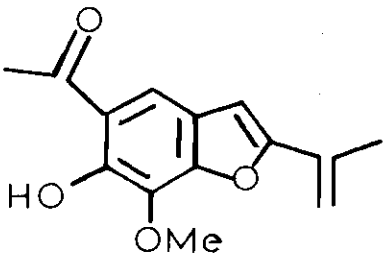
Table 6. Examples of Benzofuran Mass Spectrum Fragmentation Patterns.



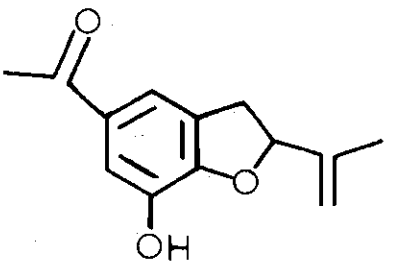
M^+ 245
M^+-15 233 (100%)



M^+ 274
M^+-15 259
m/e 43 base peak



M^+ 246 (100%)
M^+-15 231 (43%)



M^+ 218 (80%)
M^+-15 (100%)
M^+-CH_3CO 175 (60%)

the benzofurans isolated from Isocoma wrightii is shown in Table 7. Interpretation of the mass spectrum fragmentation pattern for toxyl angelate (23a) is shown in Figure 2.

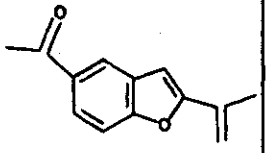
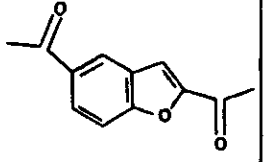
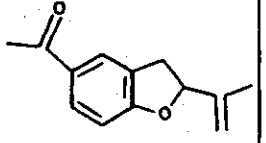
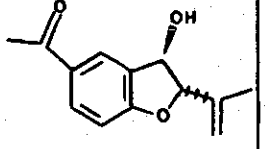
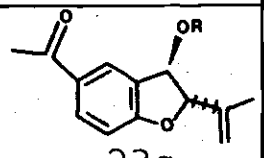
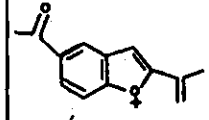
Isolation and Identification of Phytol, Hentriacontane and Squalene

The non-ketone fraction (33.9 g) obtained as shown in Charts 4 and 5 was chromatographed on 300 g of alumina. The column was eluted with solvents of increasing polarity yielding 11 fractions (shown on Chart 7). A GC study (Col I, program temp: 150°C, held for eight min, then programmed at 20°C/min to 245°C and held at 245°C) of the fractions is shown in Appendix 1, p. 96.

The GC trace of fraction 4A showed that it contained one major component. Chromatography of this fraction (4.08 g) on alumina yielded 420 mg of a clear oil (see Chart 8). The IR and ^{1}H-NMR spectra of the oil were identical with those reported in the literature for phytol (18).[36] The mass spectrum of phytol shown in Appendix 2, p. 117 also agrees with the published spectrum of phytol - m/e 41 (27%), 43 (39%), 55 (26%), 57 (35%), 69 (24%), 71 (100%), 123 (24%) and 296 (3%).[36] Therefore, the clear oil was identified as phytol.

Phytol (18) is rarely reported as being free in plants.[64] It is usually attached as a side chain to the

Table 7. Major Peaks from the Mass Spectra of the Benzofurans from *Isocoma wrightii*.

	M^+ m/e		$[M^+-CH_3]$	$[M^+-CH_3-C\equiv O]$	$[CH_3-C\equiv O^+]$
	202 (91%)		m/e 187 (53%)	m/e 159 (50%)	m/e 43 (100%)
	200 (52%)		m/e 185 (100%)	m/e 157 (41%)	m/e 43 (26%)
	202 (41%)		m/e 187 (100%)	m/e 159 (17%)	m/e 43 (14%)
	218 (28%)	m/e % 187 55 185 43 163 34 162 50	m/e 203 (32%)	m/e 175 (16%)	m/e 43 (100%)
 23a	300 (3%)	 m/e 200 (100%)	m/e 200-CH_3 m/e 185 (80%)	m/e 200-CH_3-C≡O m/e 157 (19%)	m/e 43 (58%)

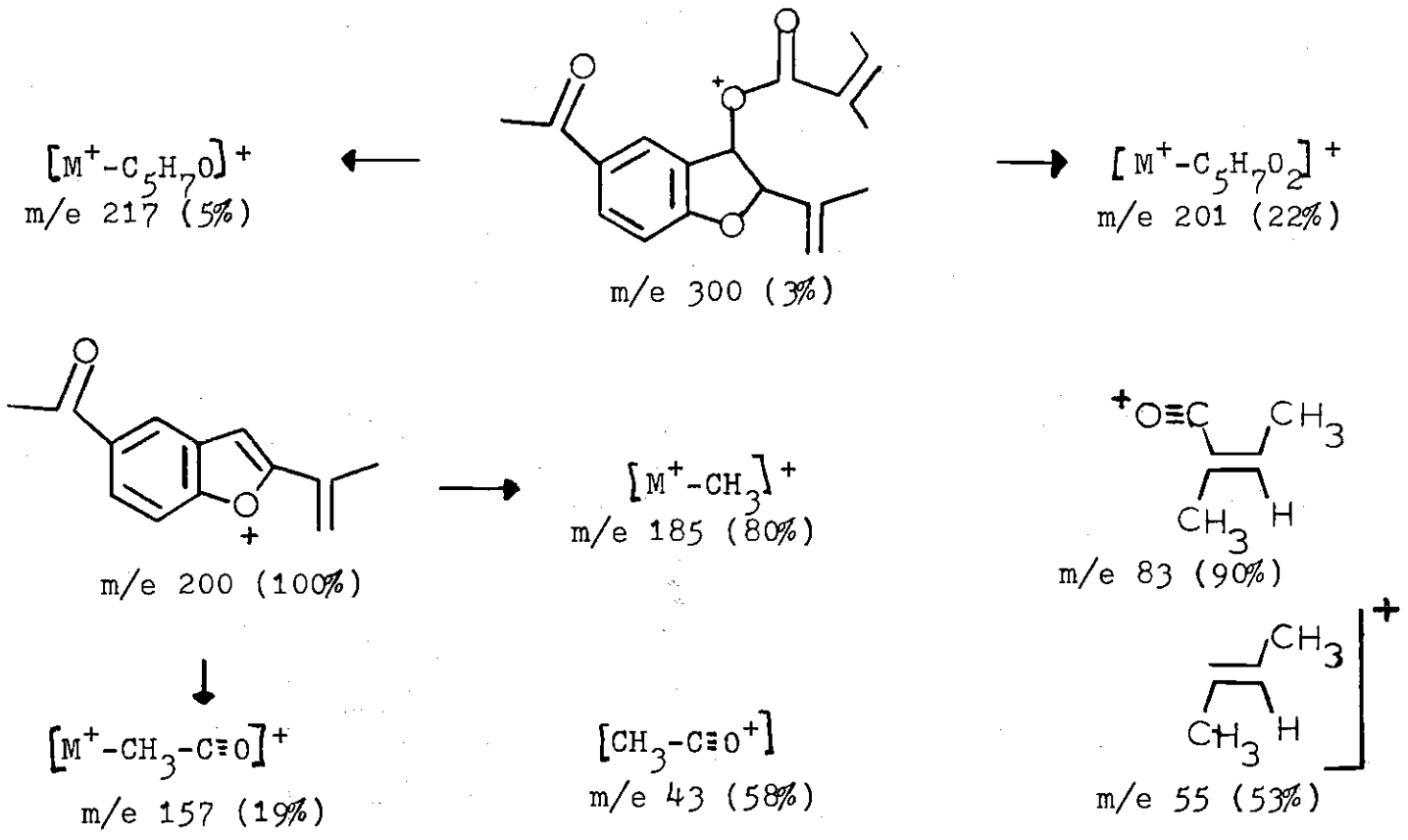


Figure 2. Fragmentation Scheme of Toxyl Angelate (23a).

chlorophyll molecule. Saponification of chlorophyll yields phytol - see Figure 3. Therefore the phytol obtained in this isolation was probably not present in the free form since the isolation procedure used a saponification step.

The GC trace of fraction 1A of Chart 7 showed three major components (Col I, same temp program as before). The retention time of the peaks were 15.6 min, 16.5 min and 19.0 min. Chromatography of this fraction was done on alumina. The components with R_t of 16.5 and 19.0 min came off in the first fraction (1C) as a white waxy substance. The component with the R_t of 15.6 min came off in the third fraction as a yellow oil (see Chart 9). The first fraction (1C) was chromatographed on alumina. The seventh fraction (7F) was composed mostly of the component with the R_t of 19.0 min. This fraction (291.1 mg) was chromatographed on alumina to give 14 mg of a white waxy solid shown to be 95% pure by GC (Col IV, program temp). This component had a R_t of 19.0 min. Its IR and ^{1}H-NMR spectra indicated that it was a hydrocarbon but were not much help from a structural point of view. Its mass spectrum showed a $M^+ = 436$ (4%) and had a pattern characteristic for straight chain hydrocarbons, i.e. the gradual increase from $(M-15)^+$ to m/e 57 in abundance of C_nH_{2n+1} fragments.[65] The MP of the waxy solid was 66-68°C. Therefore, from the mass spectrum, which indicated a

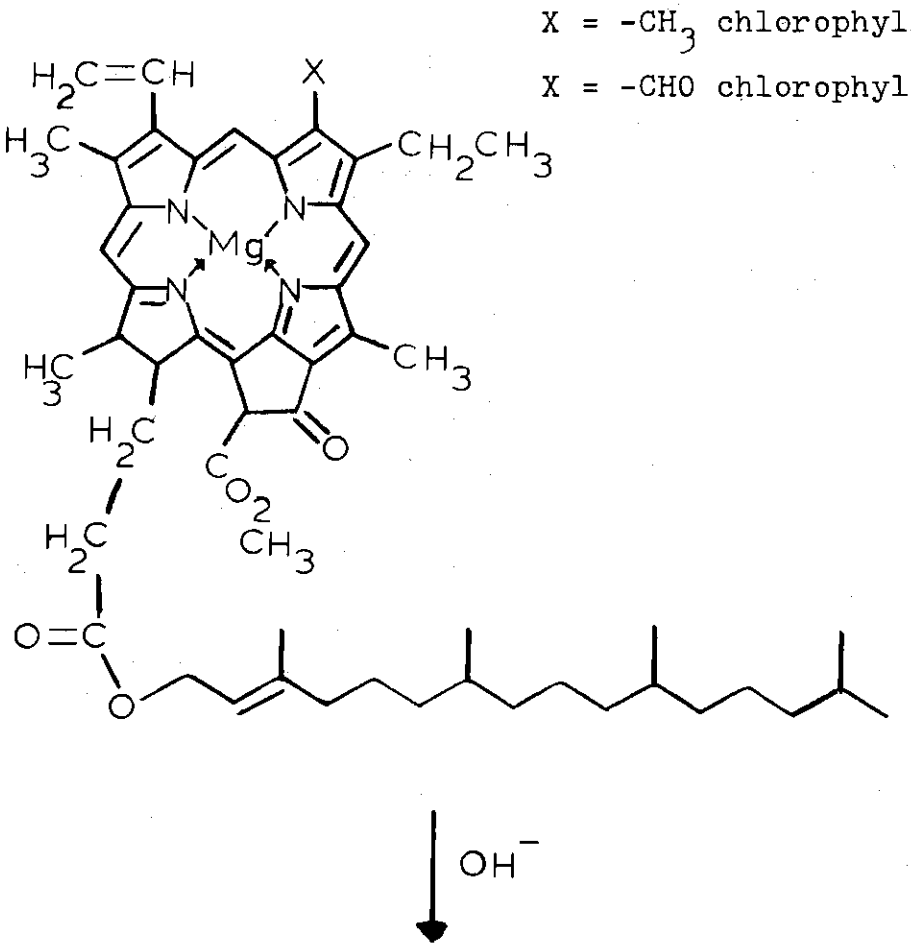


Figure 3. Saponification of Chlorophyll

straight chain hydrocarbon of molecular formula - $C_{31}H_{64}$ whose reported MP = 67°C, the waxy substance was identified as hentriacontane (19). This identification was confirmed by a mass spectral comparison of the fragmentation patterns of C_{30}, C_{31}, and C_{32} hydrocarbons (shown in Appendix 2, p. 118) and mixed injection (GC Col IV, 290°C) of the three hydrocarbons (see Appendix 1, p. 109).

Alkanes of this type are not rare in nature.[66] The content of odd-numbered alkanes is usually greater than that of even-numbered alkanes by a factor of more than ten. The major constitutent of the alkane fraction is usually C_{27}, C_{29}, C_{31}, or C_{33}.[66] The component of fraction 7F with the R_t = 16.5 min was shown by GC study to be C_{29} - nonacosane.

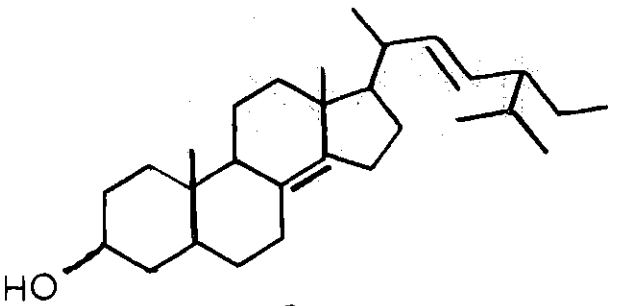
Fraction 3C (156.7 mg) from Chart 9 was chromatographed on alumina to yield 47.6 mg of a clear viscous oil (see Chart 10). This oil had the same IR, ^{1}H-NMR and mass spectra as those reported for squalene.[38] Therefore, the oily liquid was identified as squalene (20).

Squalene is not usually isolated from plants, but can be isolated in large quantities from animals. Squalene is important in the biosynthesis of triterpenes and steroids.

Isolation and Identification of the Steroids

Fraction 7A from Chart 7 exhibited three peaks

with R_t = 19.7 (A), 20.4 (B), and 21.4 (C) min (Col I, program temp, see Appendix 1, p. 102). Chromatography of this fraction on alumina (activity I) failed to separate the mixture. Chromatography on activity III alumina gave a white solid (3G) which was 90% pure (see Chart 11). This component had a R_t = 20.4 min (B). The other two components did not separate. This solid (B) had the same spectral and physical properties as those of stigmasta-8(14), 22-dien-3β-ol (9).[32]

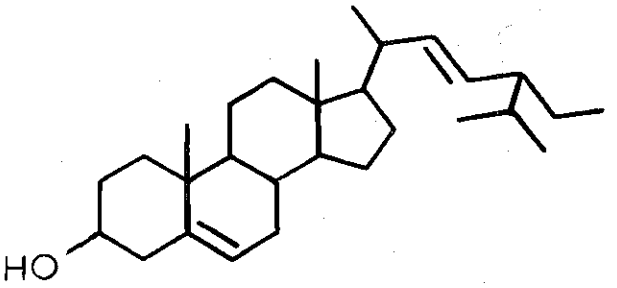


9

Separation of the other two components by chromatography on silica gel and silica gel impregnated with silver nitrate failed. The two components were finally separated by preparative gas chromatography (Col V, 290°C). The component with the R_t of 19.7 min (Col I, program temp) (A) had the same physical and spectral properties of an authentic sample of stigmasta-5,22-dien-3β-ol (21).

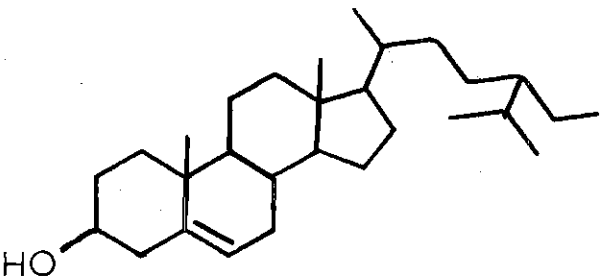
This steroid (21) is one of the most common plant sterols (phytosterols) found. Stigmasta-8(14),22-dien-3β-ol, however, is rarely found even though it has the more

thermodynamically stable double bond.

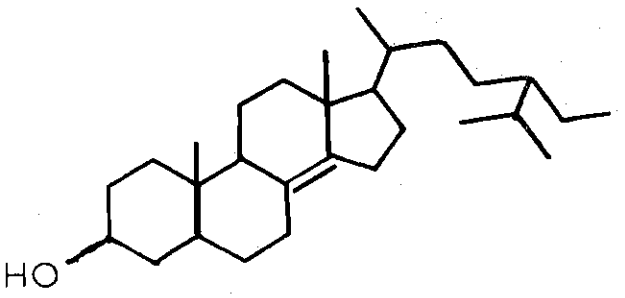


21

The other component (C) (R_t = 21.4 min, Col I, program temp) showed a molecular ion of 414 (100%) in its mass spectrum. It showed a peak at m/e 273 (27%) due to $M^+-C_{10}H_{20}$ and at m/e 255 (44%) due to $M^+-C_{10}H_{20}$, $-H_2O$. Its IR spectrum was similar to both IR spectra of 21 and 9, and therefore, it was assumed that (C) was a stigmasterol with one double bond - and that the double bond was in the steroid nucleus and not in the side chain. Since the two steroids isolated from the plant have either a double bond at $\Delta 5$,(β-sitosterol 30) or at $\Delta 8(14)$, (stigmasta-8(14)-en-3β-ol 22), it was assumed that (C) was one of these two.



30



22

In their ^{1}H-NMR spectra, the C-18, C-19 methyl groups appear at δ 0.70 and δ 1.01 respectively in Δ5,22 stigmasterol (21) and at δ 0.55 and δ 0.80, respectively in Δ8(14),22 stigmasterol (9). In (C) the C-18, C-19 methyl groups appear at δ 0.54 and δ 0.80, respectively. Therefore, (C) seems to be 22. The reported MP of 22 is 108-111°C; The MP observed for (C) was 109-111°C. Also an authentic sample of Δ5-stigmasterol (β-sitosterol 30) had a longer R_t than (C) - (Col V, 290°C). Therefore (C) was identical to 22 - stigmasta-8(14)-en-3β-ol.

Results of Anti-Tumor Testing

Since this investigation was conducted in order to find the agent responsible for the anti-tumor activity of the chloroform extract, the results obtained from this testing will now be discussed.

In general, an increase in survival of the treated animals as compared to the control animals resulting in

a T/C % of 125 or more is required by NIH for further testing in their anti-tumor program.

The results of this testing done on P 338 lymphocytic leukemia tumors in mice for the chloroform extract is shown below:

	Dose/Inj.	T/C %
1st Test	400	152
	200	136
	100	122
2nd Test	600	104
	400	134
	265	160
	175	138

The results shown above are very good, i.e. the chloroform extract exhibits good anti-tumor activity.

Since it was doubtful that squalene, hentriacontane, phytol or the steroids would have any anti-tumor activity these were not sent for testing. However, the benzofurans were sent. Unfortunately, none showed significant anti-tumor activity. The results are shown below.

	T/C %
toxyl angelate	111
toxol	103
tremetone	tested by different method, but no activity
2,5 diacetylbenzofuran	97

A sample of dehydrotremetone has been sent recently for testing.

CHAPTER V

CONCLUSIONS AND RECOMMENDATIONS

A reinvestigation of the toxic plant Isocoma wrightii (rayless goldenrod) has led to the isolation of the previously identified benzofurans - toxol (5), tremetone (1), dehydrotremetone (2), and 2,5 diacetylbenzofuran (6) and to the identification of a new benzofuran - toxol angelate (23). Three compounds, previously undentified as occurring in this plant, were isolated and identified - phytol (18), hentriacontane (19), and squalene (20). A series of three phytosterols were isolated and identified: stigmasta-8(14),22-dien-3β-ol (9), stigmasta-5,22-dien-3β-ol (21) and stigmasta-8(14)-en-3β-ol (22); 9 had been previously isolated from the plant.

An NMR (^{1}H and ^{13}C) study was done on the benzofurans, resulting in the first reported natural abundance ^{13}C-NMR spectra on naturally occurring benzofurans.

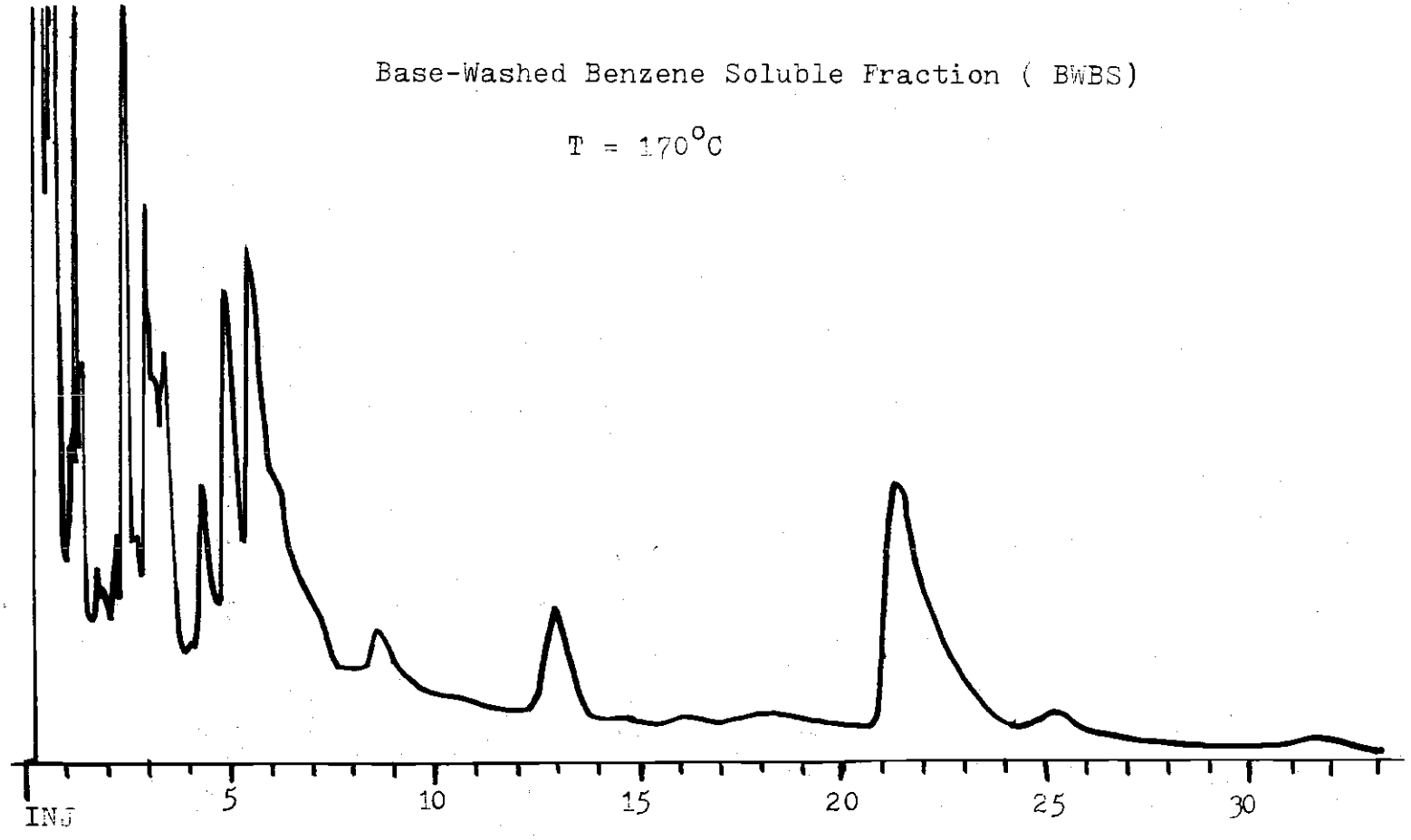
Unfortunately the anti-tumor active agent and the toxic agent in the plant have yet to be identified. The chloroform extract of the plant should be reinvestigated using modern methods of liquid chromatography, such as high pressure and medium pressure liquid chromatography.

An assay method for the toxicity of rayless goldenrod has recently been devised and this should be used for following the toxicity during chromatography.[67]

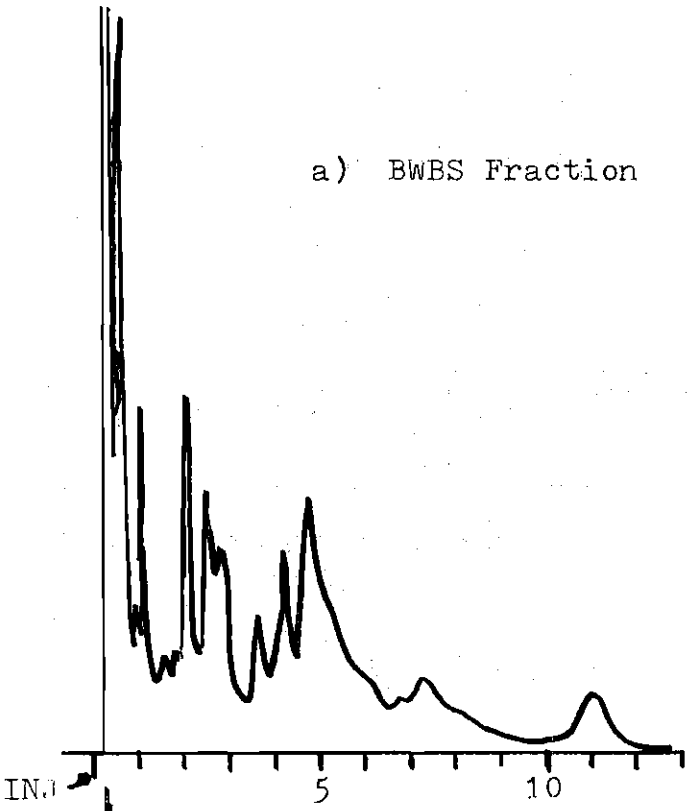
APPENDICES

APPENDIX 1

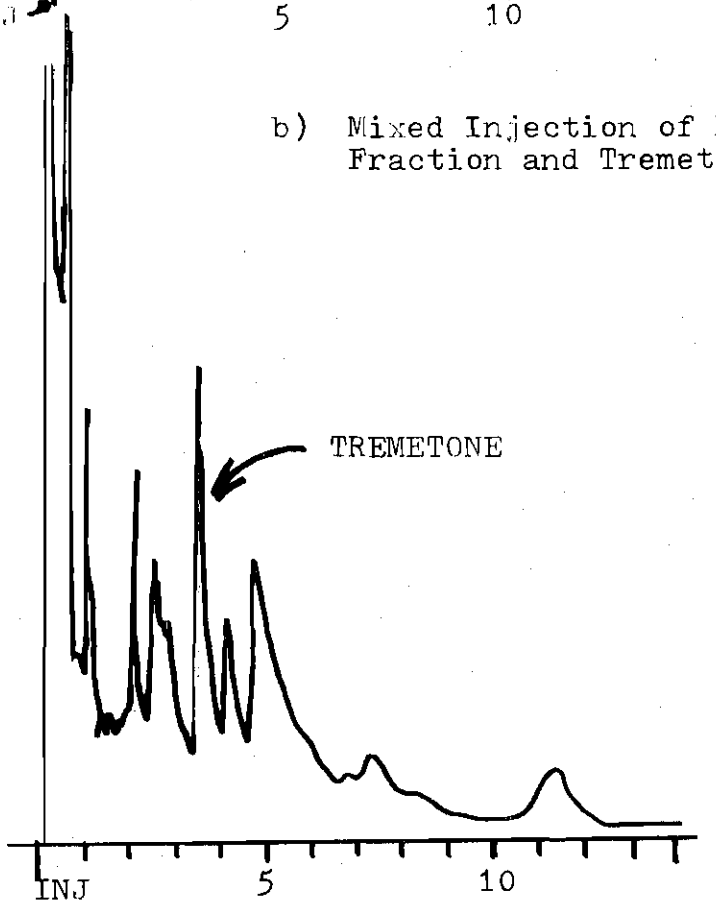
GAS CHROMATOGRAPHY TRACES

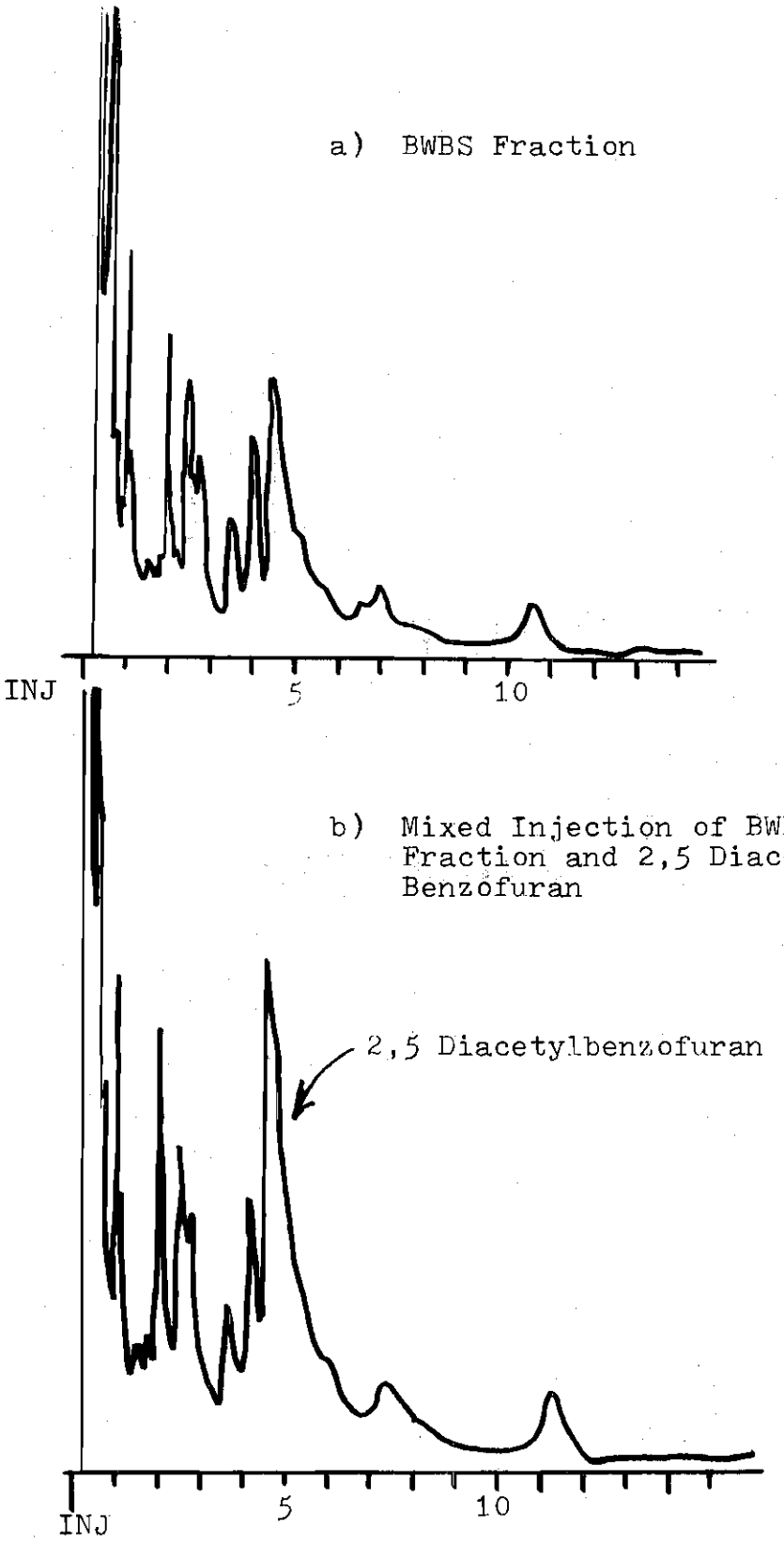

Base-Washed Benzene Soluble Fraction (BWBS)
T = 170°C
INJ
5
10
15
20
25
30
Time in min

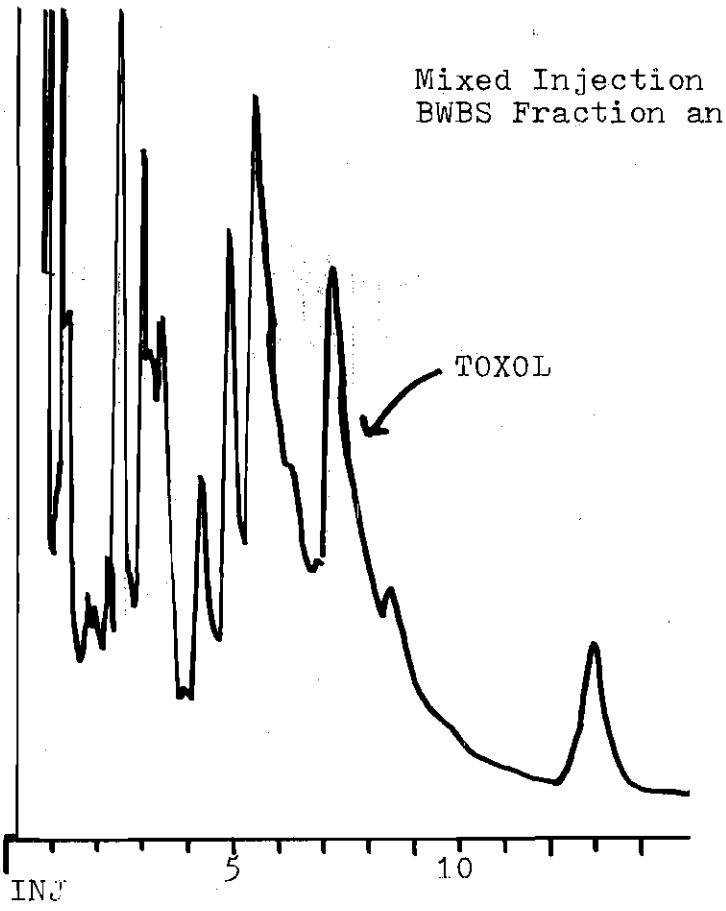
a) BWBS Fraction

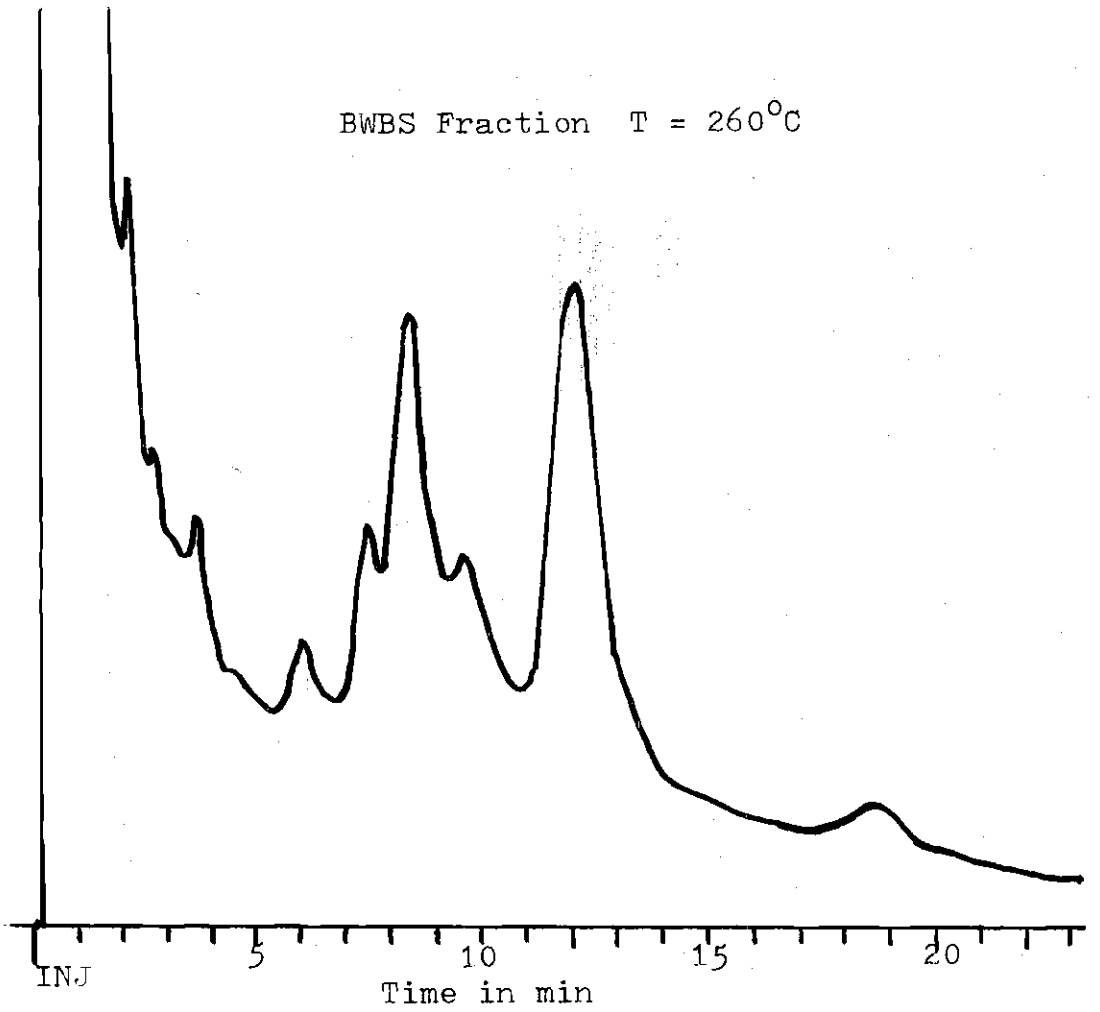


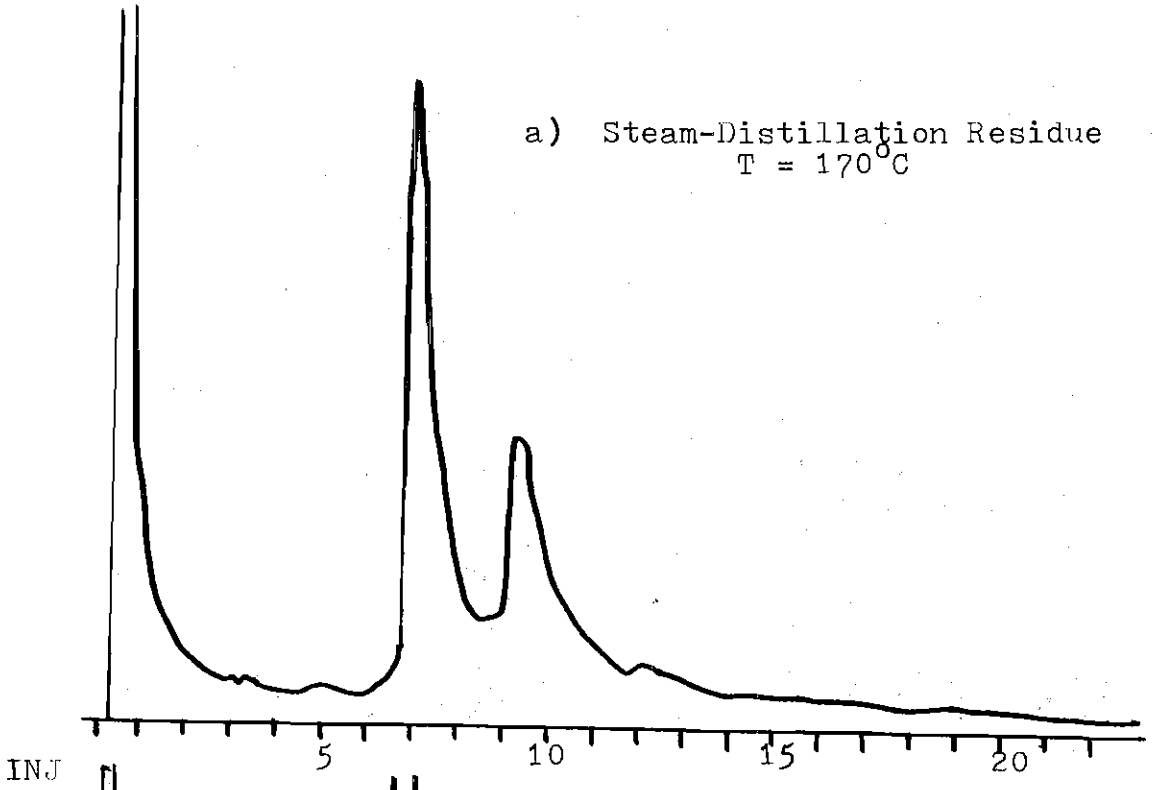
b) Mixed Injection of BWBS Fraction and Tremetone



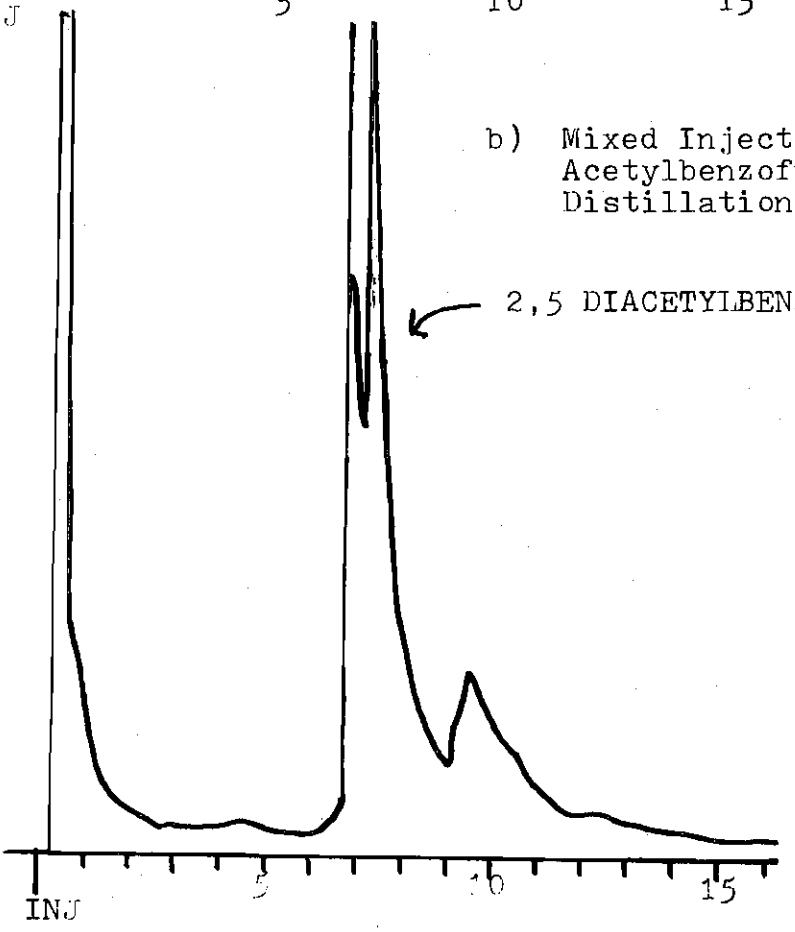

a) BWBS Fraction
INJ
5
10
b) Mixed Injection of BWBS Fraction and 2,5 Diacetyl-Benzofuran
2,5 Diacetylbenzofuran
INJ
5
10


Mixed Injection of
BWBS Fraction and Toxol
TOXOL
INJ
5
10

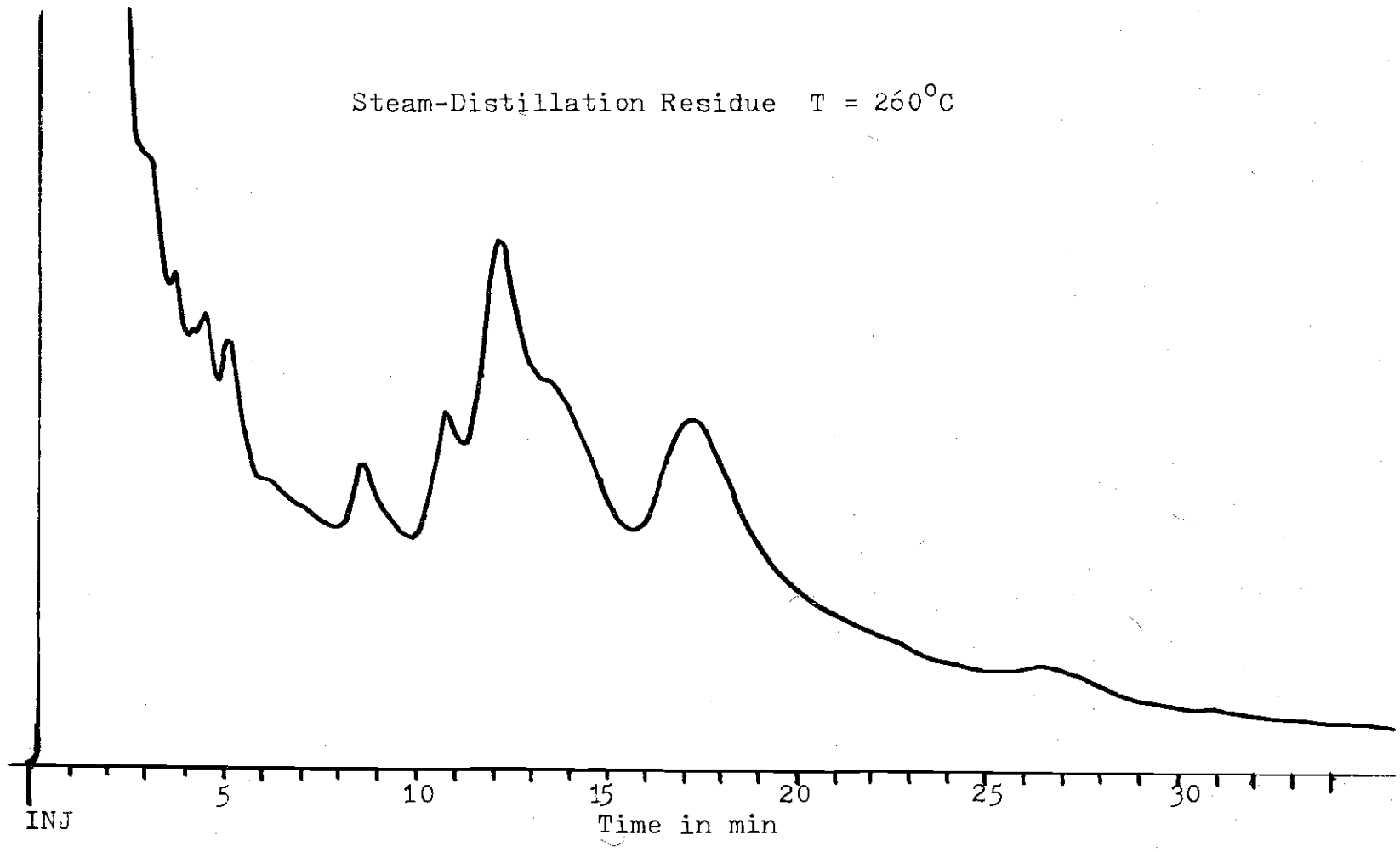

BWBS Fraction T = 260°C
INJ
5
10
15
20
Time in min

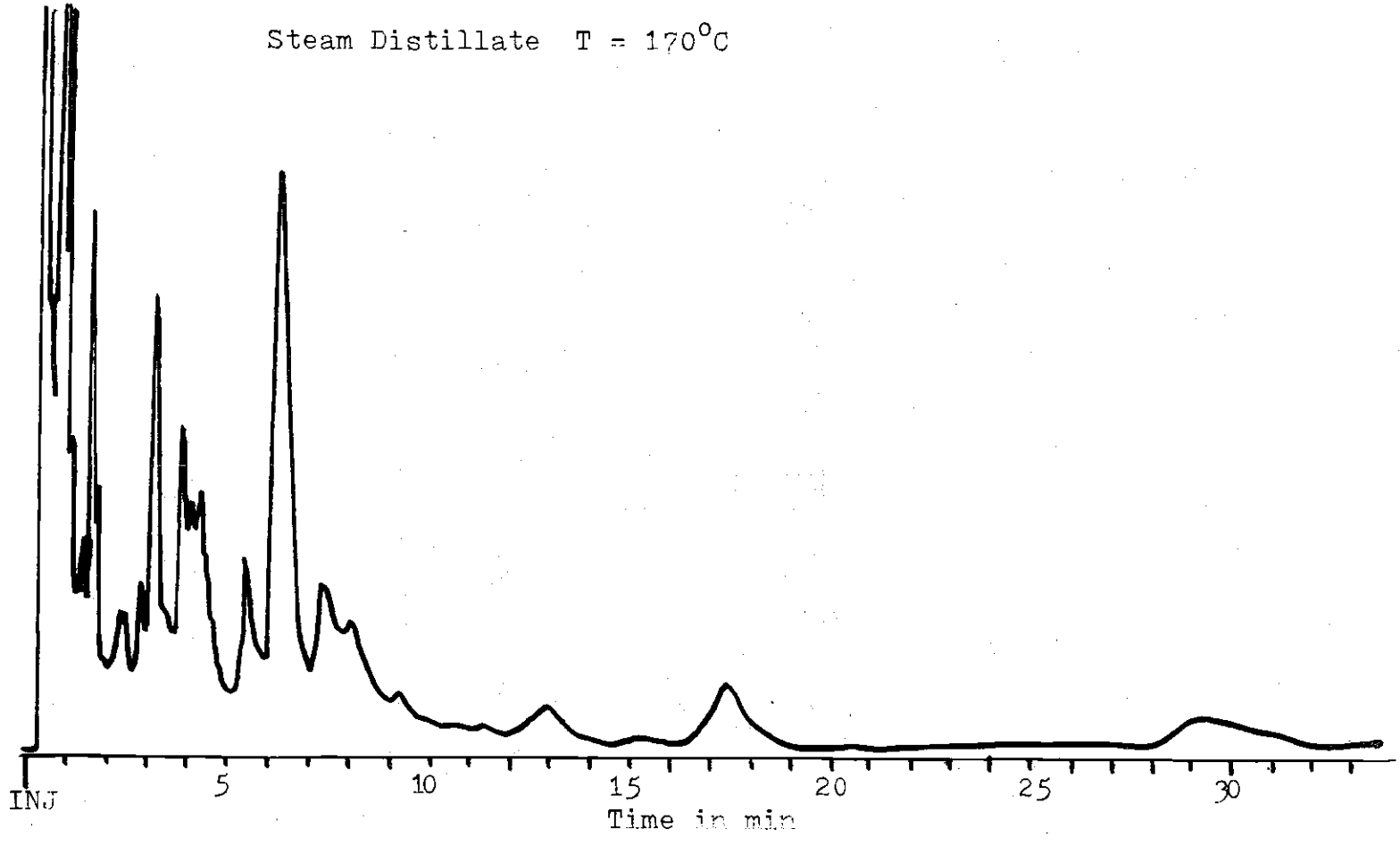


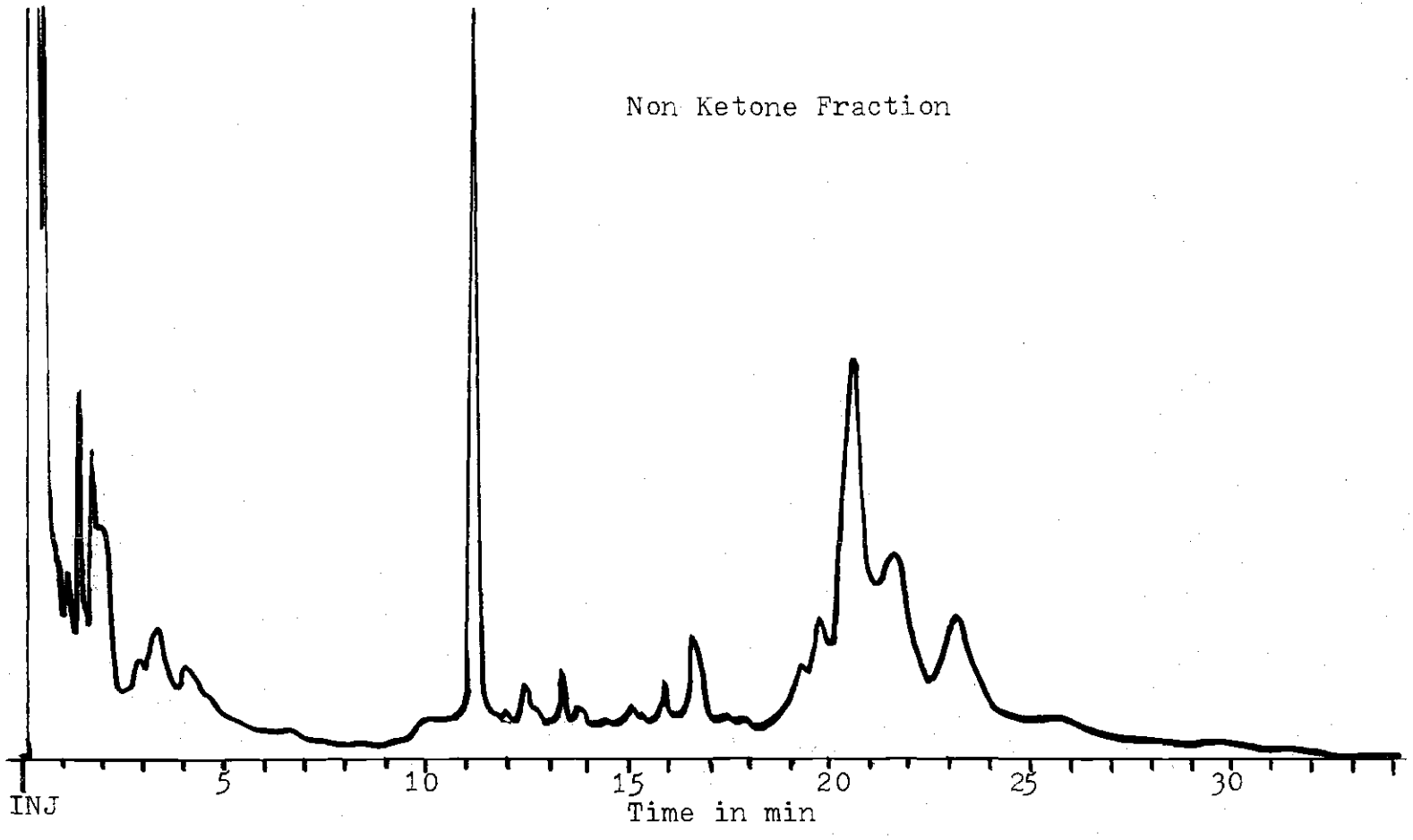
a) Steam-Distillation Residue
T = 170°C



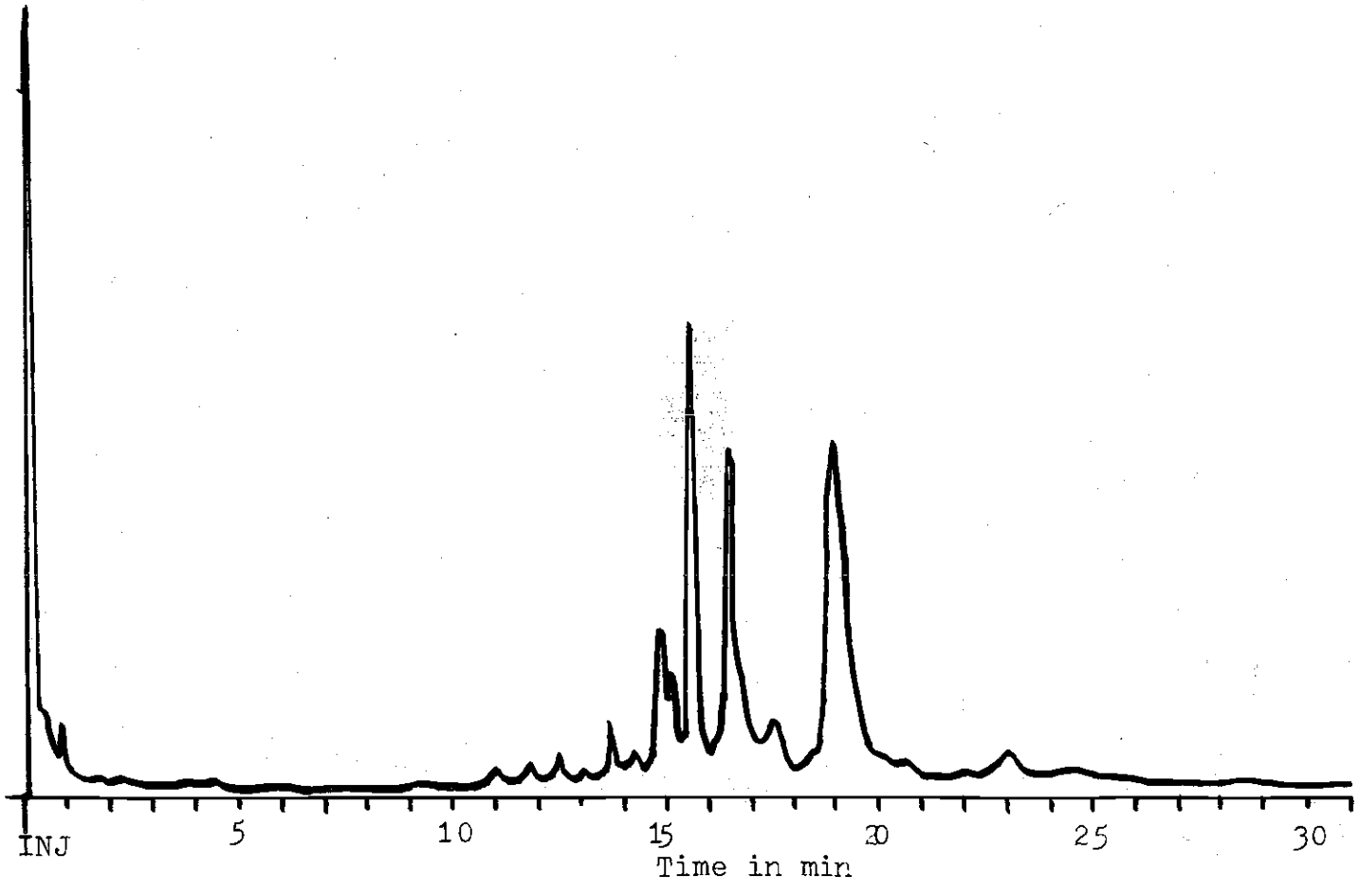
b) Mixed Injection of 2,5 Di-Acetylbenzofuran and Steam-Distillation Residue

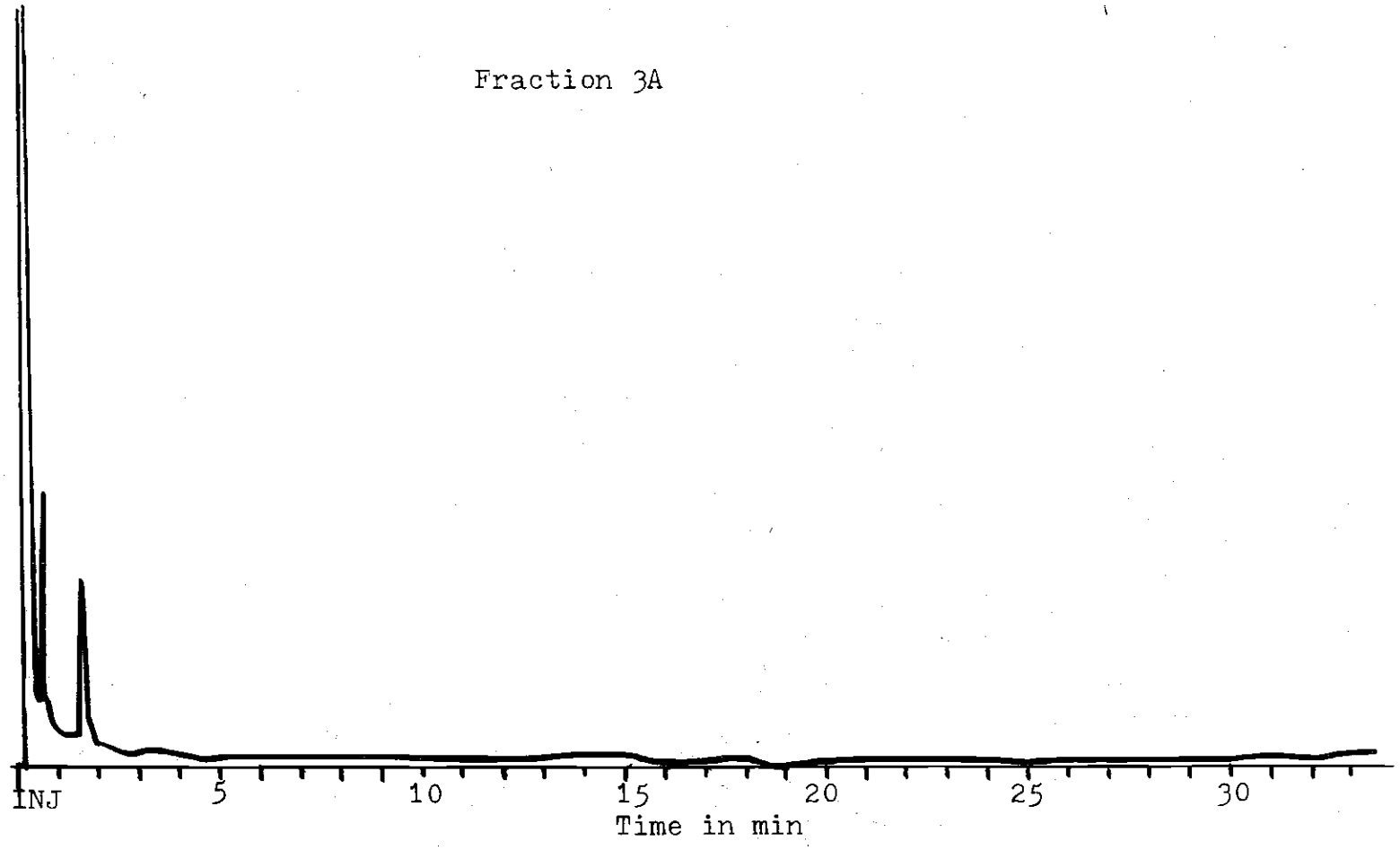

Steam-Distillation Residue T = 260°C
INJ
5
10
15
20
25
30
Time in min

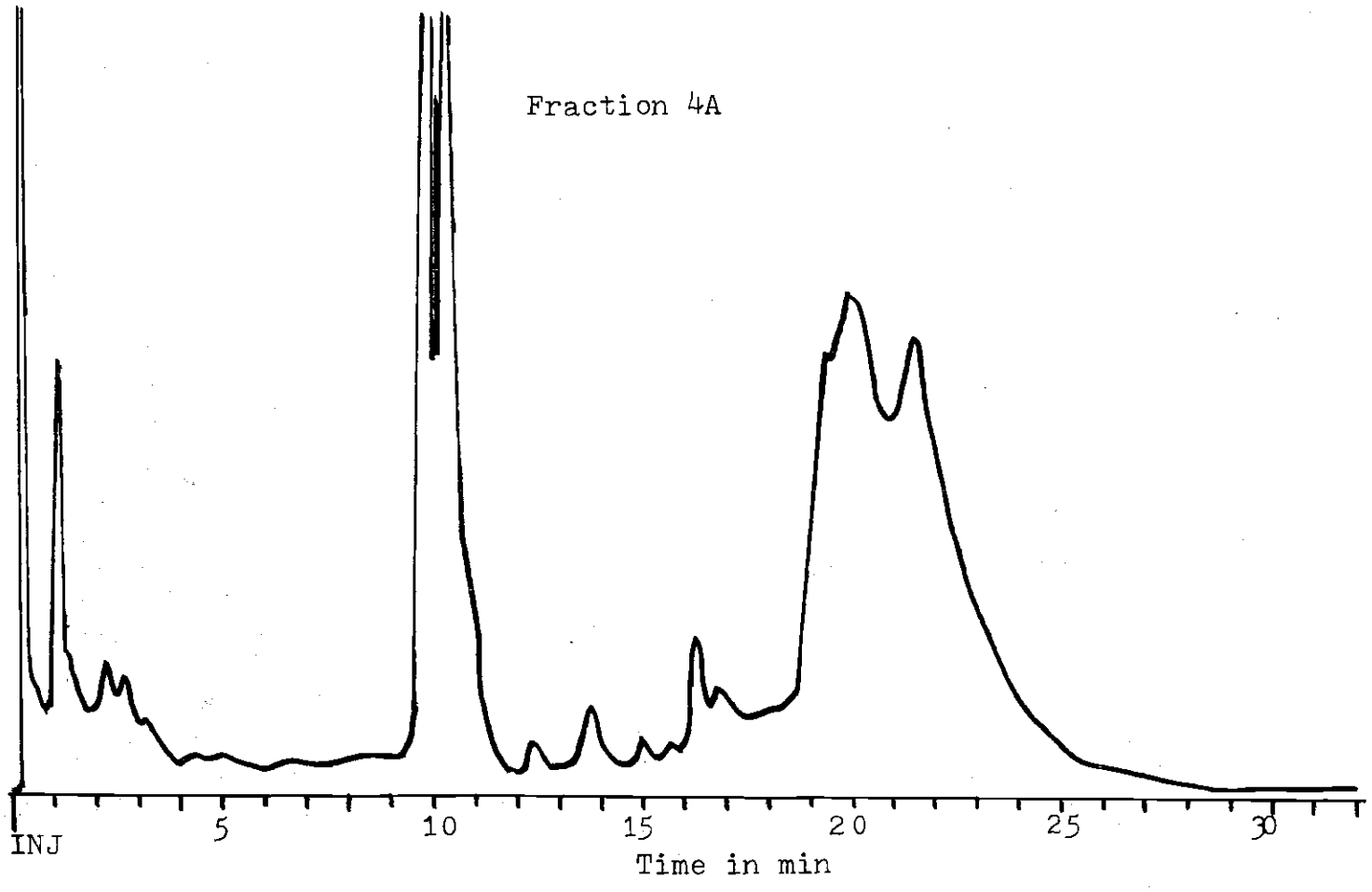

Steam Distillate T = 170°C
INJ
5
10
15
20
25
30
Time in min

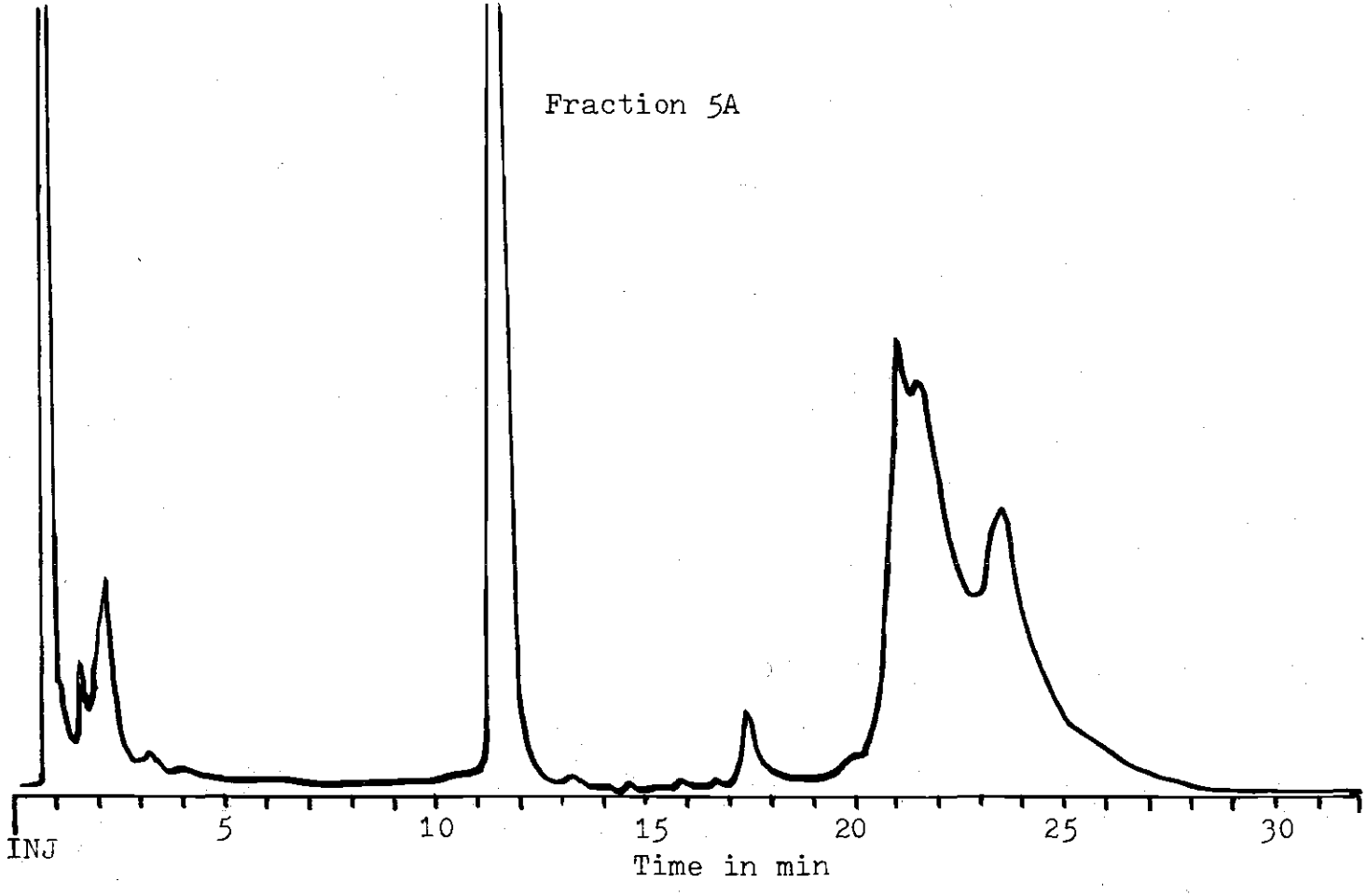

Non Ketone Fraction
INJ
5
10
15
20
25
30
Time in min

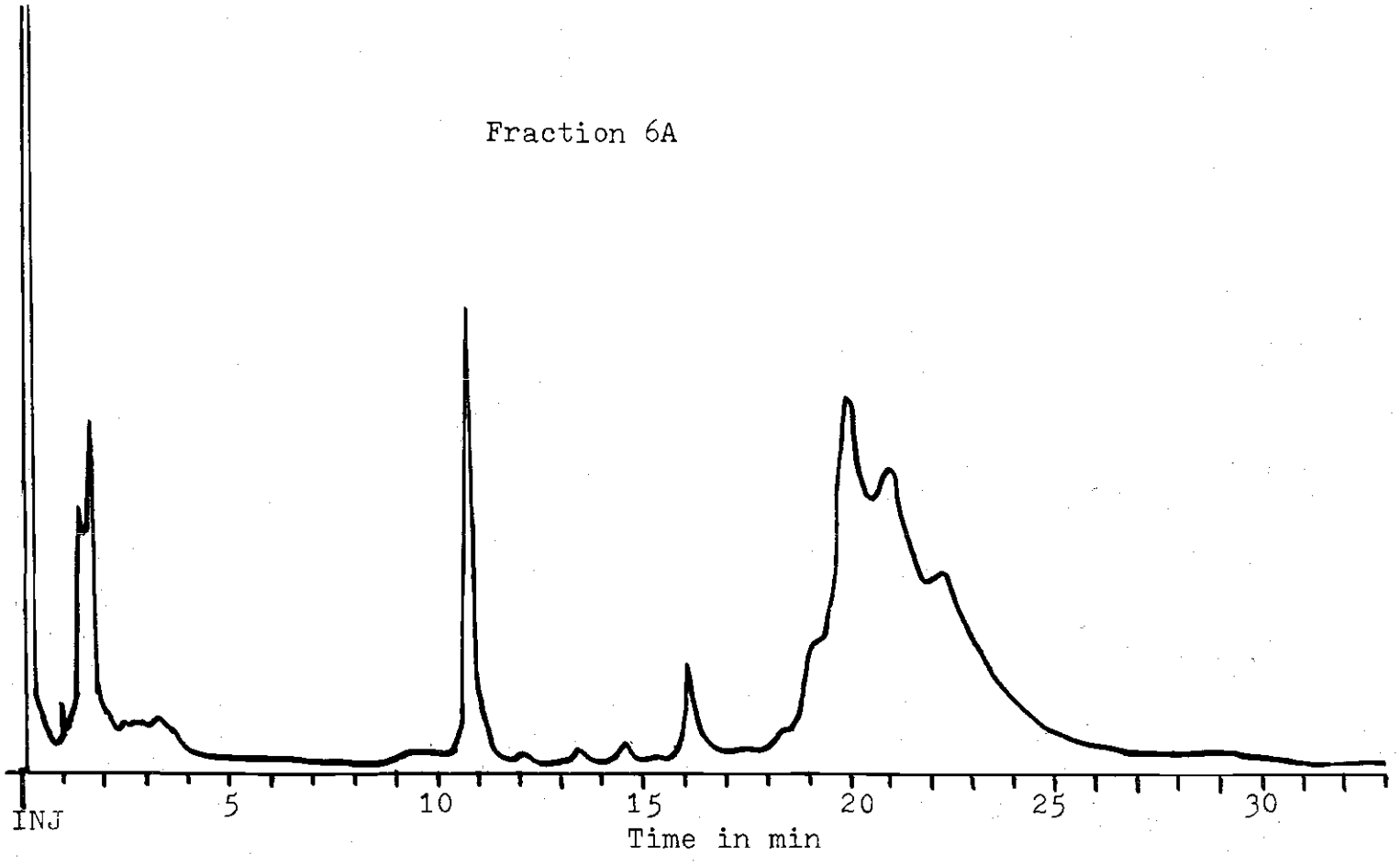
Fraction 1A

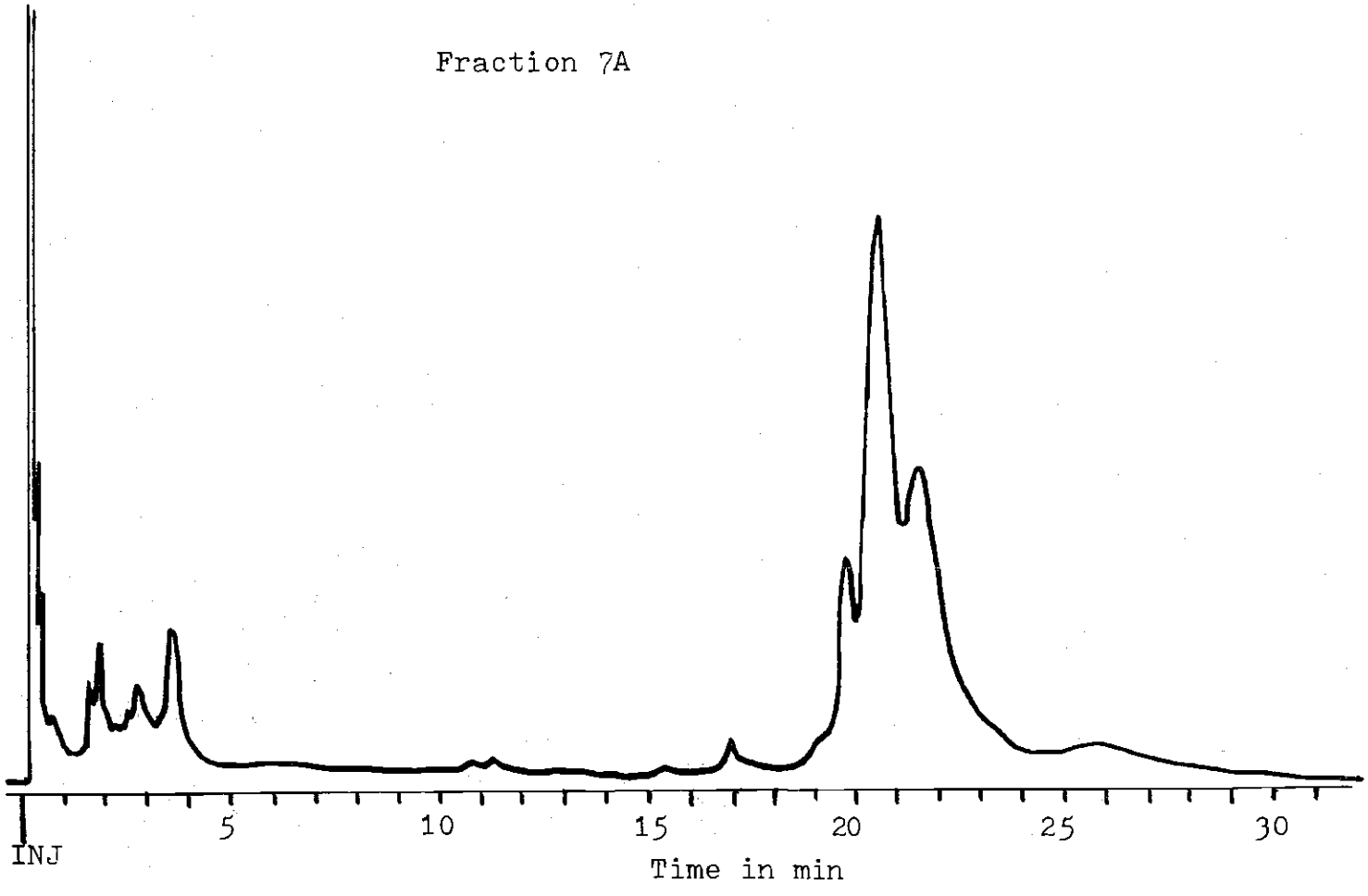


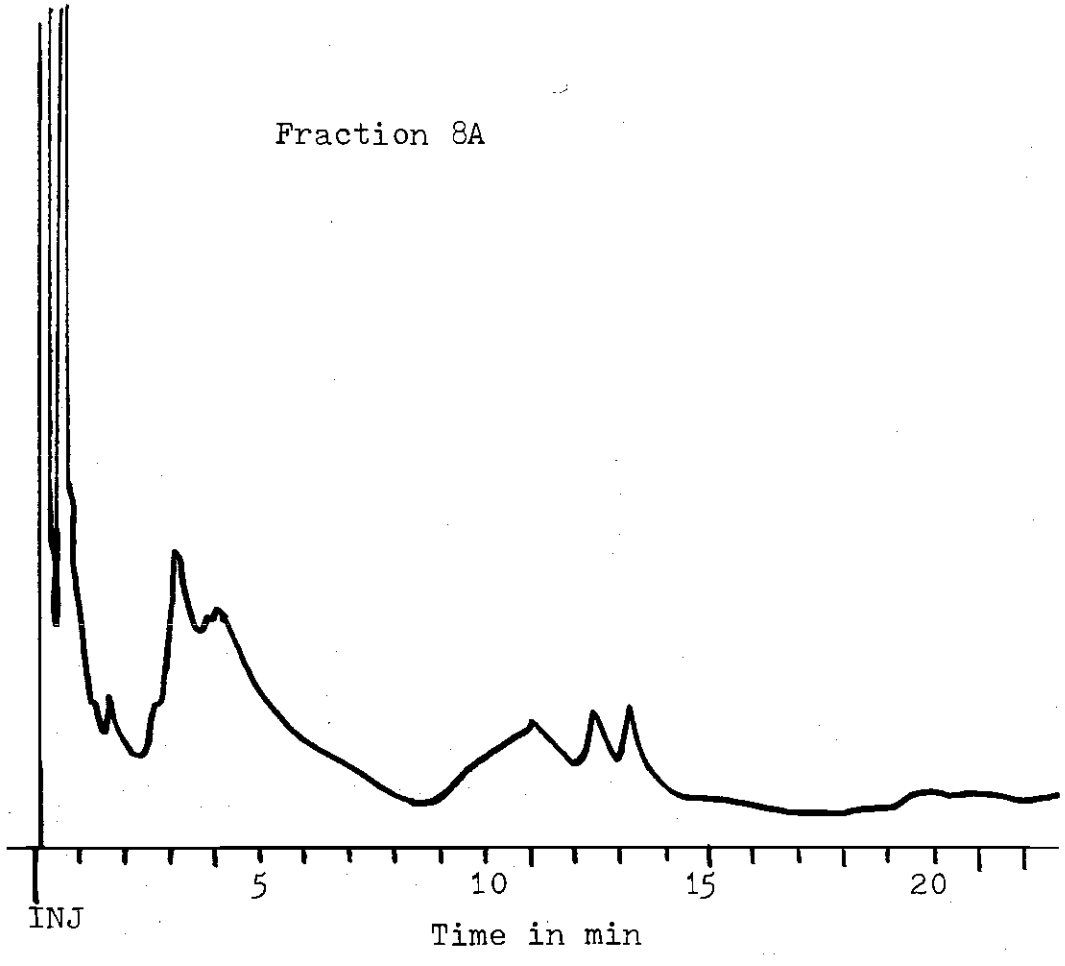

Fraction 3A
INJ
5
10
15
20
25
30
Time in min

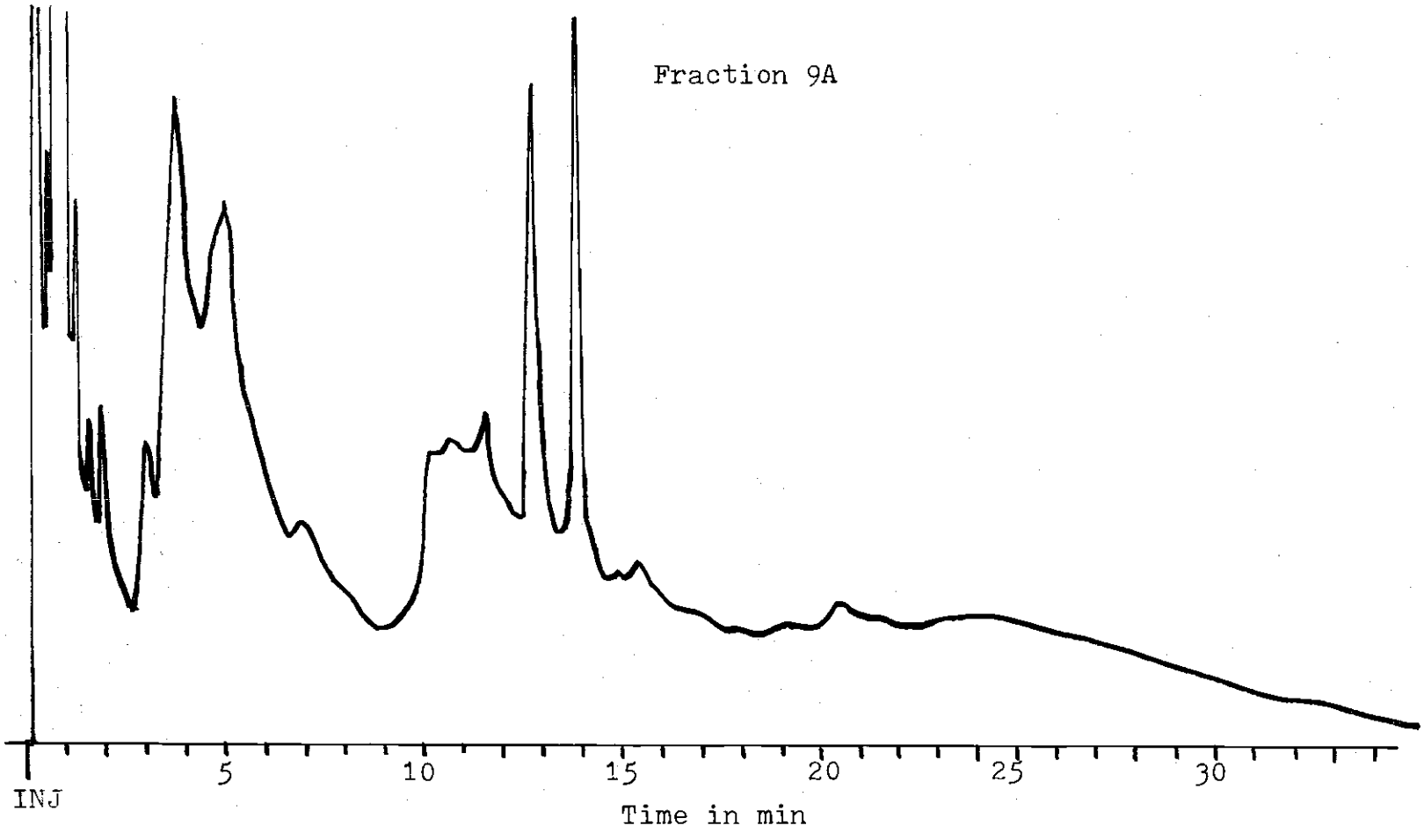

Fraction 4A
INJ
5
10
15
20
25
30
Time in min


Fraction 5A
INJ
5
10
15
20
25
30
Time in min

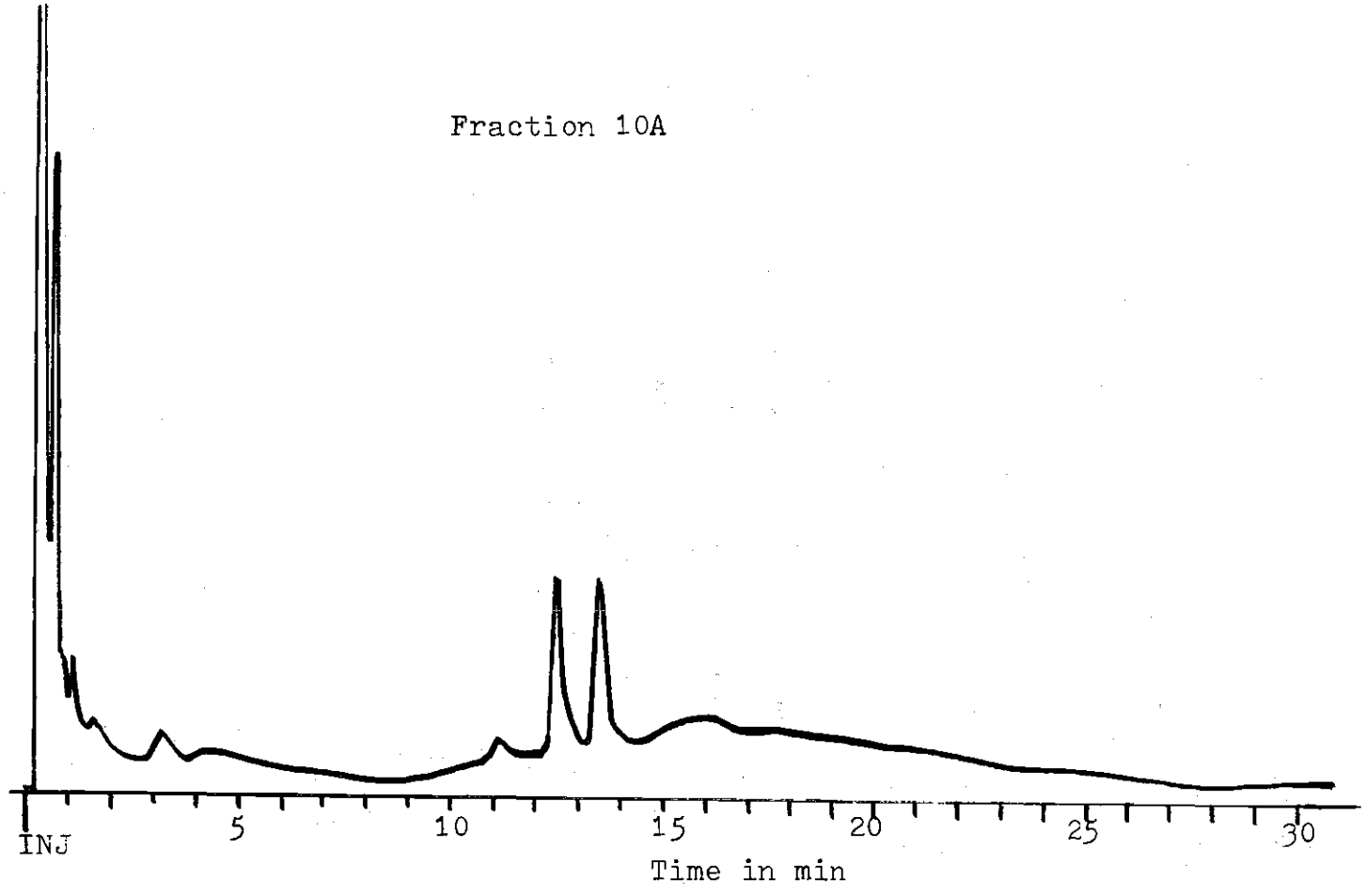

Fraction 6A
INJ
5
10
15
20
25
30
Time in min

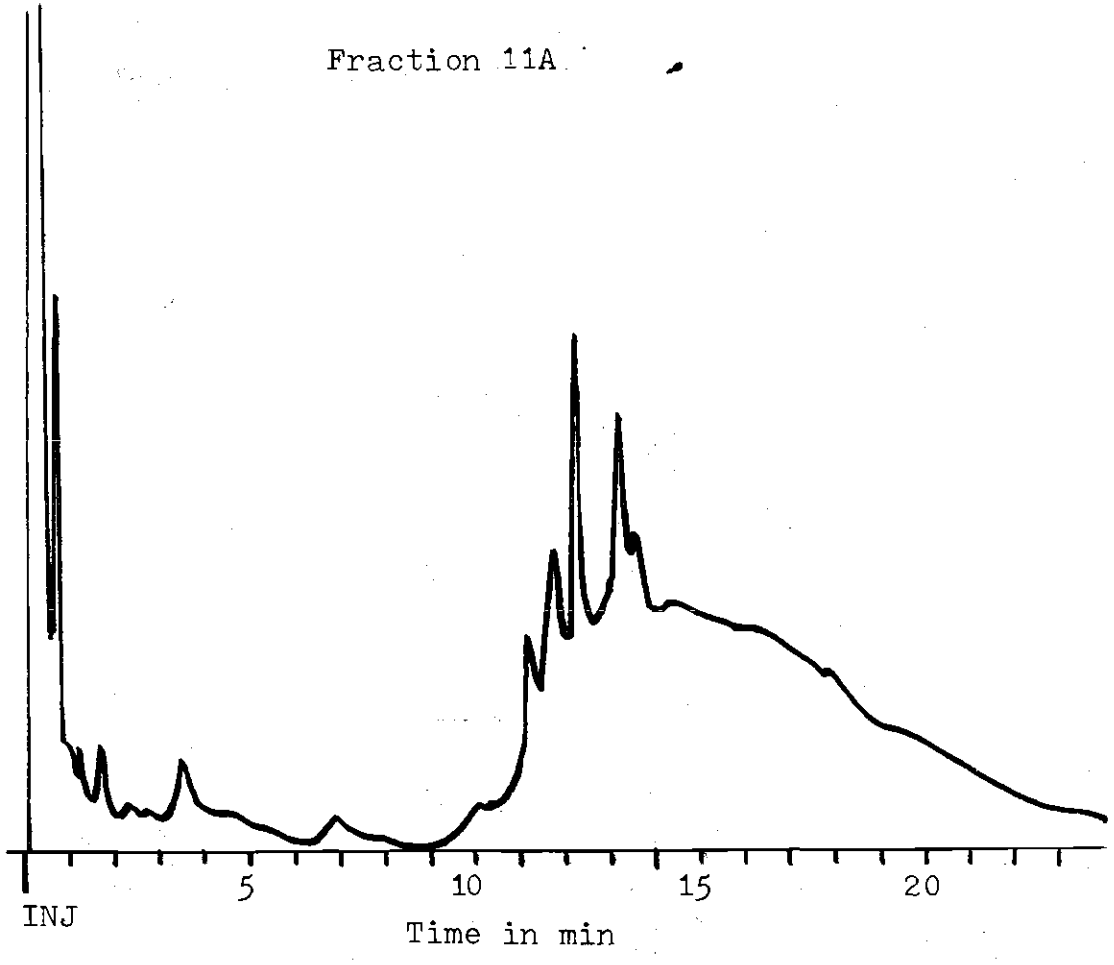

Fraction 7A
INJ
5
10
15
20
25
30
Time in min

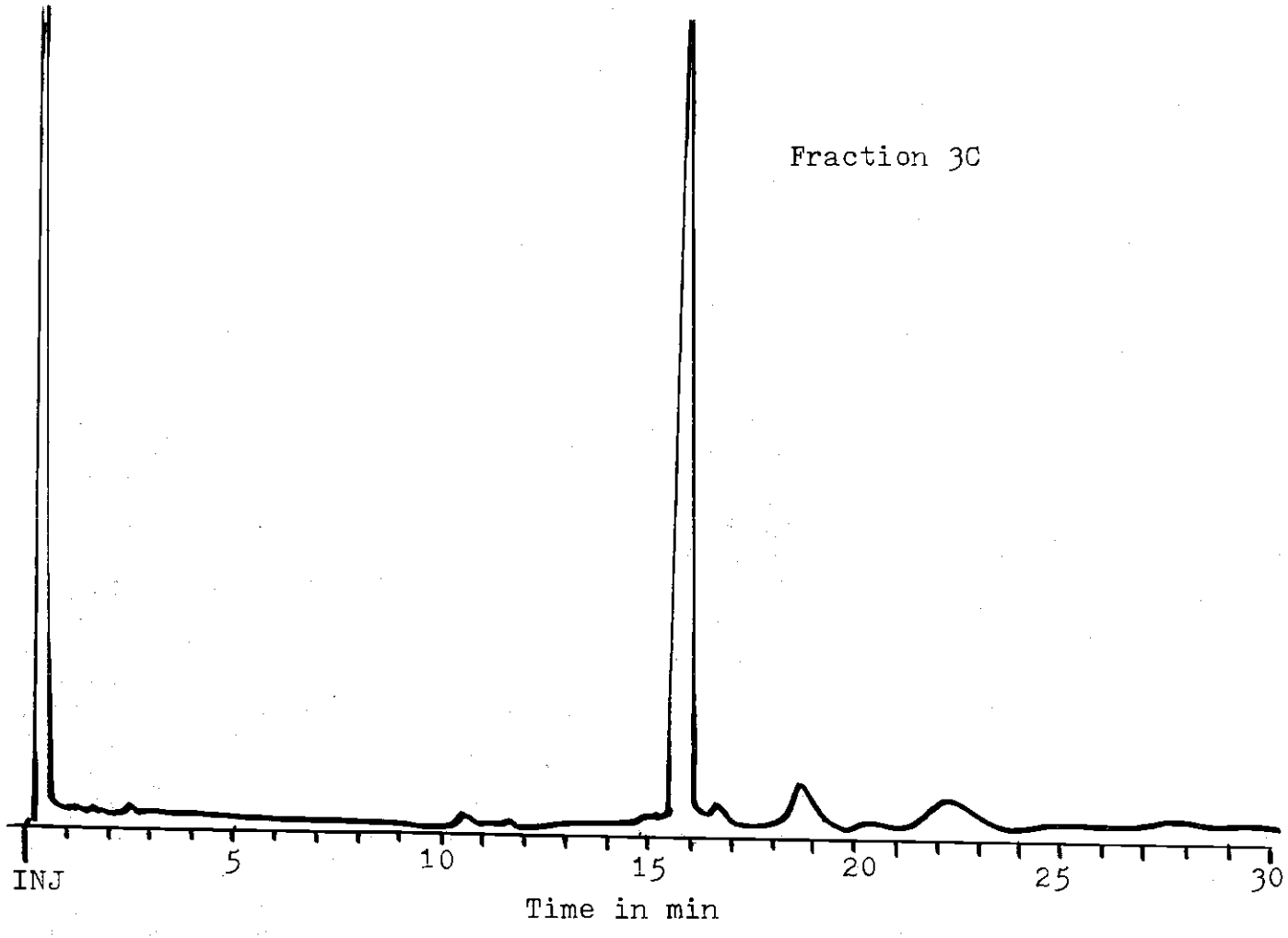

Fraction 8A
INJ
5
10
15
20
Time in min

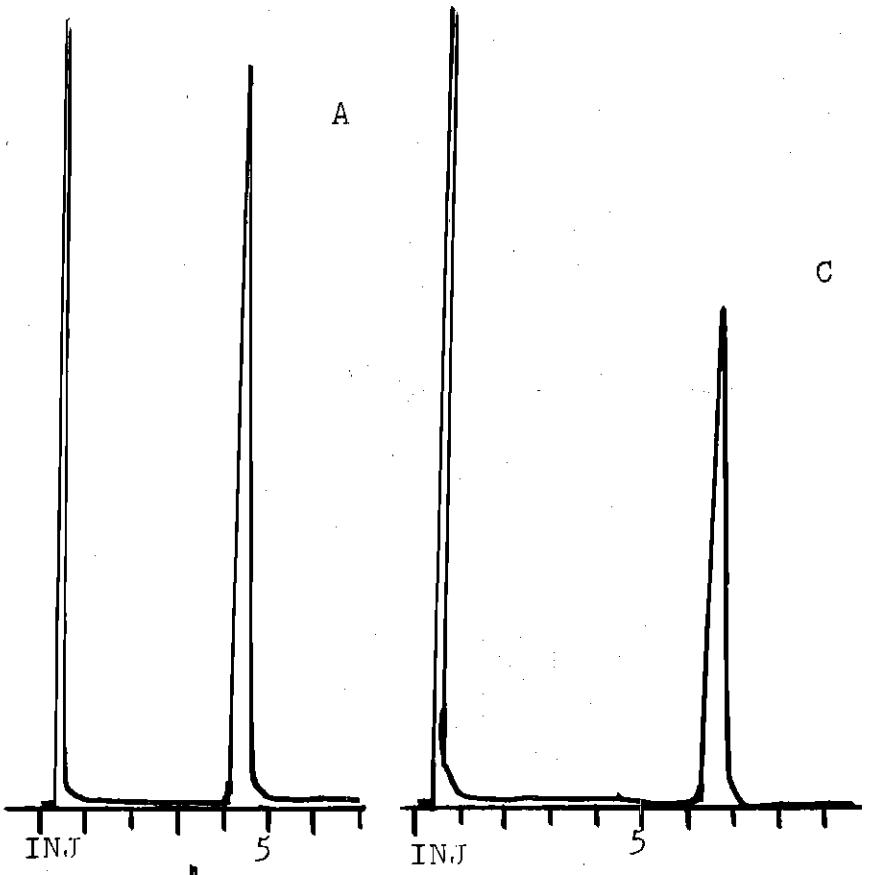

Fraction 9A
INJ
5
10
15
20
25
30
Time in min

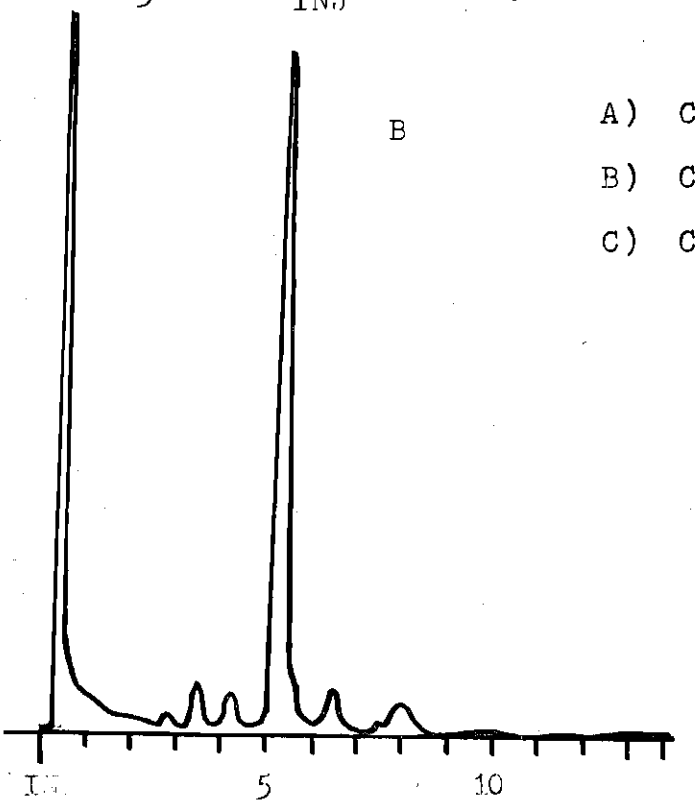
Fraction 10A




Fraction 11A
INJ
5
10
15
20
Time in min


Fraction 3C
INJ
5
10
15
20
25
30
Time in min

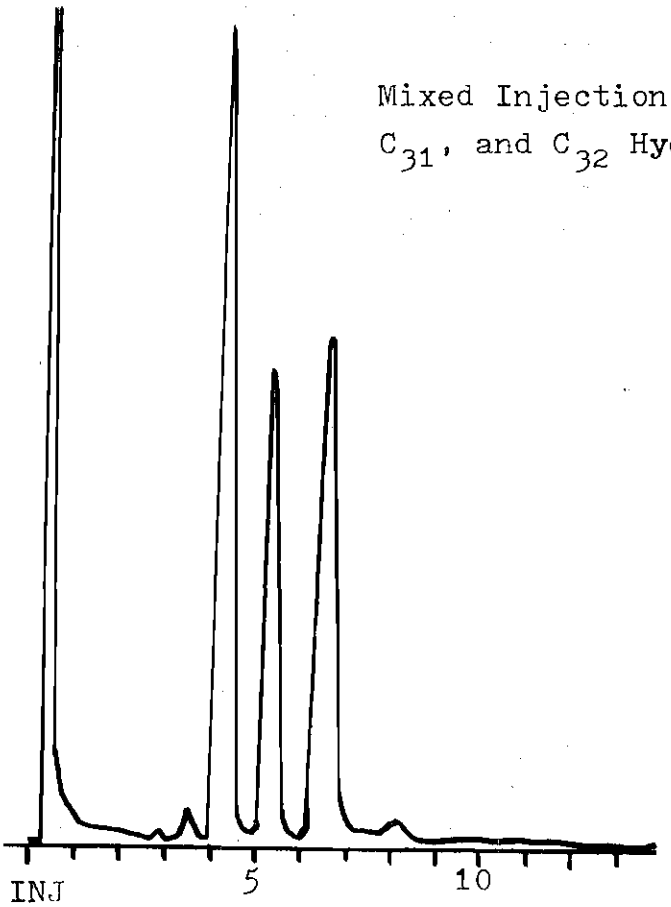




A) C_{30}

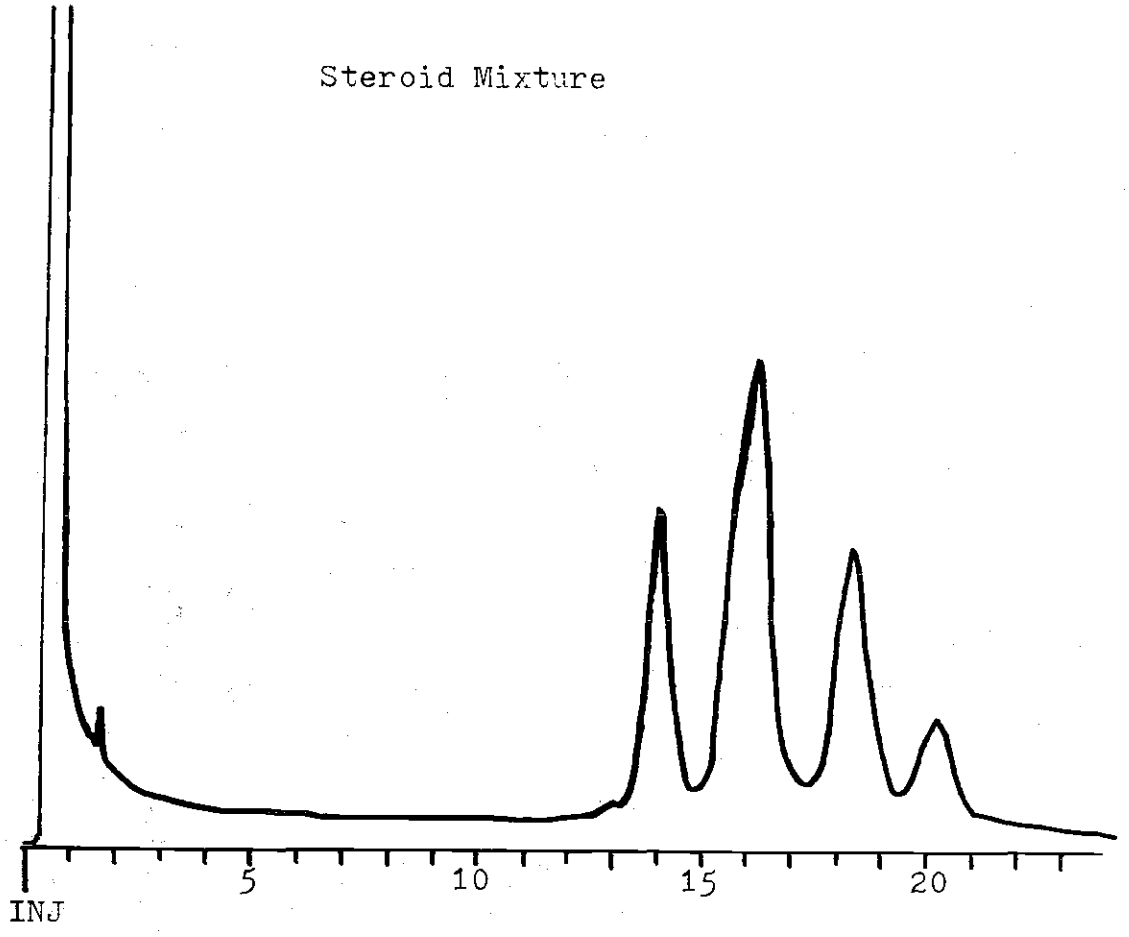
B) C_{31}

C) C_{32}



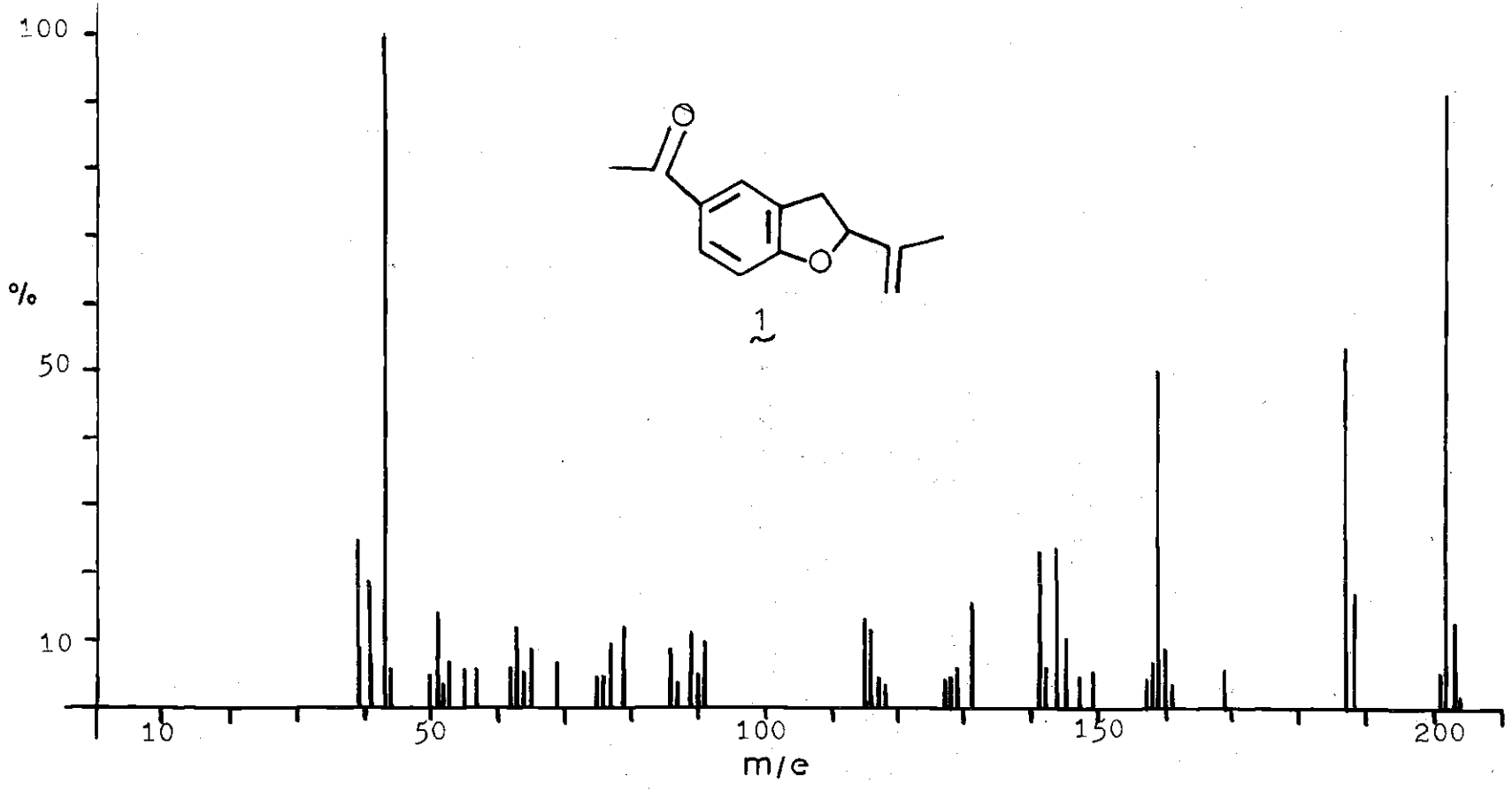
Mixed Injection of C_{30}, C_{31}, and C_{32} Hydrocarbons

Steroid Mixture

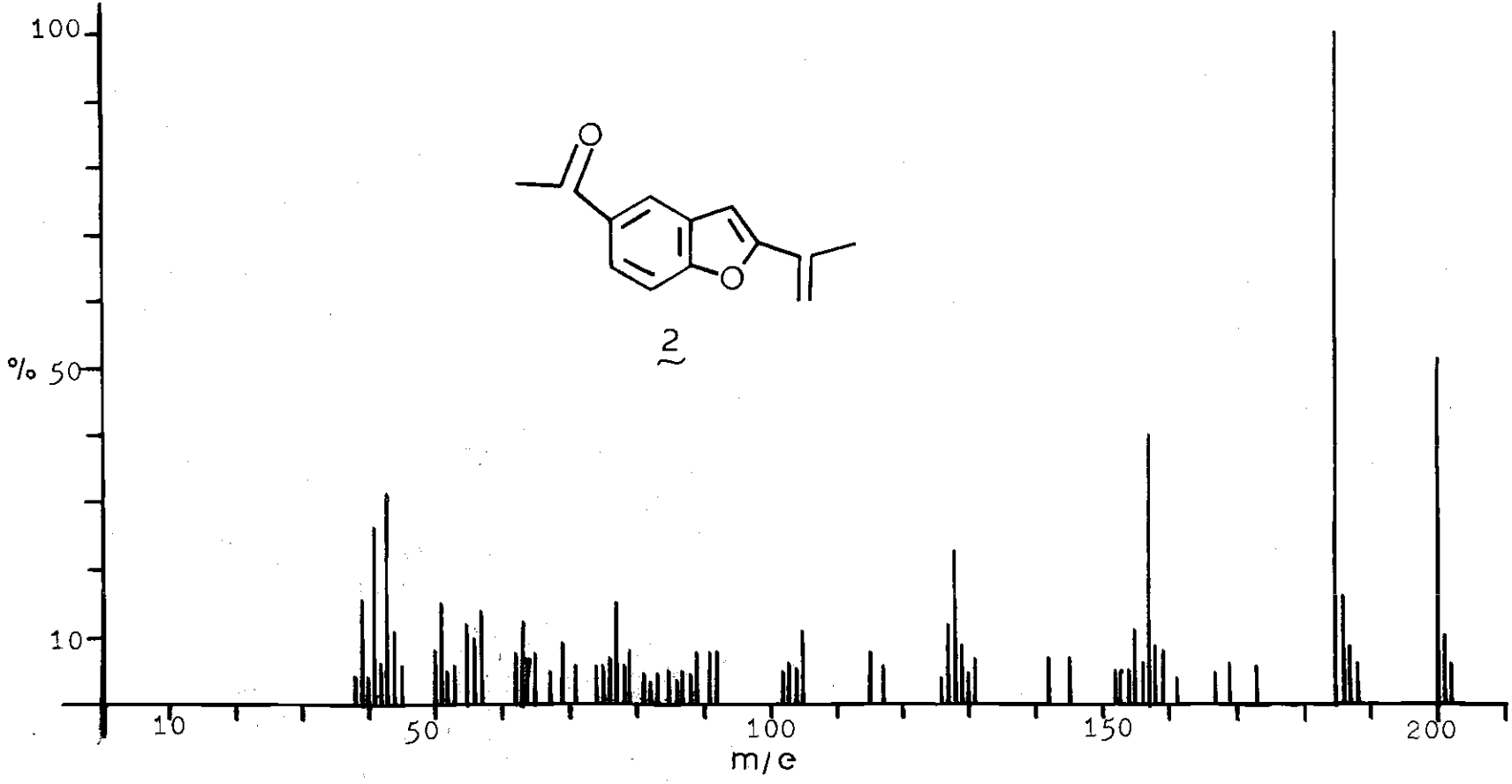


APPENDIX 2

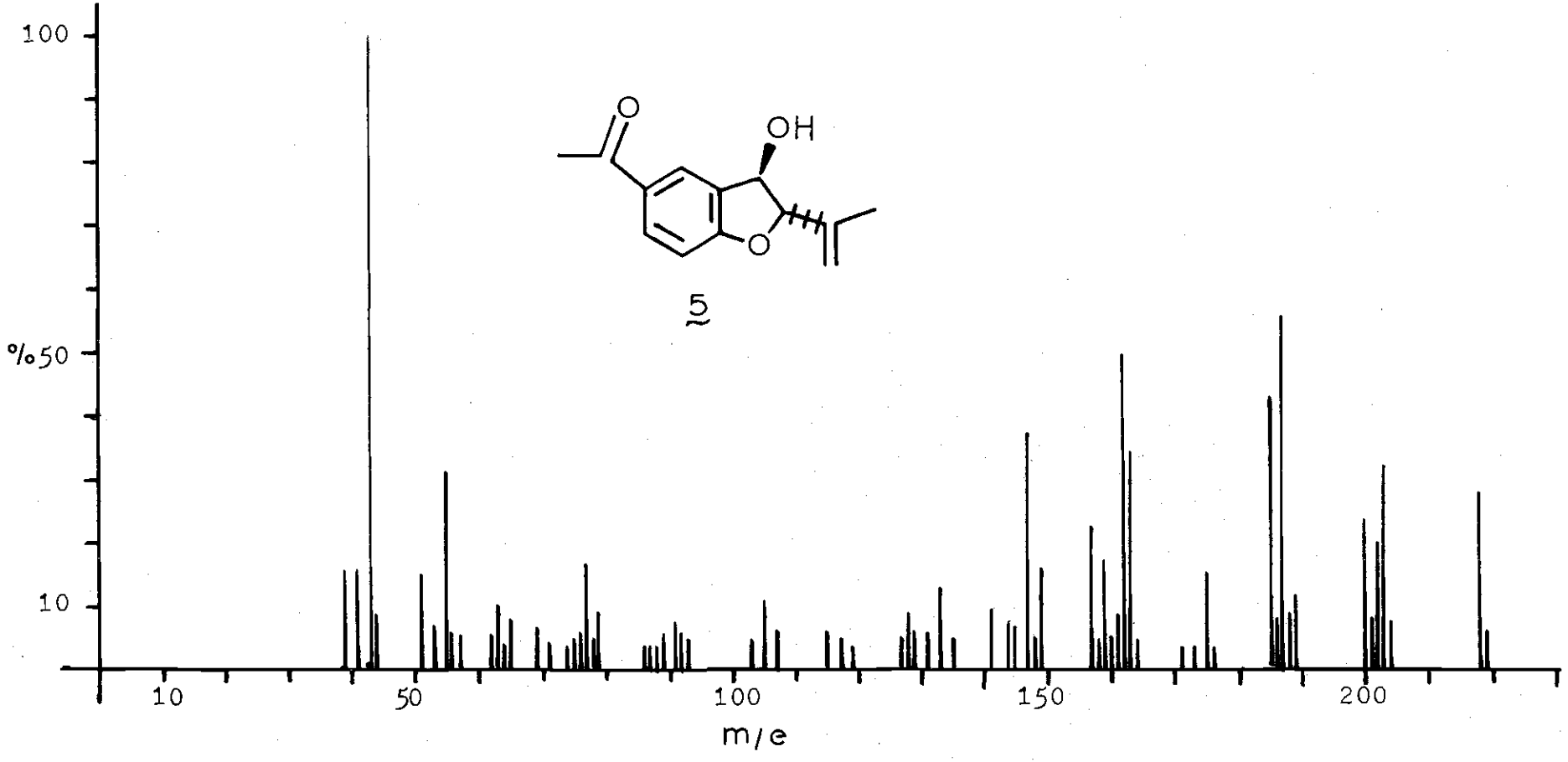
MASS SPECTRA



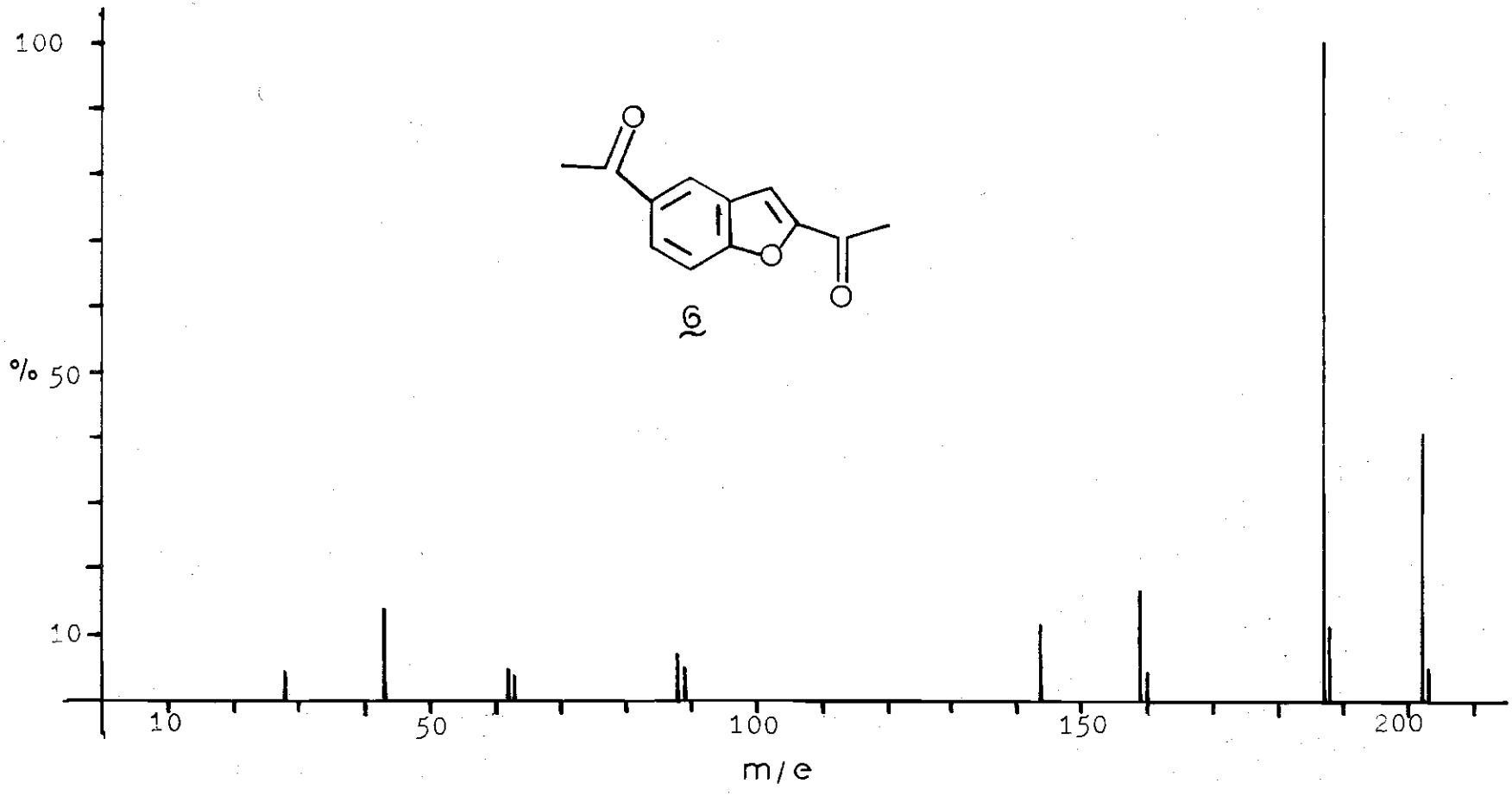
1



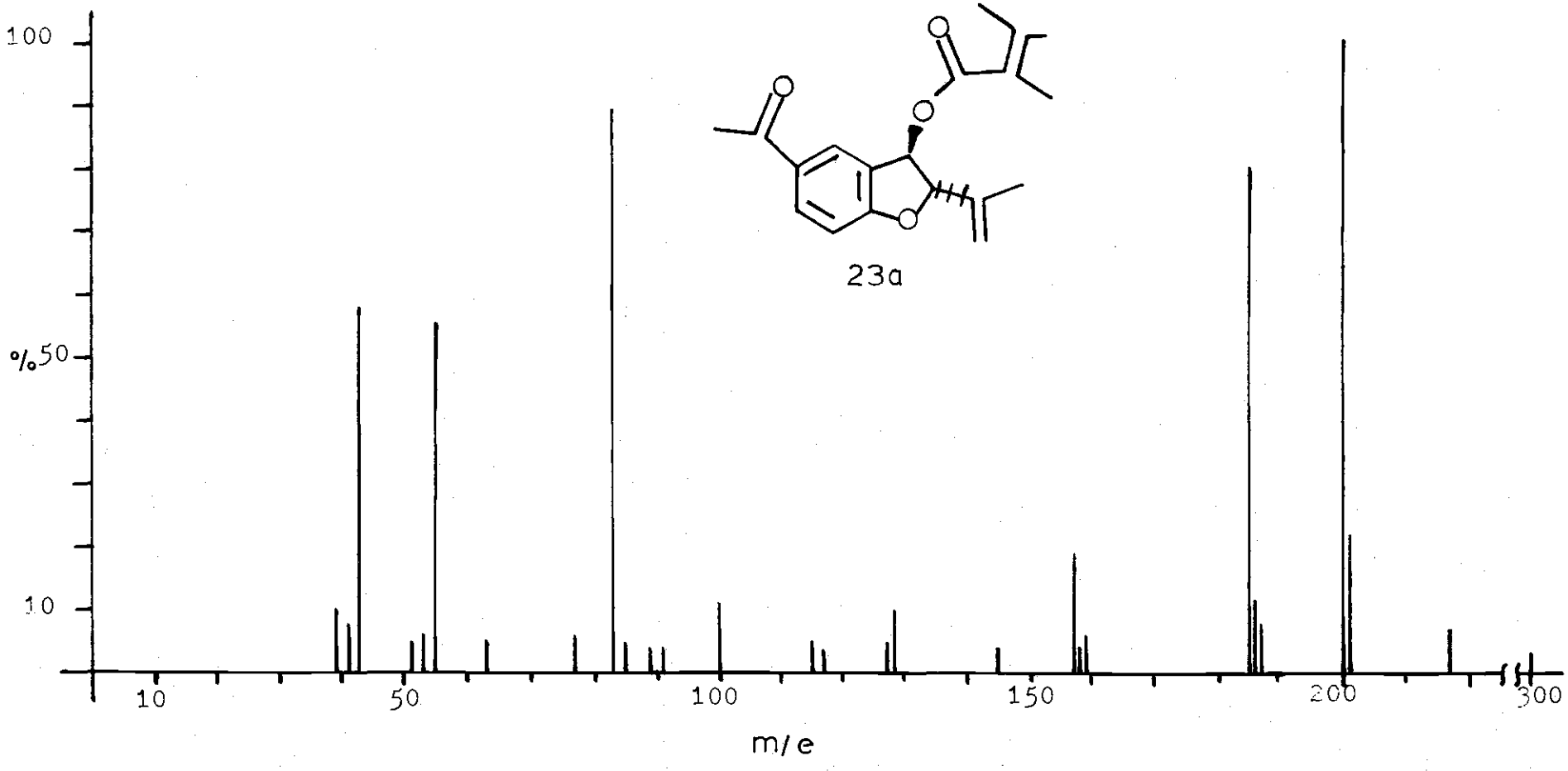
2



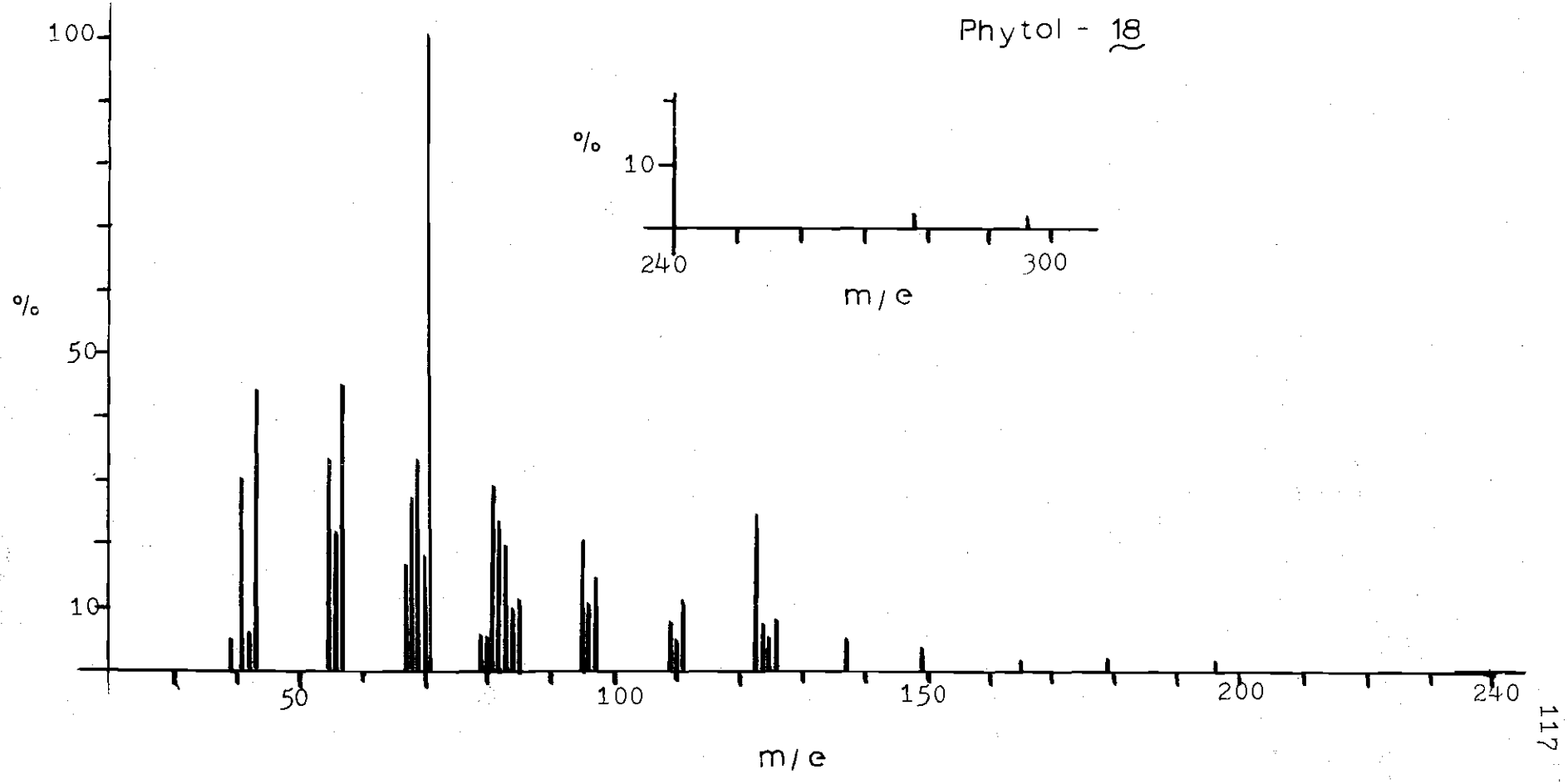
5

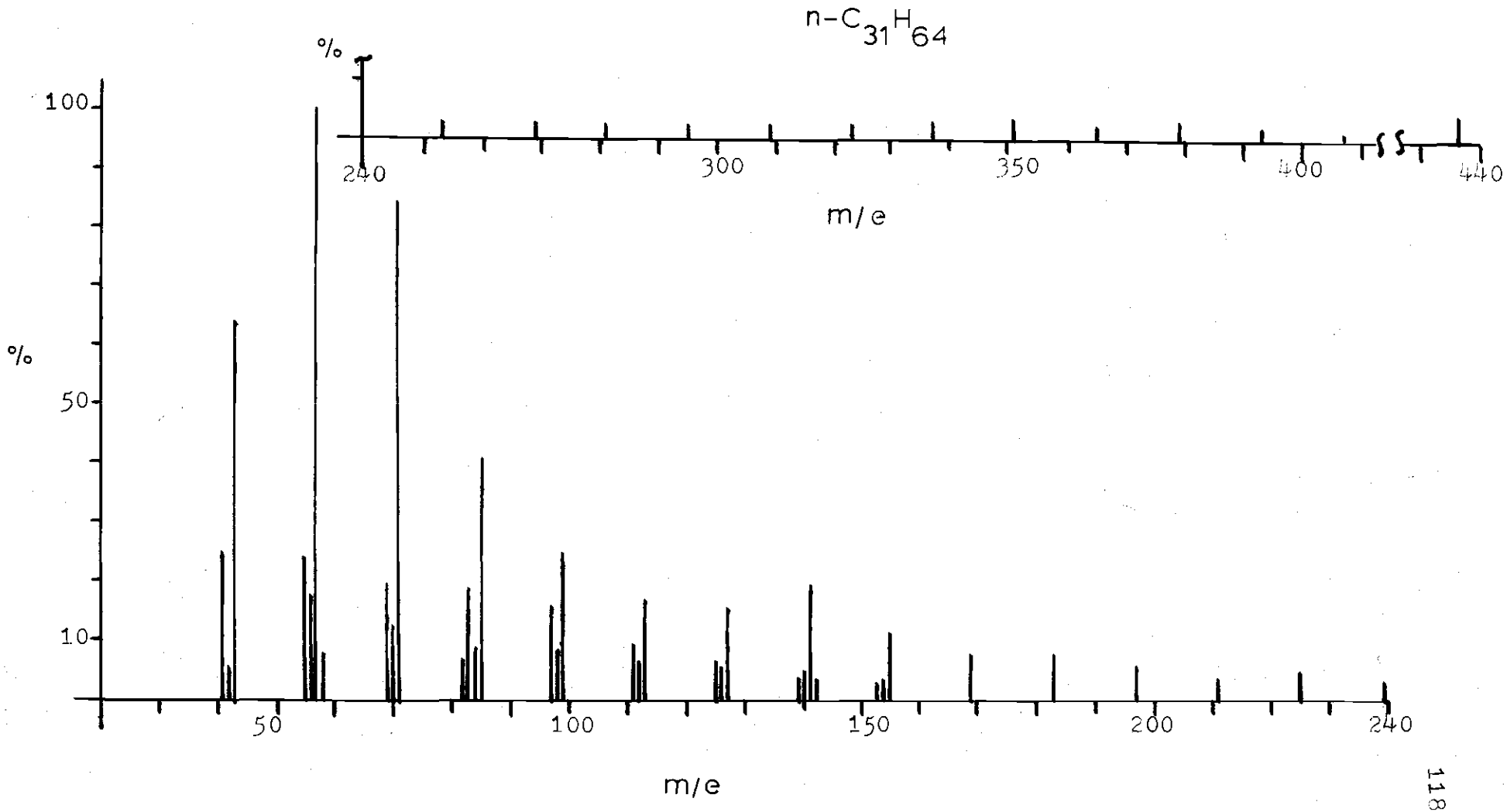


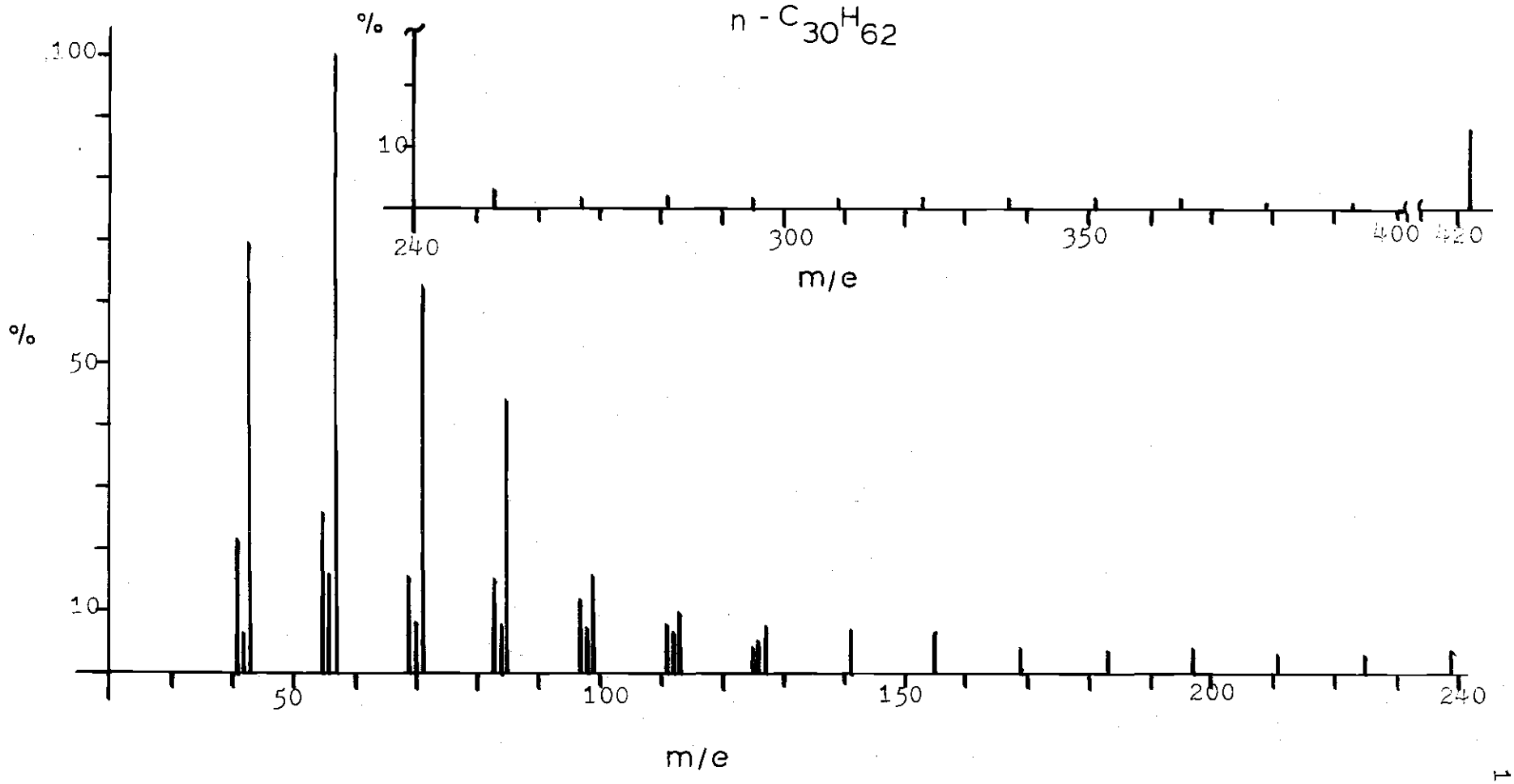
6

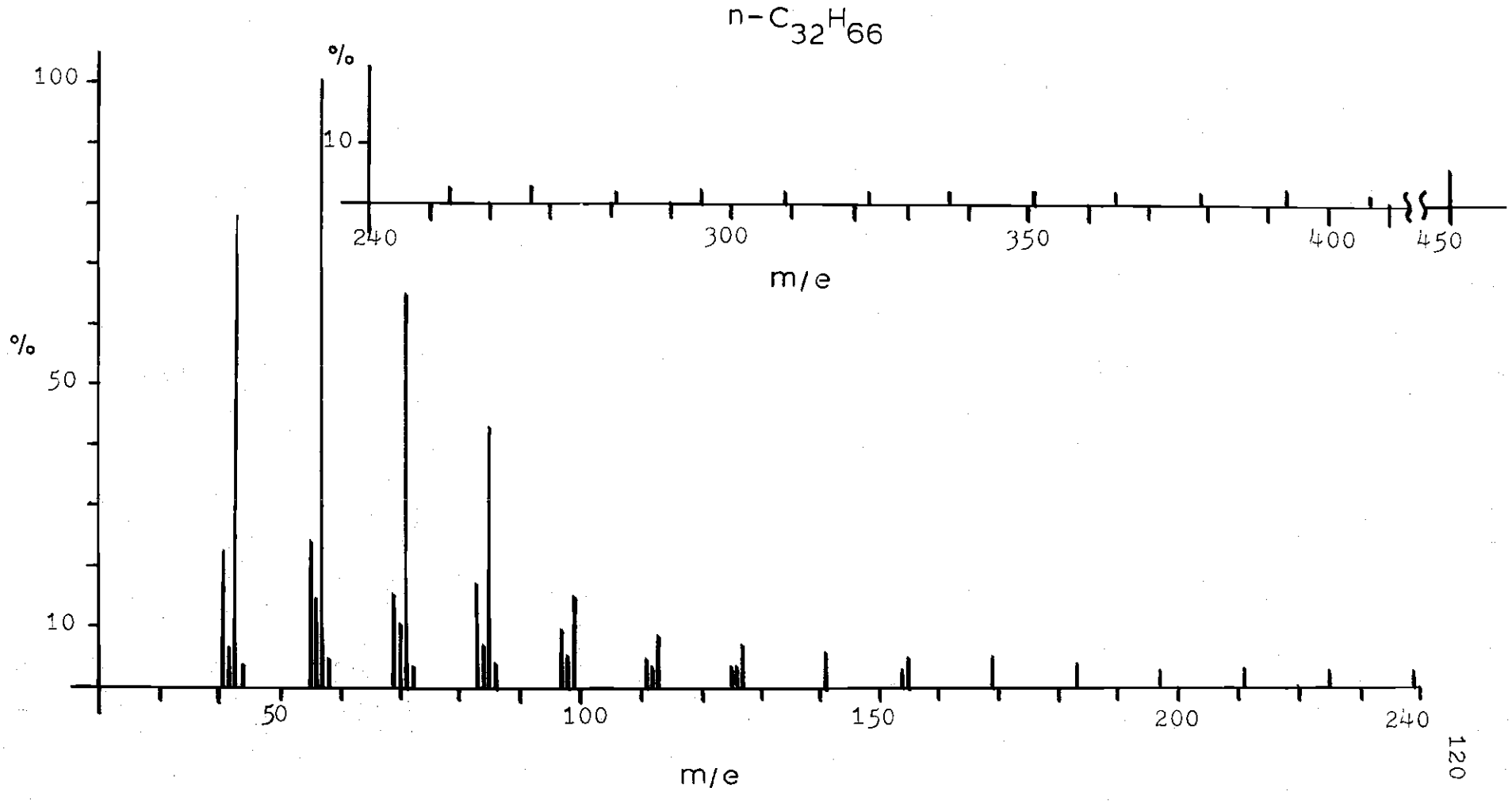


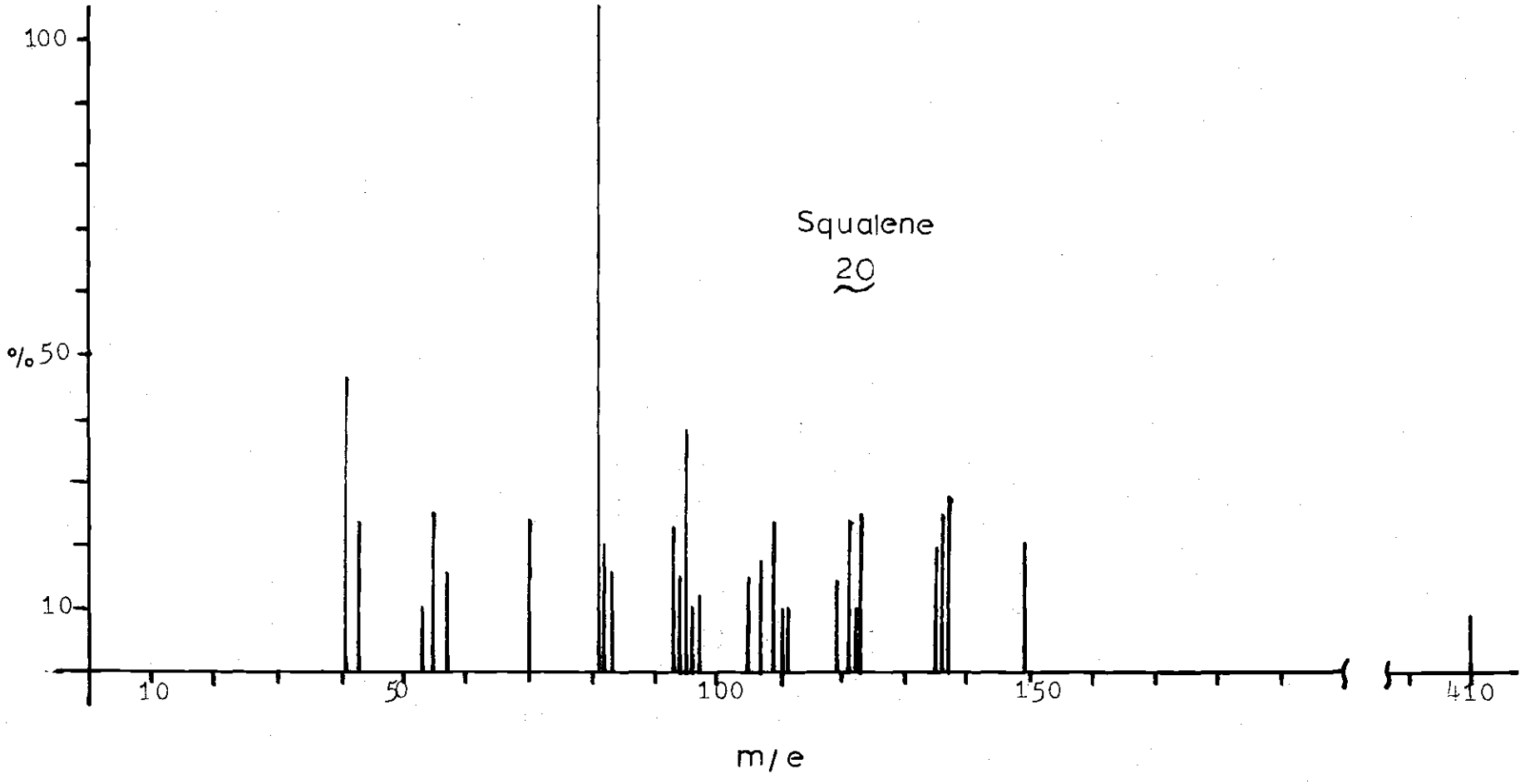
23a

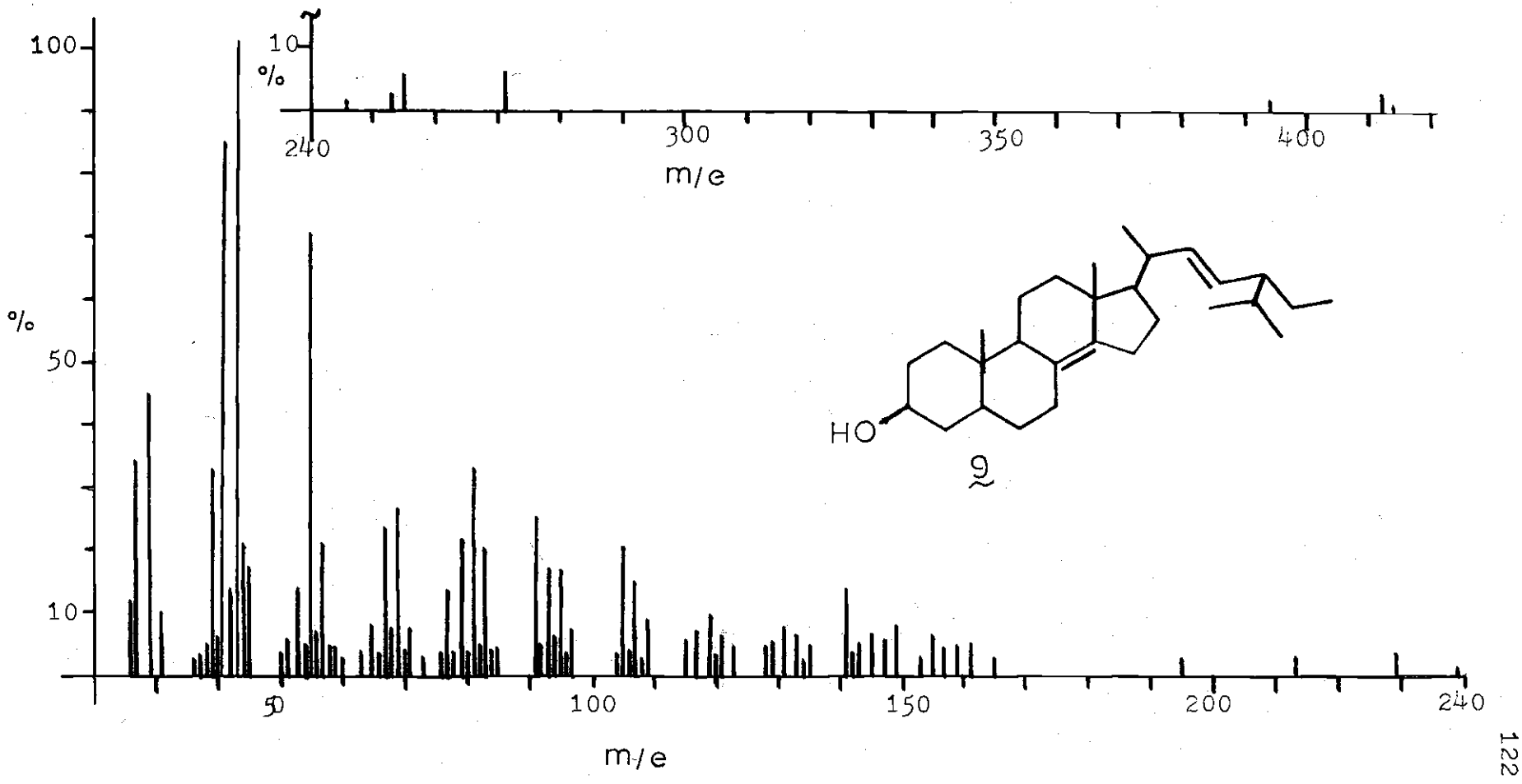

Phytol - 18
100
50
10
%
50
100
150
200
240
m/e
%
10
240
300
m/e

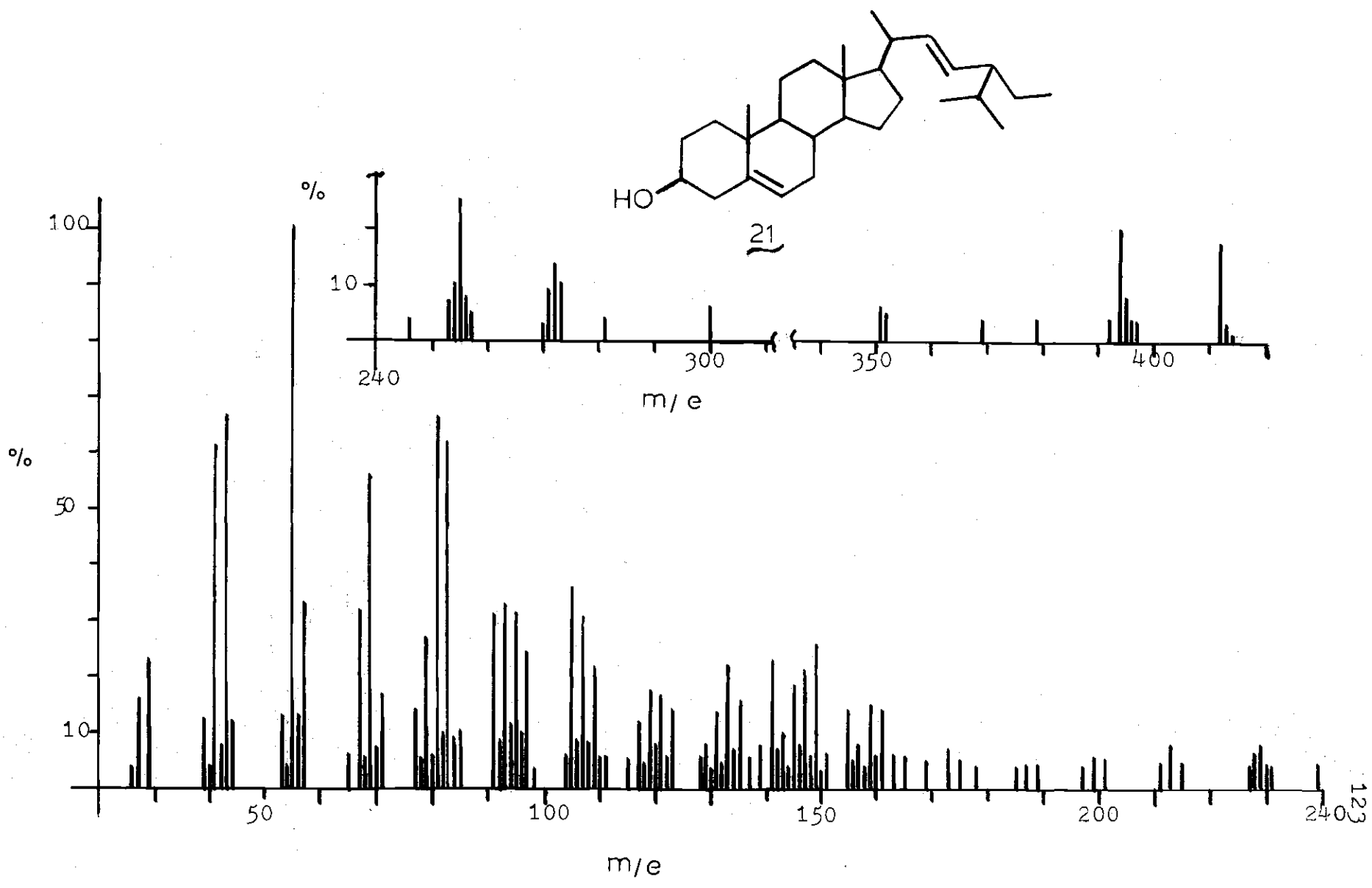

n-C31H64
%
100
50
10
50
100
150
200
240
m/e
%
240
300
350
400
440
m/e

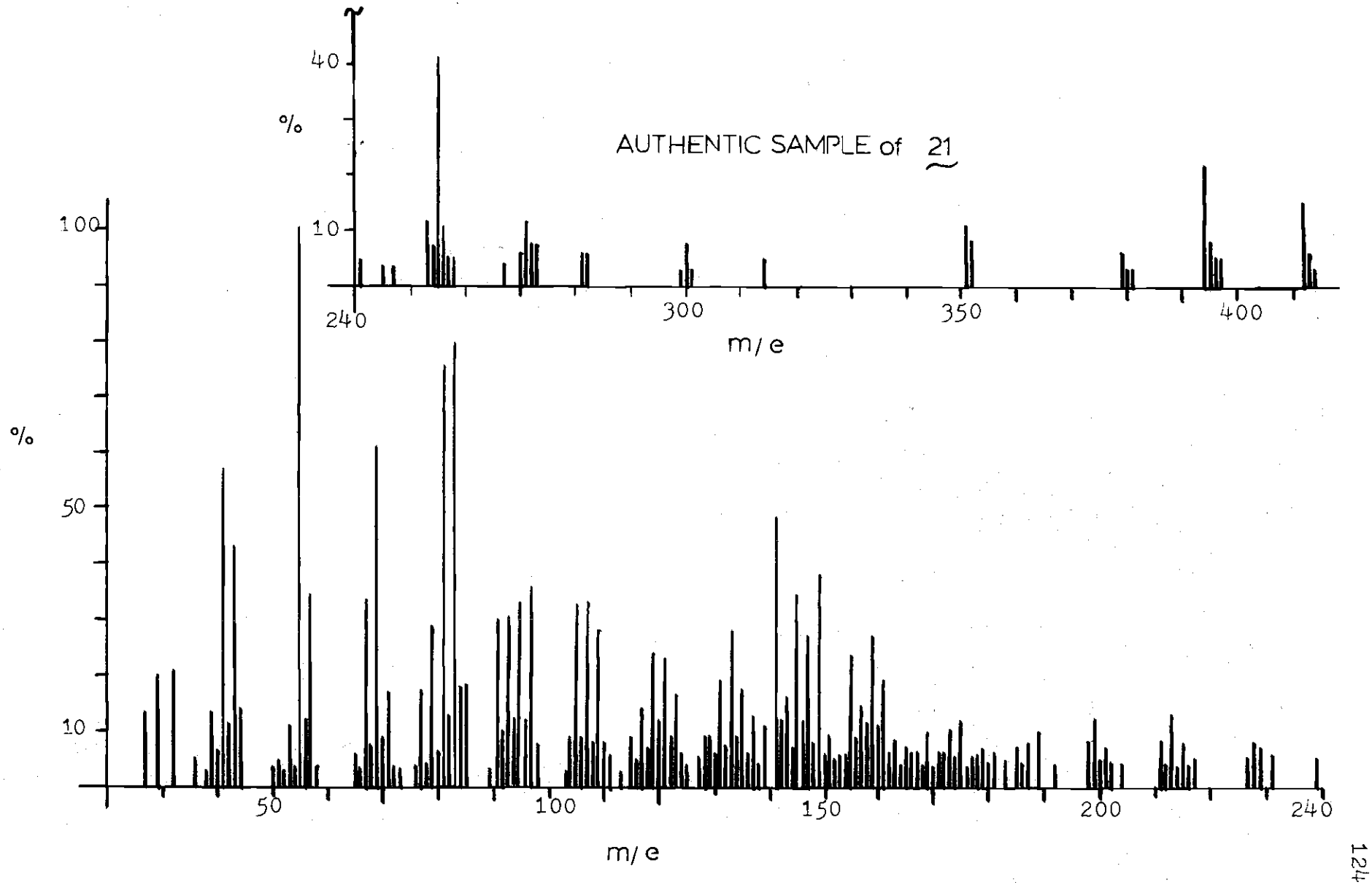

n - $C_{30}H_{62}$
%
100
50
10
240
300
350
400
420
m/e
%
10
50
100
150
200
240
m/e

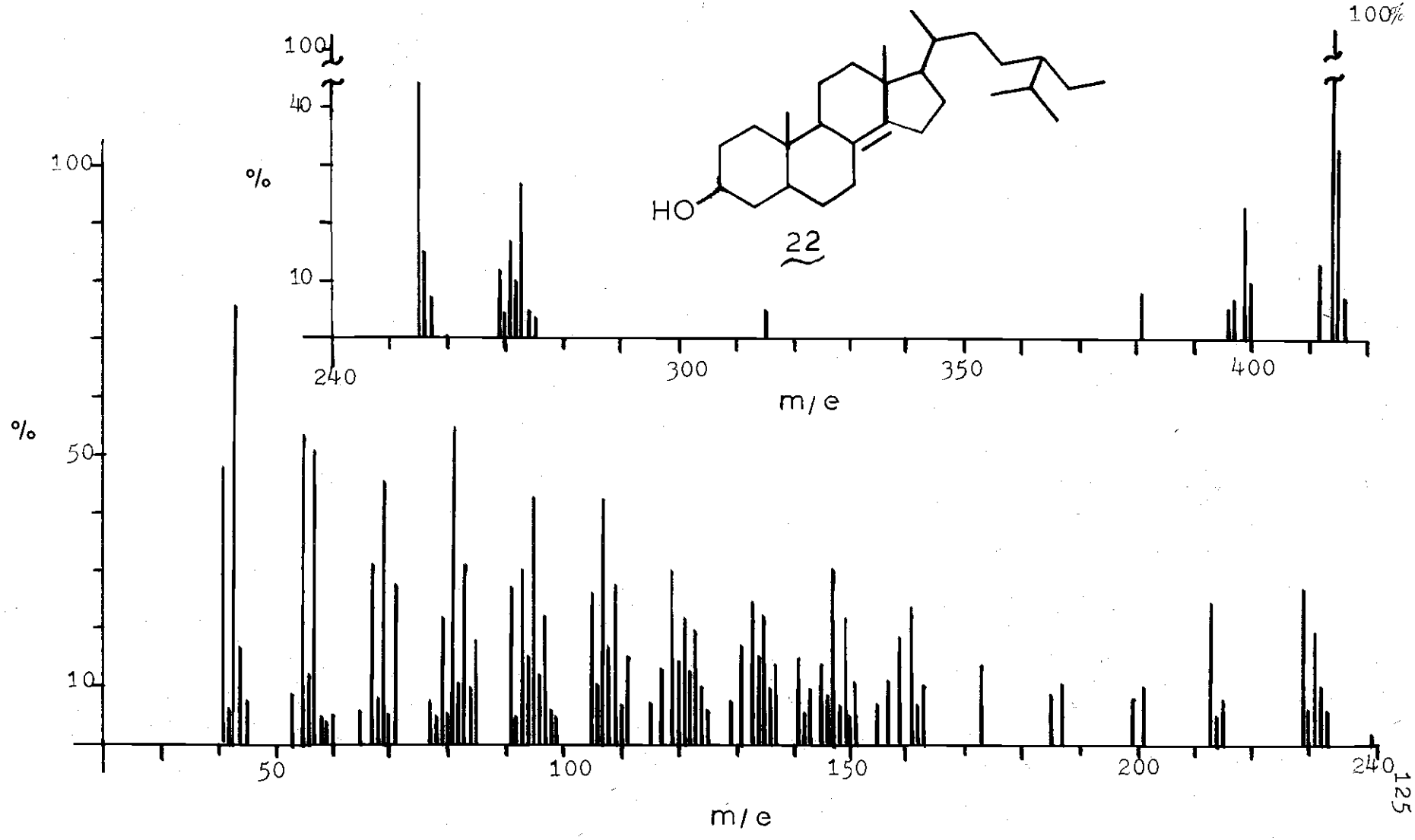

n-C32H66
%
100
10
240
300
350
400
450
m/e
%
50
10
50
100
150
200
240
120
m/e


100
%50
10
Squalene
20
10
50
100
150
410
m/e


100
%
50
10
50
100
150
200
240
m/e
10
%
240
300
350
400
m/e
HO
9


HO
21
%
100
50
10
50
100
150
200
240
m/e
123
%
10
240
300
350
400
m/e


AUTHENTIC SAMPLE of 21
%
40
10
240
300
350
400
m/e
%
100
50
10
50
100
150
200
240
m/e


100%
100
40
10
%
240
300
350
400
m/e
HO
22
100
%
50
10
50
100
150
200
240
125
m/e

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VITA

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Mr. Novak attended Temple University in Philadelphia, where he received his Bachelor of Arts degree with a major in chemistry in June, 1972.

In September, 1972, he began graduate study in the School of Chemistry at the Georgia Institute of Technology, Atlanta, GA under the direction of Dr. Leon H. Zalkow.

In October, 1976, he married Jo-Ann Stout of Atlanta, GA.

Mr. Novak is a member of the American Chemical Society.