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I. COMPARATIVE STUDY OF LIPIDS OF SHARK LIVERS. II. COMPARATIVE BIOCHEMICAL STUDY OF SOME GORGONIANS

The University of Oklahoma PH.D. 1980

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THE UNIVERSITY OF OKLAHOMA

GRADUATE COLLEGE

I. COMPARATIVE STUDY OF LIPIDS OF SHARK LIVERS

II. COMPARATIVE BIOCHEMICAL STUDY OF SOME GORGONIANS

A DISSERTATION

SUBMITTED TO THE GRADUATE FACULTY

in partial fulfillment of the requirements for the

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MUHAMAD WIRAHADIKUSUMAH

Norman, Oklahoma

1980

I. COMPARATIVE STUDY OF LIPIDS OF SHARK LIVERS
II. COMPARATIVE BIOCHEMICAL STUDY OF SOME GORGONIANS

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DEDICATIONS

To my wife, Anon, and to my children, Jaka, Dian, Ratna and Sari.

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To the memory of

my father and my mother

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CHAPTER I

COMPARATIVE STUDY OF LIPIDS OF SHARK LIVERS

INTRODUCTION

In areas where coral reefs are associated with continental shelves and where the depth of the water does not change sharply, sharks are usually found in the shallow waters of the coral reefs only at night and in the deeper waters most of the time. The sharks move into and out of the shallow coral reef areas.

In the Pacific where the coral atolls occur there are very sharp, steep dropoffs into very deep water. There seem to be two populations of sharks, one associated with the relatively shallow lagoons and the other with deep water.

In the course of a visit to the Pacific Marine Laboratory at Enewetak Atoll in the Marshall Islands in May 1978, Dr. L. S. Ciereszko was able to obtain livers of a number of species of sharks caught in shallow waters. According to Dr. J. E. Randall, these sharks do not move periodically from deeper to shallow water. They form a

shallow water population.

The livers of sharks make up a very large proportion of the total body weight and are rich in "oil." The low density of the oil rich and large liver is thought to endow the shark with nearly neutral bouyancy and thus to facilitate the movement of sharks from great to small depths. The triterpenoid hydrocarbon squalene is found in shark liver oil, often in relatively large amounts, and because of its very low density would be expected to contribute heavily to the buoyancy of the shark.

It was of interest to examine the lipid composition of the livers of shallow water sharks which move relatively little vertically. One of the shallow water sharks, *Triaenodon obesus*, the whitetip reef shark, was pregnant when caught and yielded two unborn pups, permitting a comparison of the lipid composition of the liver of the unborn young (embryo) with that of its mother.

Sharks (class Chondrichthyes, subclass Elasmobranchii, order Selachii) (1,2) are called cartilaginous fish because the skeletal elements lack the architecture of true compact bone with included living bone cells. They are usually fusiform and always have their gill slits either more or less above the plane of the pectoral fins or in advance of them, whereas the batoids or rays are flattened forms with ventral gill slits and pectoral fin attached at their forward ends to the sides of the head.

About 300 species of shark are known. More than half are either restricted to depths greater than 200m or enter lesser depths rarely and briefly. Sharks have no swim-bladder and tend to sink to the bottom if not supported by the hydrodynamic lift given to the swimming shark by fins and body surfaces that may act as planes. The tendency to sink is offset to a varying extent by the presence of a large amount of fat stored as oil in the liver with specific gravity around 0.9. Shark livers vary greatly in content of low density oil with species, size and physical condition of the shark, and they vary greatly in terms of percentage of total weight of the fish. Baldrige (3, and references therein) reported the liver to be 3-25% of total weight of *Galeocerdo cuvieri* (the tiger shark), *Carcharhinus leucas* (the bull shark) and *C. obscurus* (the dusky shark), and 10-15% for *C. milberti* (the brown shark). He found that the total oil content of the liver of Florida sharks ranged from only a few percent by weight in juvenile *Negaprion brevirostris* (the lemon shark) to 75% in adult *C. milberti*. The oil showed specific gravities between 0.91 and 0.92. A lower specific gravity, between 0.89 and 0.90, was exhibited by the liver oil of *G. cuvieri*. It was found that deep-sea sharks maintain essentially constant whole-animal density during growth by means of compensatory increases in densities of other tissues as the liver lipid content increases, re-sulting in a positive correlation between percentage liver

in the shark and body density. It has been reported, however, that the common shark of New England, Odontaspis taurus (3,4), gulps air at the surface and holds it in its stomach for varying periods to offset the insufficient buoyancy provided by its relatively dense liver oil.

An early suggestion of the function of fat as a buoyancy mechanism in shark was made by Denton (5) in the case of a Portugese shark, Centroscymnus coelolepis (the deep sea shark). The buoyancy of this fish is due to the very large amount of fat of low density (about 0.88 g/ml) in the liver. The liver accounts for about 30% of the total volume of the fish and contains over 80% fat. The fat has a large unsaponifiable fraction containing the hydrocarbon squalene as a major constituent. This fraction with its density of about 0.85 g/ml is over 70% more efficient at giving lift than the more usual animal fat, triacyl glycerol. This suggested that one of the principal functions of the liver was the high content of lipid in making the fish neutrally buoyant.

The possible role of squalene in buoyancy was studied by Heller et al. (6) using sharks caught from the west and east coasts of Florida, the Caribbean and the Gulf of Mexico. It was found that there was no significant difference in squalene content of the liver samples, as a function of sex, size of shark or habitat. Of 18 shark liver samples, only 2 contained huge amounts of squalene;

70% and 90% squalene in _Dalatias_ _licha_ and _Centrophorus_ _uyato_ shark liver oils, respectively. The rest of the liver oils, obtained from 16 shark samples, including the tiger shark, _Galeocerdo_ _cuvieri_ and _C_. _leucas_, contained less than 1% squalene. The function of squalene, in relation to the buoyancy mechanism, is unclear. However, it is clear that one of the principal functions of the liver must be to give buoyancy and that its oil content must be regulated to give neutral buoyancy. On the other hand, many teleosts bring themselves close to neutral buoyancy by using a gas-filled swimbladder or by the fat which their tissues or sometimes swimbladders, contain (7).

The study of deep-sea shark liver oils of the family Squalidae by Corner _et al_. (7) indicated that their livers made up 19 to 29% of the weight of the animals. The oil accounted for 80-86% of weight of the liver, with specific gravities around 0.88, and squalene accounted for around 42% of the oil. For comparison, the basking shark liver oils contained 7 to 45% squalene. They suggested that if the function of the oil were to give buoyancy, it would be much more economical, in terms of acetate as the precursor for the biosynthesis, to make squalene in place of the more common fish oil rich in oleic acid.

In addition to squalene, other hydrocarbons have been isolated and characterized from shark liver oils: C_{19} monoolefins (8), pristane (8,9,10,11), C_{20} diolefins (8),

n-paraffins with C_{15} to C_{38} (9), octadecane (10), nonadecane (10), and phytane (8,10).

Glyceryl ethers with various long-chain groups (saturated and unsaturated C_{16} and C_{18} chains) have been found in some liver oils from Suruga Bay sharks (*Centroscyllium kamohari*, *Chimaera barbouri* and *Scapanorhynchus owstoni*) (12,13). Six shark liver oils of the so-called "Kuroko-zame" from Sanriku, Japan (*Triakis scyllia*, *Squalus aqanthias* (the spiked dogfish), *Apristurus macrorhinchus*, *Centroscyllium ritteri*, *Centrophorus spp*. and *Cetorhinus maximus* (the basking shark), were studied by Kayama *et al*. (14). The content of glyceryl ethers varied widely from 0.4% of *C*. *maximus* liver oil to 11% of *S*. *acanthias* liver oil. The major glyceryl ethers were chimyl, batyl and selachyl alcohols, accounting for from 48% to 72% of the total glyceryl ethers. The unusual hydrocarbon chains of glyceryl ether found were carbon chains shorter than C_{14} and odd-numbered, branched and diunsaturated chains present in only small amounts. Methoxy- and 2-oxy-substituted glyceryl ethers with saturated and monounsaturated C_{14}, C_{16}, and C_{18} chains have been reported in the unsaponifiable fraction of Greenland shark liver oil (15).

Fatty acid composition of shark liver oils varied widely with species and location. Shimma *et al*. (12), in work on sharks from Suruga Bay, found between 9.9 and 13.1% of $C_{22:6}$ and $C_{22:5}$ fatty acids (13). They also found

the same fatty acid pattern in the egg oil of a deep-sea shark. In South American basking shark liver oils Gelpi and Oro (10) found normal fatty acids from C_{14} to C_{22} with the major components palmitic, oleic, monounsaturated eicosenoic and monounsaturated docosenoic acids. From six of the so called "Kuroko-zame" shark liver oils described above (14), Kayama et al. obtained fatty acids having C_{14} to C_{24} chains with palmitic (8.9-24.3%) and oleic (8.1-32.7%) acids as two of the major components. The percentages of eicosenoic and docosenoic homologs equaled or exceeded the oleic acid present, except in T. scyllia which were much lower. Major amounts of highly unsaturated fatty acids, including $C_{20:5}$ and $C_{22:6}$ acids, did occur.

The biosynthetic and structural study of triacylglycerol of dogfish (Squalus acanthias) liver oil, by Malins and Robisch (16), using radioactive fatty acids as the biosynthetic precursor, indicated that palmitic acid and the C_{20} and C_{22} polyenoic acids accumulated preferentially on position 2 of the liver triacylglycerol, whereas stearic acid and the monoenoic acids ($C_{18:1}$, $C_{20:1}$ and $C_{22:1}$) accumulated on positions 1 and 3.

It was reported by Gershbein and Singh (9) that sterol content of oil from the basking shark liver was up to 460 mg/100 g oil, and Shimma et al. (13) reported that cholesterol contents of egg oil, from deep-sea sharks of Suruga Bay, ranged between 33 and 59 mg/g.

Buteau et al. (17) examined the lipids of the Pacific and Atlantic coast triakid sharks by studying the lipid content of the cestodes collected from the leopard shark Triakis semifascinata and the brown smooth-hound shark Rhinotriakis henlei. Cestodes have been known to be incapable of synthesizing fatty acids de novo or of interconverting absorbed fatty acids. The fatty acids of the cestodes (Lacistorhynchus tenuis, Orygmatobothrium musteli and Calliobothrium verticillatum) reflect to varying degrees the fatty acids of host intestinal contents, and their liver oils. Neutral lipids comprised about one-half the total lipids of L. tenuis and about three-fourths of those in O. musteli. The fatty acid composition of the total lipids of all cestode species were similar, with high contents of $C_{18:1}$, $C_{20:5}$ and $C_{22:6}$ fatty acids. $C_{20:5}$ and $C_{22:6}$ together comprised about 50% of the fatty acids. Other unsaturated fatty acids together accounted for 20 to 25%, whereas the saturated acids constituted 15 to 20%.

My research work involved the comparative study of lipids of seven shark liver samples. Three of them were livers from the whitetip reef shark Triaenodon obesus (family Triakidae): one from an unborn young, one from an adult male and one from a mother shark. Three others were from the genus Carcharhinus (family Carcharhinidae): the grey reef shark Carcharhinus amblyrhinchos, the silvertip shark C. albimarginatus, and the Galapagos shark (C. gala-

pagensis). One was from the tiger shark Galeocerdo cuvieri (family Carcharhinidae). Triaenodon obesus and C. amblyrhinchos are common sharks on coral reefs of the tropical and subtropical Indo-Pacific region (18). They are relatively small species (between 82 and 165 cm total length), well adapted for life in the shallow reef environment. C. albimarginatus, C. galapagensis, and Galeocerdo cuvieri are larger sharks, considered as shore fishes. They are voracious predators of other smaller sharks (18). According to Gilbertese (19), T. obesus and G. cuvieri are among the most dangerous species of sharks to use as food due to the toxicity of their livers. Eating these sharks especially the liver caused ciguatera poisoning (18,19).

RESULTS AND DISCUSSION

A. Comparison of Lipids of Shark Livers derived from Unborn Young with that from Mother

Sample livers were collected by Dr. L. S. Ciereszko from whitetip reef shark *Triaenodon obesus*, one sample from the unborn young (the embryo) and the other from the mother.

Total Lipids

Lipids were extracted from the samples by chloroform-methanol (1:1, v/v) using a Soxhlet extractor for 4 and 8 h4. Extracts were evaporated to dryness using a rotary evaporator. The mother shark liver gave 92.9% of total lipids, density 0.895 g/ml, and the liver from the unborn young, 89.7%, density 0.898 g/ml (shown in Table 1). These values (92.9% and 89.7%) are higher than those of Florida shark livers, 75%, studied by Baldridge (3) and those of deep-sea shark liver oils studied by Corner *et al.* (7), 86%. However, the densities of my shark liver oils are lower than those of Florida shark liver oils, between 0.91 and 0.92 g/ml, and higher than those of the deep-sea shark liver oils (around 0.88 g/ml).

Squalene

Squalene was isolated from the total lipids and

determined spectrophotometrically by the method of Rothblat *et al*. (20) after it was chromatographed using hexane as eluting solvent and Florisil as adsorbent. Squalene was identified by tlc, ir and nmr spectrometry. The tlc, ir and nmr spectra of squalene isolated from the shark liver oil were identical with those of squalene standard. The tlc showed R_f 0.62 (0.25 mm Silica Gel H with hexane as eluting solvent) and R_f 0.93 (0.25 mm Silica Gel H with chloroform as eluting solvent). The ir spectra (Figures 2 and 4) indicated C-H stretching to the right of 3000 cm^{-1}, CH_2 bending at 1460 cm^{-1} and CH_3 bending at 1375 cm^{-1}. The nmr spectra (Figures 3 and 5) indicated signals at δ1.70 (vinylic methyl), δ2.0 and δ5.16. Table 2 shows that 95 to 100% recovery was obtained using the analytical procedure for squalene as described in the experimental section. Table 3 shows that the mother shark liver yields 0.36% squalene, while the embryo contains 0.53% squalene in the liver (0.39% squalene in the mother shark liver oil and 0.58% in the embryo shark liver oil).

Total and Free Cholesterol

Total and free cholesterol were determined quantitatively by the method of Courchaine *et al*. (22) as described in the experimental section. A standard curve was made using solutions of purified cholesterol (Figure 6).

Table 1

Lipids extracted from shark liver samples: percent total lipid (by weight), density (g/ml) and iodine value

Shark liver oil	Amt. of liver used for lipid extraction (g)	Total lipid (%)	Density (g/ml)	Iodine value
1. Whitetip reef shark, mother shark (*Triaenodon obesus*)	200	92.9	0.895	74.02
2. Whitetip reef shark, embryo (*T. obesus*)	60	89.7	0.898	55.5
3. Whitetip reef shark, adult male (*T. obesus*)	100	73.3	0.810	75.88
4. Grey reef shark (*Carcharhinus amblyrhinchos*	100	92.6	0.880	65.16
5. Silvertip shark (*Carcharhinus albimarginatus*)	100	87.6	0.895	52.9
6. Galapagos shark (*C. galapagensis*)	78.6	72.8	0.855	109.02
7. Tiger shark (*Galeocerdo cuvieri*)	100	58.1	0.843	50.08

Table 1a

Total lipid, squalene, and sterol contents from various shark liver oil

Shark species	Origin	% oil in the liver	% squalene in the liver oil	Reference
Carcharhinus milberti	Florida	75		(3)
Centroscymmus coelolepsis	Bay of Biscay	80		(5)
Eulamia milberti			<1	(6)
Carcharhinus leucas			<1	(6)
Sphyrna tiburo			<1	(6)
Narcine brasiliensis			<1	(6)
Negaprion brevirostis	Florida, Caribbean and Gulf of Mexico		<1	(6)
Galeocerdo cuvieri			<1	(6)
Dalatias licha			70	(6)
Centrophorus uyato			90	(6) (7)
Fam. Squalidae	Deep-Sea	80-86		(7)
Basking sharks			7-45	(7)
Basking sharks	South American		31.3	(10)

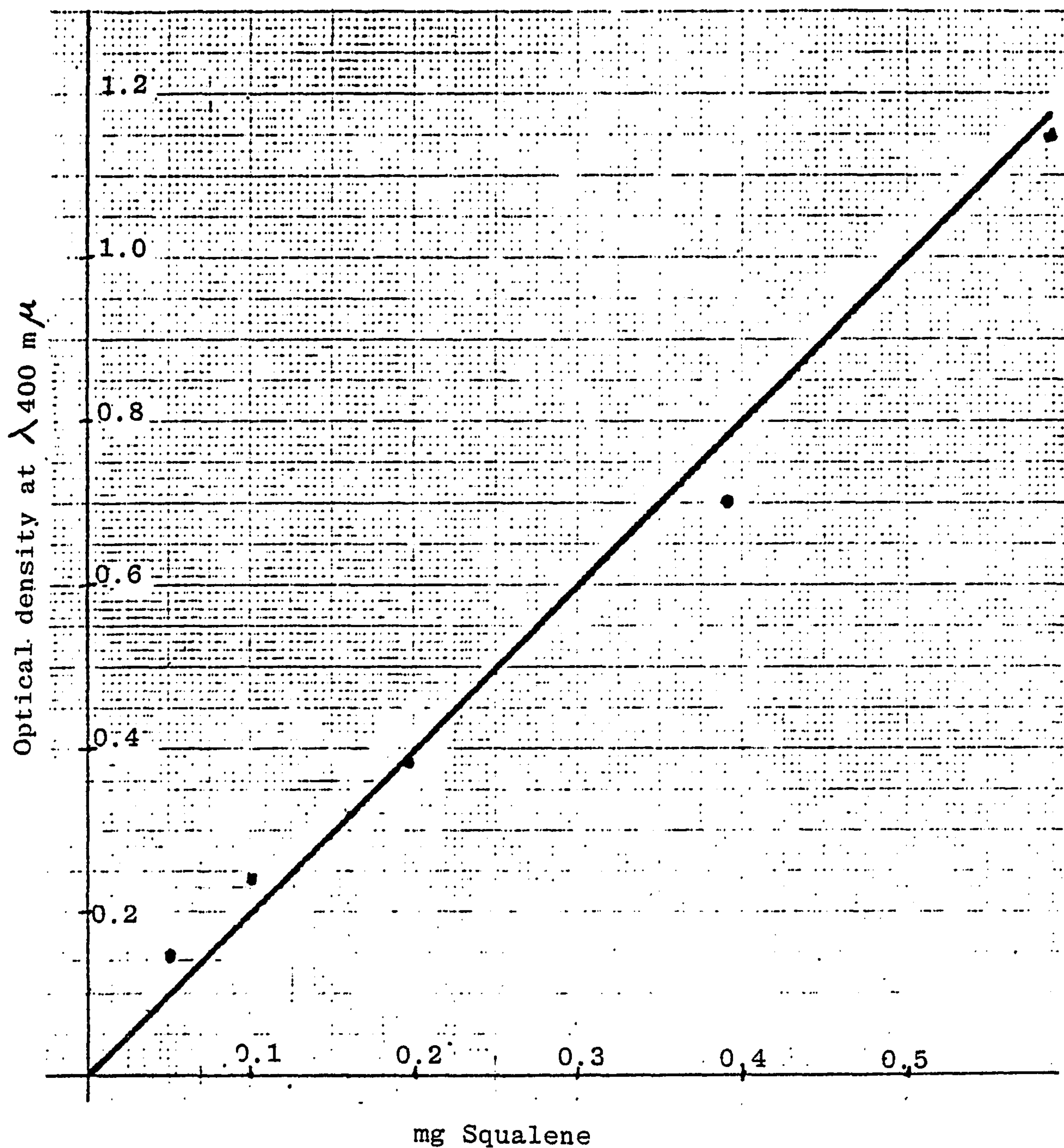

Figure 1. Standard calibration curve of squalene

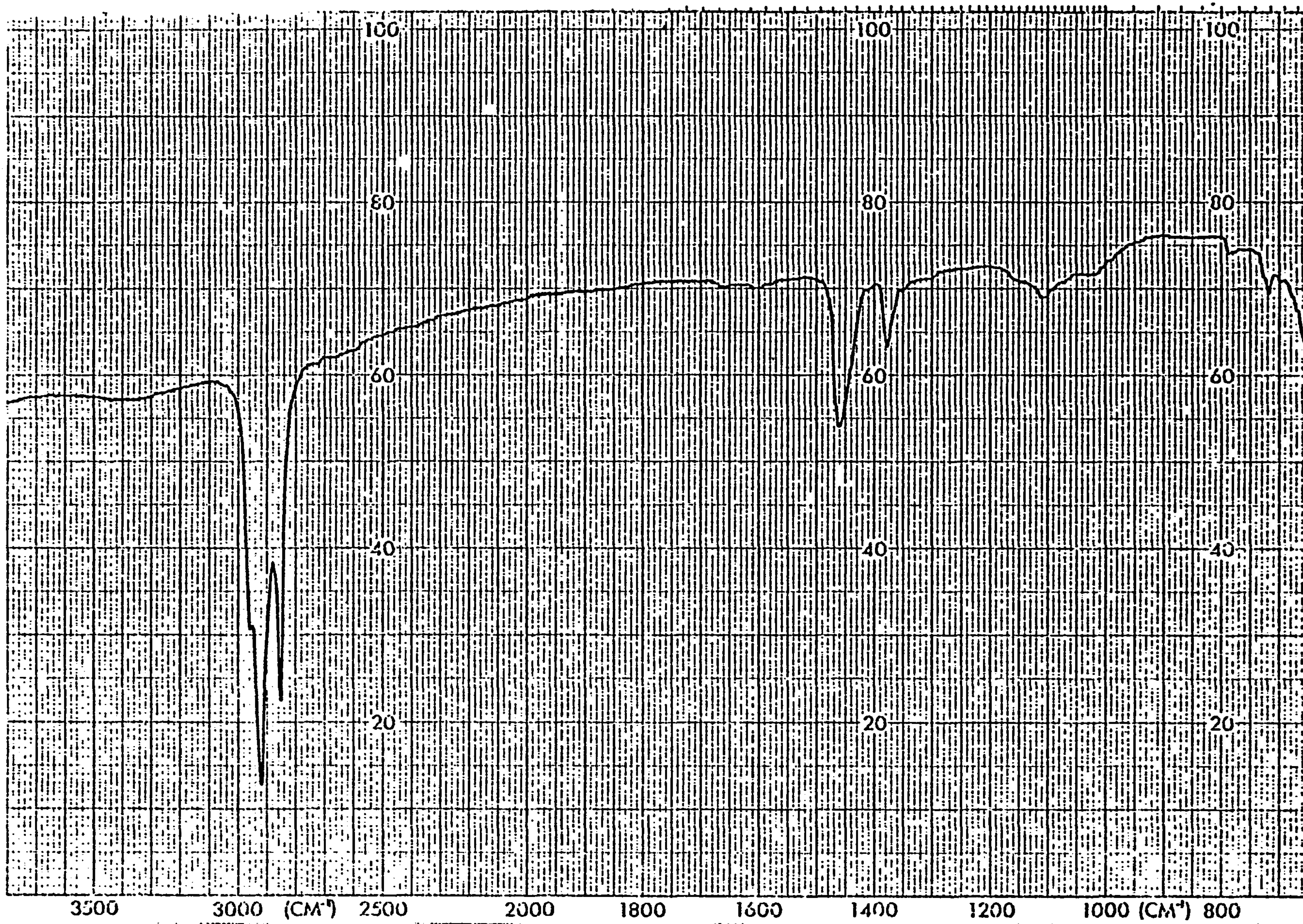

Figure 2. The ir spectrum of the squalene standard.

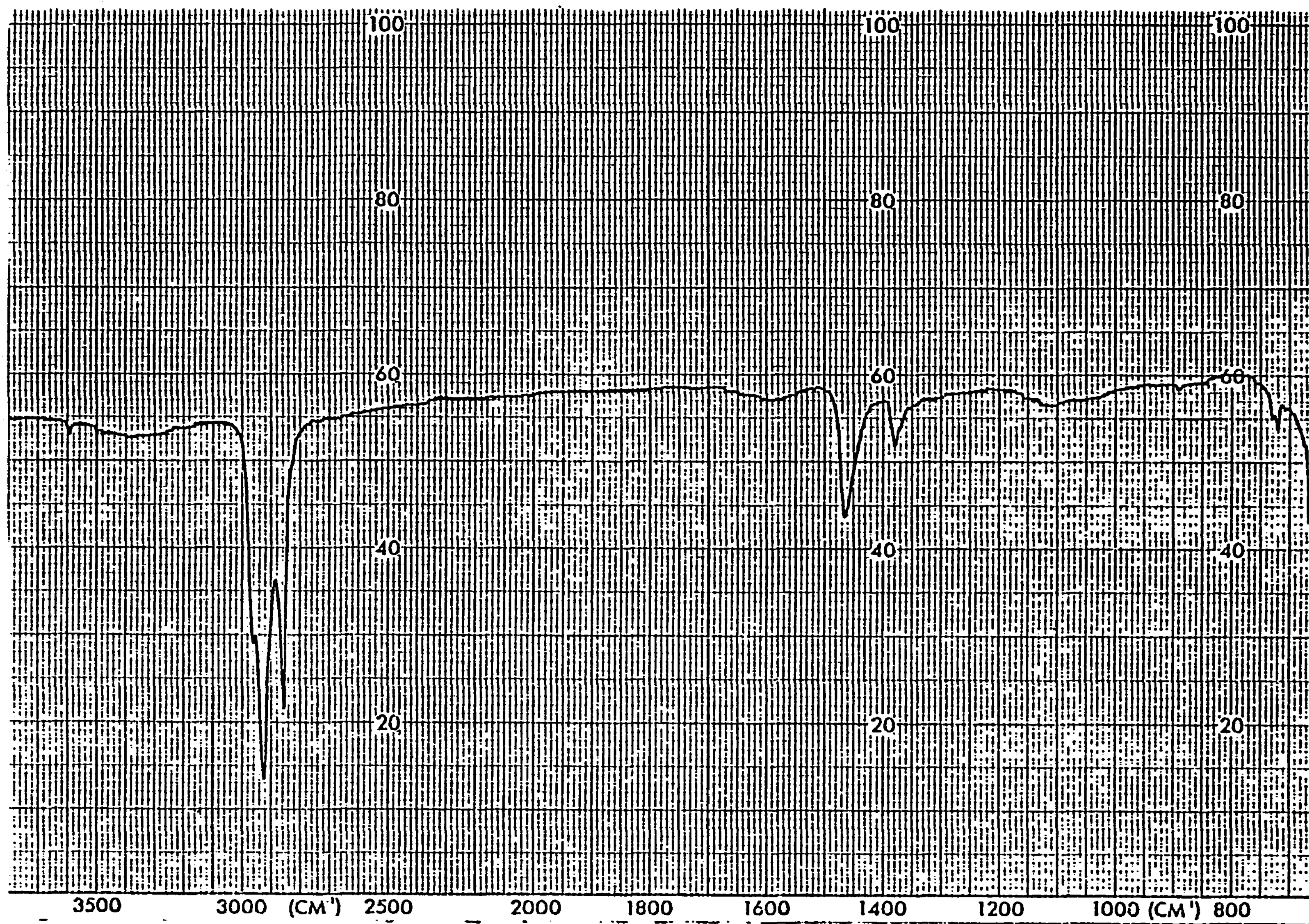

Figure 3. The ir spectrum of squalene isolated from the shark liver oil.

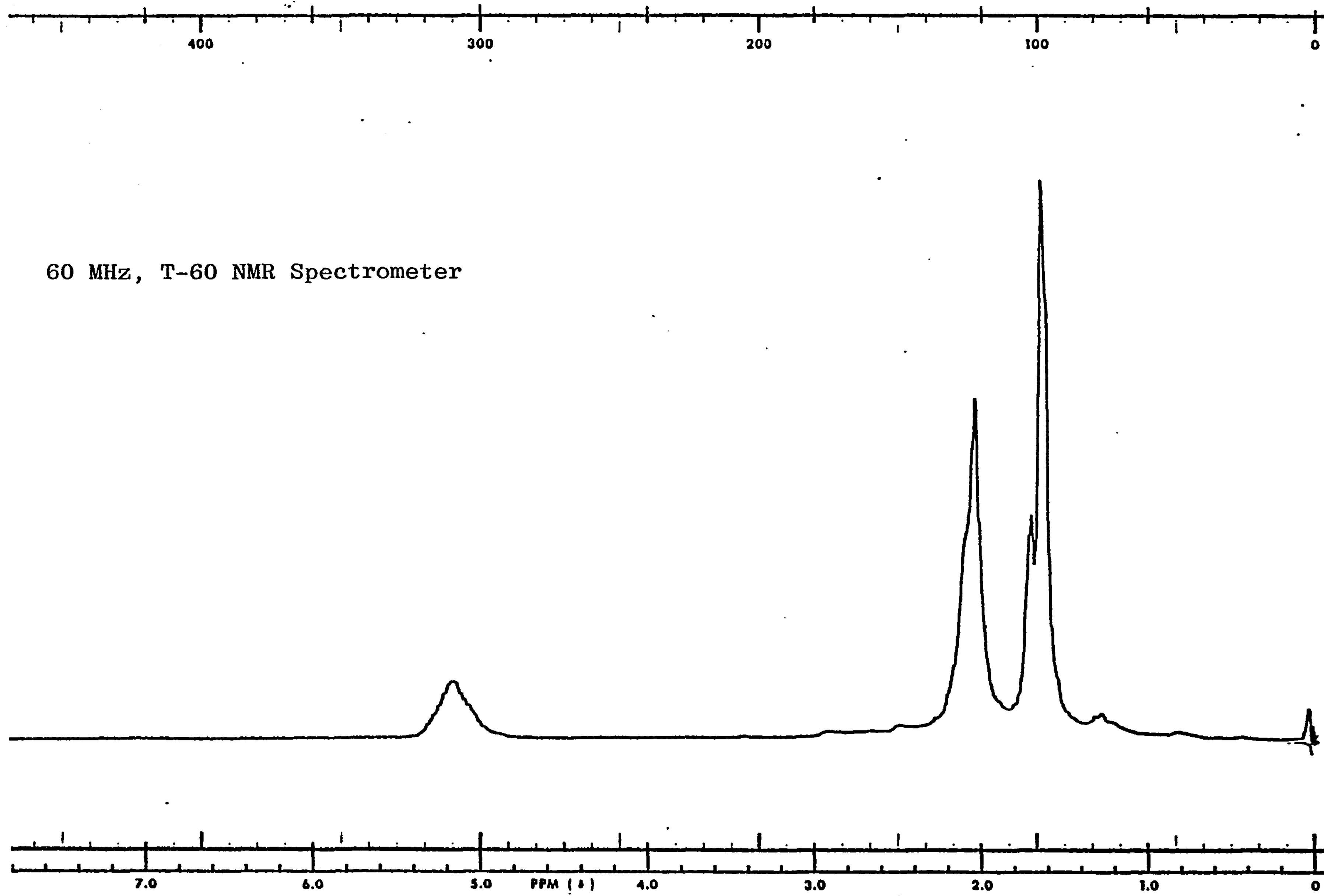

Figure 4. The nmr spectrum of the squalene standard.

60 MHz, T-60 NMR Spectrometer

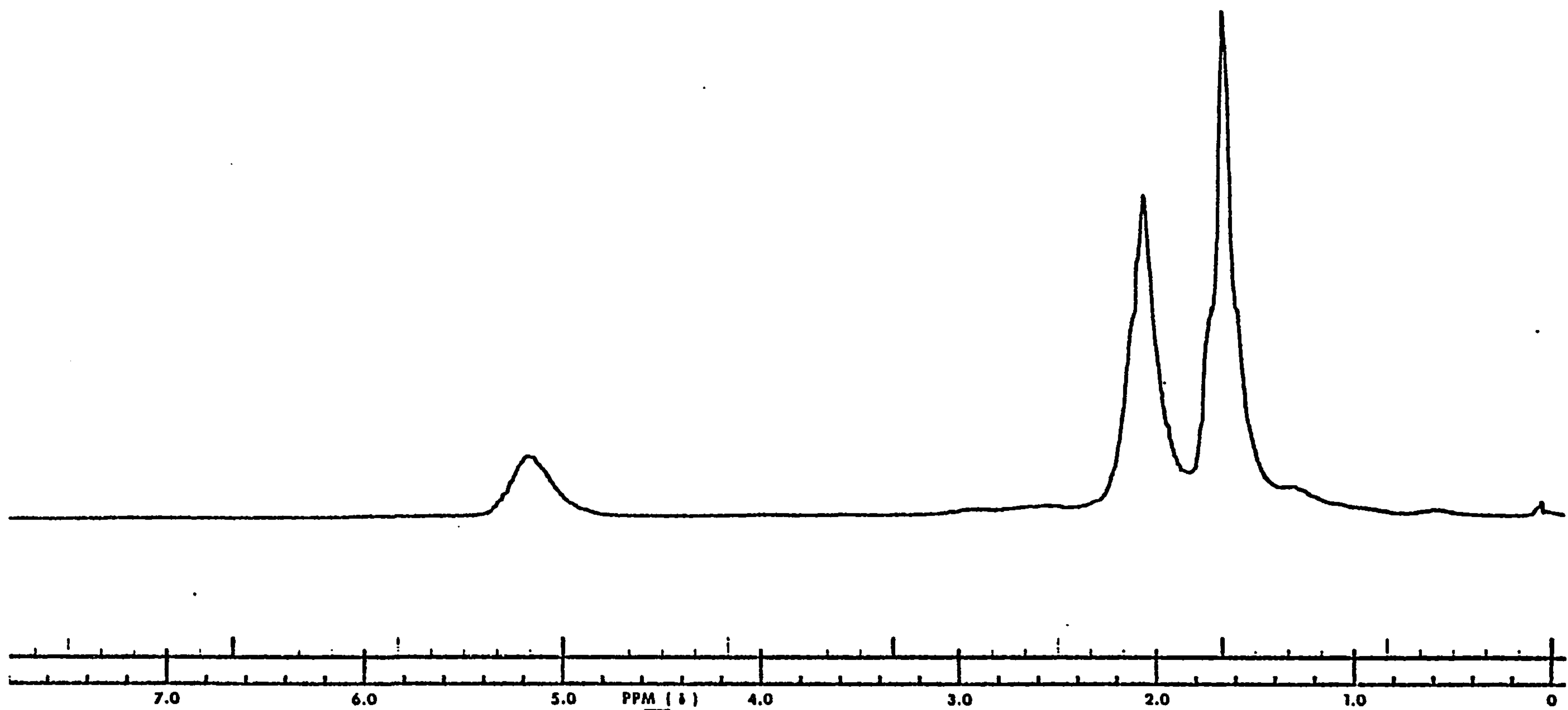

Figure 5. The nmr spectrum of squalene isolated from the shark liver oil.

Table 2

Recovery of squalene by column chromatography:
Different amounts of standard reference
squalene are added to definite amounts
of shark liver oil

Amount added to shark liver oil (mg)	Amount of squalene in the oil & added squalene (mg)	Amounts of squalene found in eluate (mg)	Recovery (%)
0	3.1*	3	97
4.9	8.0	8	100
9.8	12.9	14	108
19.6	22.7	22	97
81.0	84.1	80	95

*Amounts of squalene in 800 mg shark liver oil (mother *T. obesus*) determined spectrophotometrically.

Table 3

Squalene content of shark liver oil: (1) Determined by spectrophotometry following column chromatography;* (2) Determined by weighing samples separated by column chromatography*

Source	mg sample	Squalene content						
		1			2			Average (%) in the liver
		mg	% in oil	% in the liver**	mg	% in oil	% in the liver**	
1. *Triaenodon obesus*, mother	895	3.5	0.39	0.36	-	-	-	-
2. *Triaenodon obesus*, embryo	898	5.2	0.58	0.53	-	-	-	-
3. *Triaenodon obesus*, male	810	2.7	0.34	0.25	3.0	0.37	0.27	0.26
4. *Carcharhinus amblyrhinchos*	880	3.2	0.36	0.33	3.0	0.34	0.31	0.32
5. *C. albimarginatus*	895	2.0	0.23	0.20	3.0	0.34	0.30	0.25

Table 3 (continued)

Source	mg sample	Squalene content						Average (%) in the liver
		1			2			
		mg	% in the oil	% in the liver**	mg	% in the oil	% in the liver**	
6. *C. galapagensis*	855	3.0	0.35	0.25	2.9	0.35	0.25	0.25
7. *Galeocerdo cuvieri*	843	9.0	1.07	0.62	8.0	0.95	0.55	0.59

*Methods are described in the experimental section.

**Calculation based on the total lipid content, Table 1.

Table 4

Cholesterol content in shark liver oil: Total cholesterol and free cholesterol.

Shark liver oil*	Cholesterol, % by weight				
	Total cholesterol		Free cholesterol		Free cholesterol / Total cholesterol
	in the oil	in the liver	in the oil	in the liver	
1. _T. obesus_ (mother)	7.02	6.52	0.42	0.39	5.98
2. _T. obesus_ (embryo)	1.50	1.34	0.69	0.62	46.27
3. _T. obesus_ (adult male)	7.62	5.59	0.45	0.33	5.90
4. _C. amblyrhinchos_	6.45	5.97	0.35	0.32	5.36
5. _C. albimarginatus_	4.34	3.80	0.45	0.39	10.26
6. _C. galapagensis_	8.87	6.46	0.70	0.51	7.89
7. _G. cuvieri_	5.28	3.07	0.86	0.50	16.29

*For genus name see Table 1.

Table 4 shows that the mother shark liver contains more cholesterol, 6.52%, than does the embryo liver, 1.34%. However, the free cholesterol content is higher in the embryo liver, 0.62%, than in the mother liver, 0.39%. About 46% of the cholesterol occurs free in the embryo liver and only about 6% in the mother liver. The cholesterol ester content is 6.6% and 0.81% for the mother and the embryo livers, respectively. If one compares these cholesterol contents with those of the deep-sea sharks of Suraga Bay (13), one would see that the total cholesterol content in the mother shark *T. obesus* liver is close to the highest cholesterol content of the Suruga Bay shark liver, *i.e.*, 59 mg/g (or equal to 5.9%).

Iodine Value

Iodine values were determined iodometrically by the method of Yasuda (23) using a standardized solution of sodium thiosulfate to titrate the liberated iodine in the solution. The oil from the mother shark liver shows a higher iodine value, 74.02, than that of the embryo liver, 55.50, as shown in Table 1. Therefore, the mother shark liver contains more unsaturated lipid.

Total Phosphorus

Total phosphorus content of the lipids was determined spectrophotometrically by the method of Allen (24).

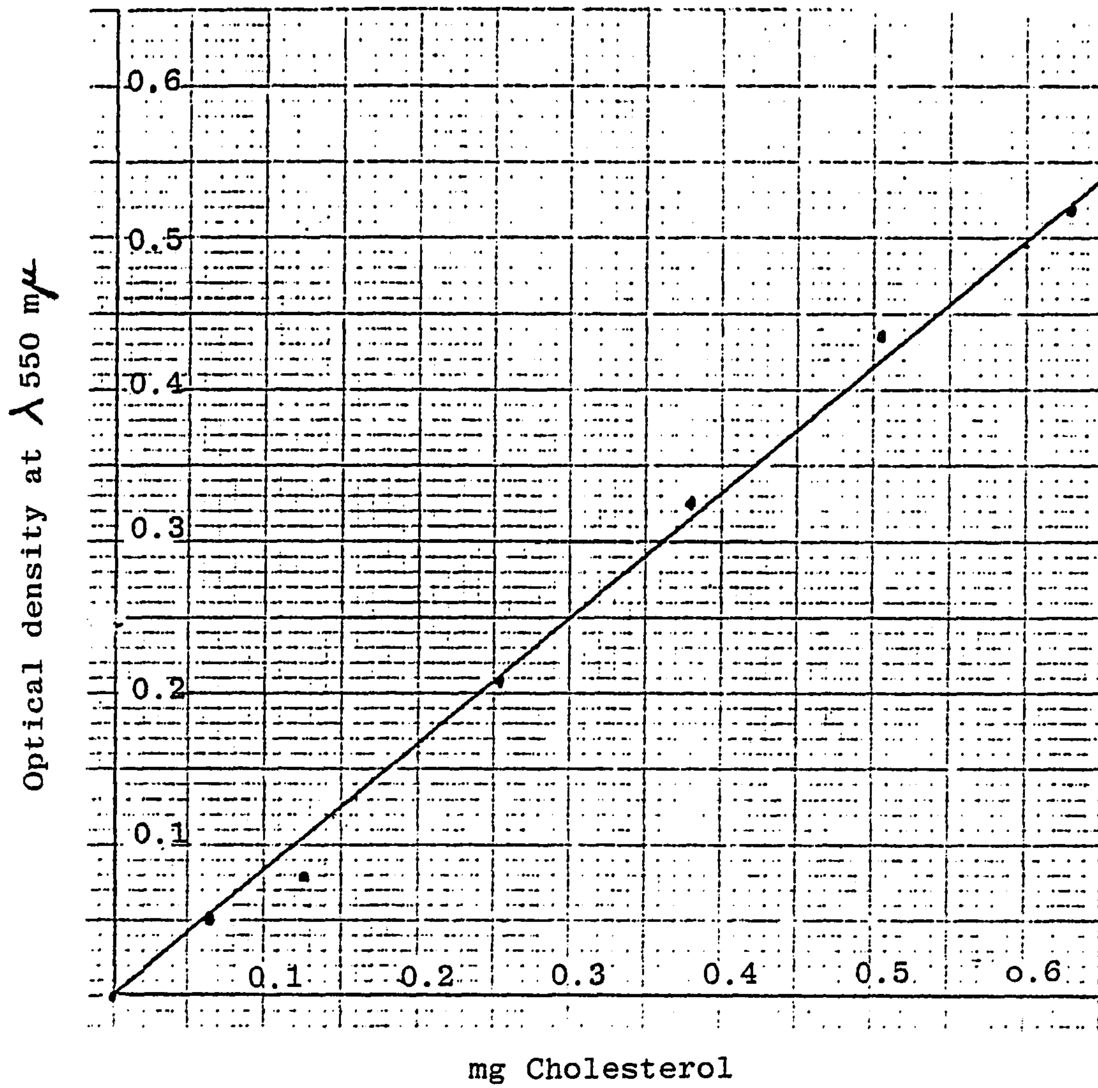

Figure 6. Standard calibration curve of cholesterol.

Standard solutions of KH_2PO_4 were used to make a standard curve for phosphorus, shown in Figure 7. The results, shown in Table 5, indicate that the total phosphorus content of the mother shark liver, 0.77%, is not much different from that of the embryo liver, 0.70%.

Neutral Lipids and Phospholipids

Total neutral lipids and total phospholipids (including non-neutral lipids) were determined quantitatively by thin layer chromatography, using specific eluting solvents, combined with the gravimetric method as described by T. M. Lowenstein (29). The results, shown in Table 5, indicate that the amount of both phospholipids and neutral lipids of embryo liver and mother shark liver are insignificantly different, *i.e.*, about 77% for total neutral lipids and 11 to 14% for phospholipids.

Fatty Acid Composition

Preparation of fatty acid methyl esters from the total lipids was carried out by the method of W. R. Morrison and L. M. Smith using boron trifluoride as the catalyst (25). The fatty acid methyl esters were identified by comparing their gas chromatographic retention times with those of reference standard methyl esters (Table 6) of C_8 to C_{24} fatty acids, including $C_{16:1}$, $C_{18:1}$, $C_{20:1}$, $C_{22:1}$, $C_{24:1}$, $C_{18:2}$ and $C_{18:3}$. The composition of fatty acids was cal-

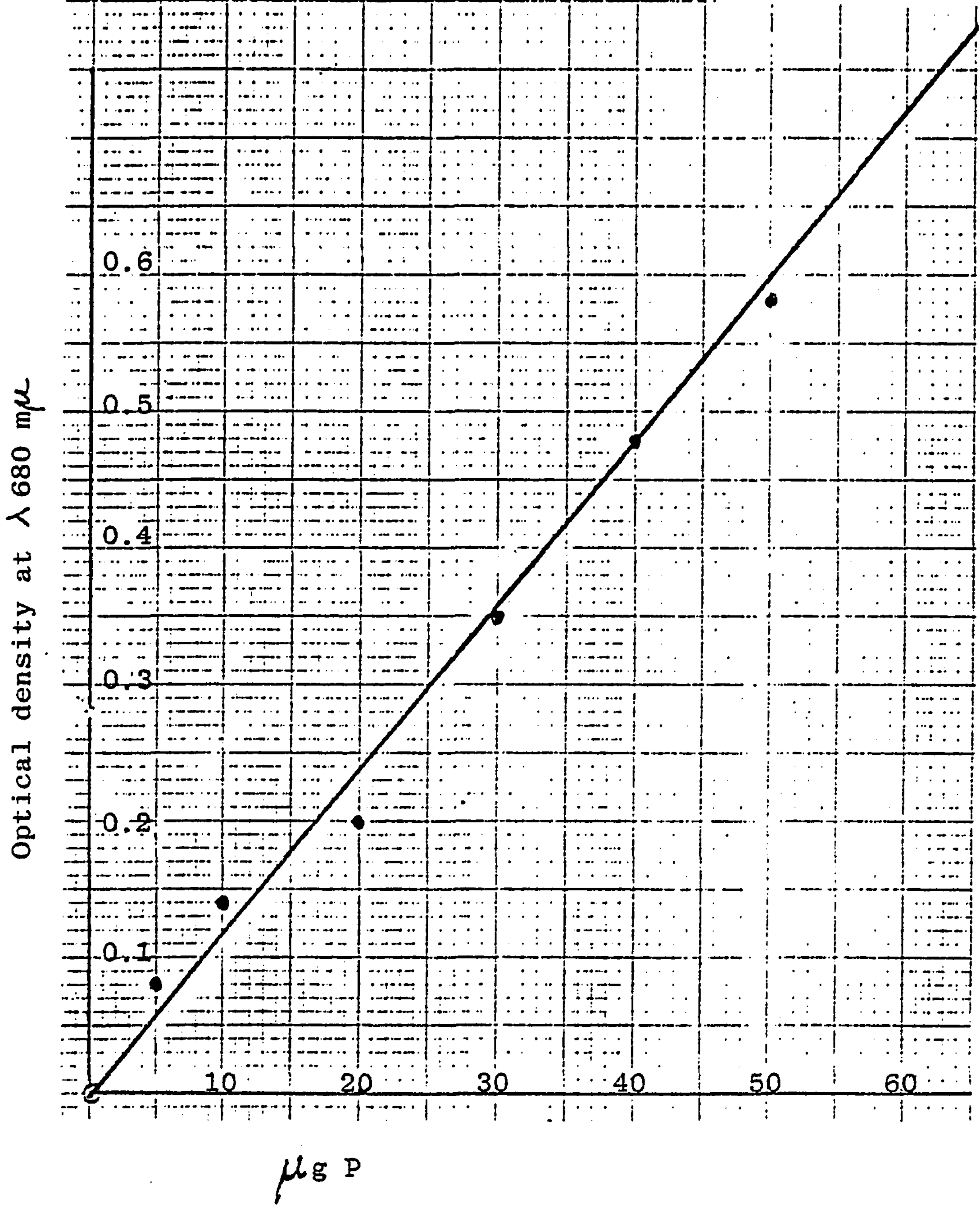

Figure 7. Standard curve of phosphate.

Table 5

Shark liver oil: Phosphorus, Phospholipids and neutral lipid content

Shark liver oil	Total P (%)		Phospholipid (%)		Neutral lipid (%)	
	in the oil	in the liver*	in the oil	in the liver*	in the oil	in the liver*
1. *T. obesus* (mother)	0.83	0.77	14.28	13.27	83.48	77.51
2. *T. obesus* (embryo)	0.78	0.7	11.83	10.61	86.26	77.38
3. *T. obesus* (adult male)	0.65	0.48	12.91	9.46	86.31	63.30
4. *C. amblyrhinchos*	0.83	0.77	10.88	10.08	86.40	80.01
5. *C. albimarginatus*	0.78	0.68	11.27	9.87	87.30	76.47
6. *C. galapagensis*	0.80	0.58	13.56	9.87	84.29	61.36
7. *G. cuvieri*	0.80	0.47	14.61	8.49	84.62	49.16

*The values were calculated from the values of total lipids in Table 1.

Table 6

Reference standard fatty acid methyl esters as determined by gas chromatography. Procedure is described in the experimental section

Fatty acid methyl ester		Retention time, RT (minutes)
$C_{8:0}$	Caprylate	3.30
$C_{9:0}$	Nonanoate	3.99
$C_{10:0}$	Caprate	4.91
$C_{11:0}$	Undecanoate	6.02
$C_{12:0}$	Laurate	7.27
$C_{13:0}$	Tridecanoate	8.50
$C_{14:0}$	Myristate	9.85
$C_{15:0}$	Pentadecanoate	11.17
$C_{16:0}$	Palmitate	12.50
$C_{16:1}$	Palmitoleate	12.21
$C_{17:0}$	Heptadecanoate	13.68
$C_{18:0}$	Stearate	14.90
$C_{18:1}$	Oleate	14.59
$C_{18:2}$	Linoleate	14.58
$C_{19:0}$	Nonadecanoate	16.46
$C_{20:0}$	Arachidate	17.32
$C_{20:1}$	Eicosenoate	17.29
$C_{21:0}$	Heneicosanoate	18.96
$C_{22:0}$	Behenate	20.46
$C_{22:1}$	Erucate	19.98
$C_{24:1}$	Nervonate	24.33

culated from the areas of the peaks in the gas chromatogram. The results, shown in Table 7, indicate that palmitic acid makes up about 50% of the fatty acids of the lipids derived from either embryo or mother shark livers, i.e., 51% and 48% for embryo and mother shark livers, respectively. The mother shark liver oil contains more oleic acid, but less C_{19} and C_{21} fatty acids, than does the embryo. I found that T. obesus contains palmitic and oleic acids as major constituents of the liver oil as was shown in the previous study of the shark liver oils (12,13,14). Two fatty acids, $C_{19:0}$ and $C_{21:0}$, found in my shark liver oils were also found in the shark liver oils studied by Shimma et al. (12) and Kayama et al. (14) as minor constituents. However, $C_{20:5}$ and $C_{22:6}$ fatty acids were not detected in the shark liver oils under my investigation.

Individual Lipid Classes

Column chromatography on Florisil was used to separate lipid classes representative of hydrocarbons, cholesterol esters, triglycerides, free sterols, diglycerides, monoglycerides, and phospholipids. The method used was that of K. K. Carroll (26). The system and order of eluting solvents is described in the experimental section. The identifications of lipid classes were done by comparing their thin layer chromatography characteristics with those of the reference standard compounds and by using specific

Table 7

Gas chromatography of fatty acid methyl esters from shark liver oils

Shark liver oil	Percent amount of the oil			
	Palmitate $C_{16:0}$	Oleate $C_{18:1}$	Nonadecanoate $C_{19:0}$	Heneicosanoate $C_{21:0}$
1. _Triaenodon obesus_, mother	48.3	35.5	6.0	10
2. _T. obesus_, embryo	51.5	27.2	8.6	12.7
3. _T. obesus_, male	34.5	65.5	-	-
4. _Carcharhinus amblyrhinchos_	63.0	36.0	-	-
5. _C. albimarginatus_	48.8	33.8	5.5	11.9
6. _C. galapagensis_	29.9	70.1	-	-
7. _Galeocerdo cuvieri_	38.5	36.4	4.9	20.2

spray reagents (27). The results are shown in Figures 8, 9 and 10, and Tables 8 and 9. Figure 8 shows the tlc (0.25 mm Silica Gel H and chloroform as eluting solvent) of the mother and the embryo shark liver oils. Seven lipid spots were detected in both the mother and the embryo liver oils. Identification of the spots (by comparing their R_f characteristics with those of the reference standard lipids and by using specific spray reagents as described in the experimental section) indicated the presence of squalene (R_f 0.93), cholesterol ester (R_f 0.89), triglycerides (R_f 0.65), sterols (R_f 0.17), diglycerides (R_f 0.15), monoglycerides (R_f 0.09), and phospholipids (R_f 0.0). Fractions 1 to 14 (40.3%) collected from the mother shark liver oil and fractions 1 to 15 (42.1%) from the embryo, were found to contain squalene, cholesterol esters and triglycerides. Fractions 15 to 27 (32.1%) from mother shark liver and fractions 16 to 30 (28.5%) from the embryo liver, were identified as triglycerides. Fractions 37 to 49 (5.4%) from the mother liver and fractions 37 to 58 (11.4%) from the embryo, contained sterols and diglycerides. Fractions 63 to 76 (7.8%) from the mother liver and fractions 72 to 95 (7.8%) from the embryo, contained diglycerides and monoglycerides. Fractions 96 to 107 (14.1%) from the mother liver and fractions 107 to 124 (10.1%) from the embryo, were identified as phospholipids. By simple calculation and using a previously determined value of cholesterol

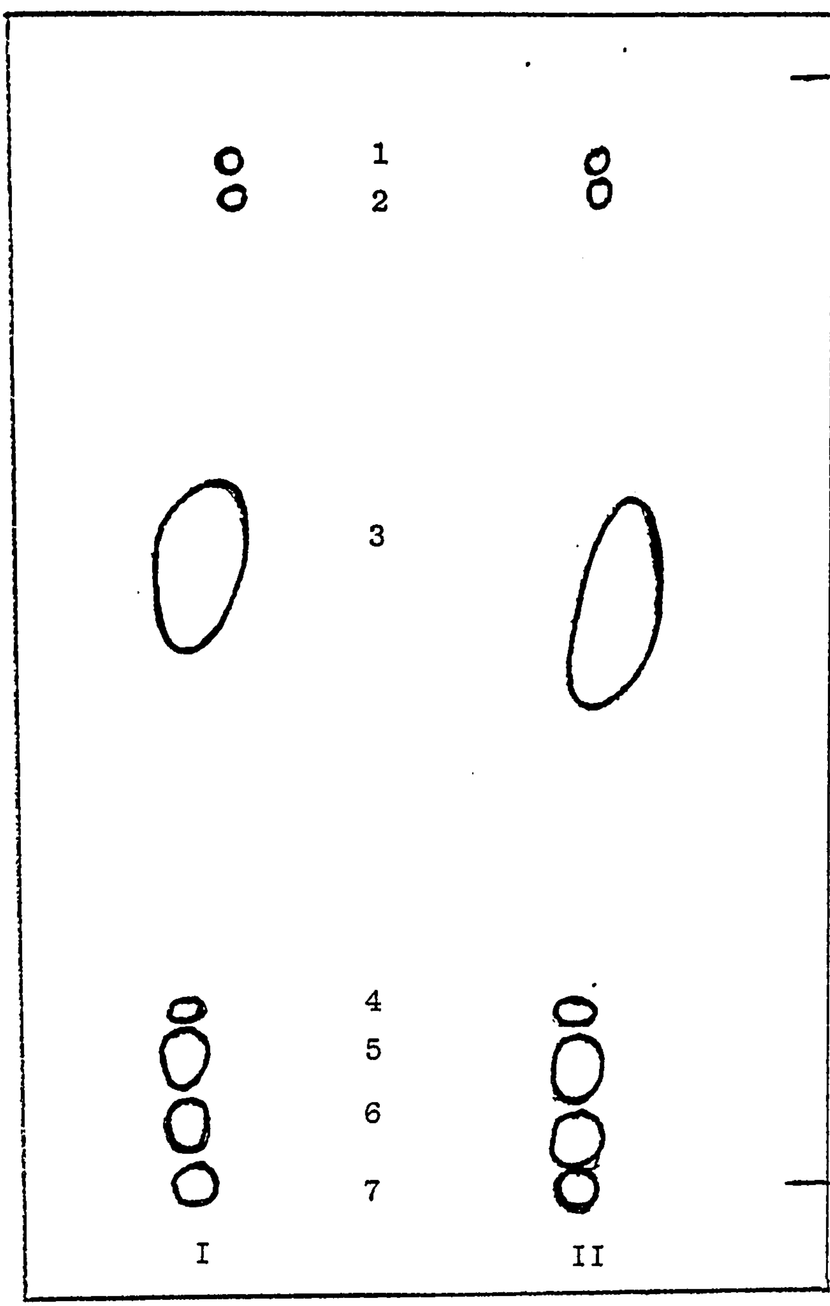

Figure 8. Thin layer chromatography (0.25 mm thick, silica gel H, chloroform as eluting solvent) of the mother (I) and the embryo (II) shark liver oils. Identification of the spots was done by comparing their R_fs with those of the reference standard lipids and by using specific spray reagents (described in the experimental section): 1, squalene, R_f0.93; 2, cholesterol ester, R_f0.89; 3, triglycerides, R_f0.65; 4, sterols, R_f0.17; 5, diglycerides, R_f0.15; 6, monoglycerides, R_f0.09; and 7, phospholipids, R_f0.0.

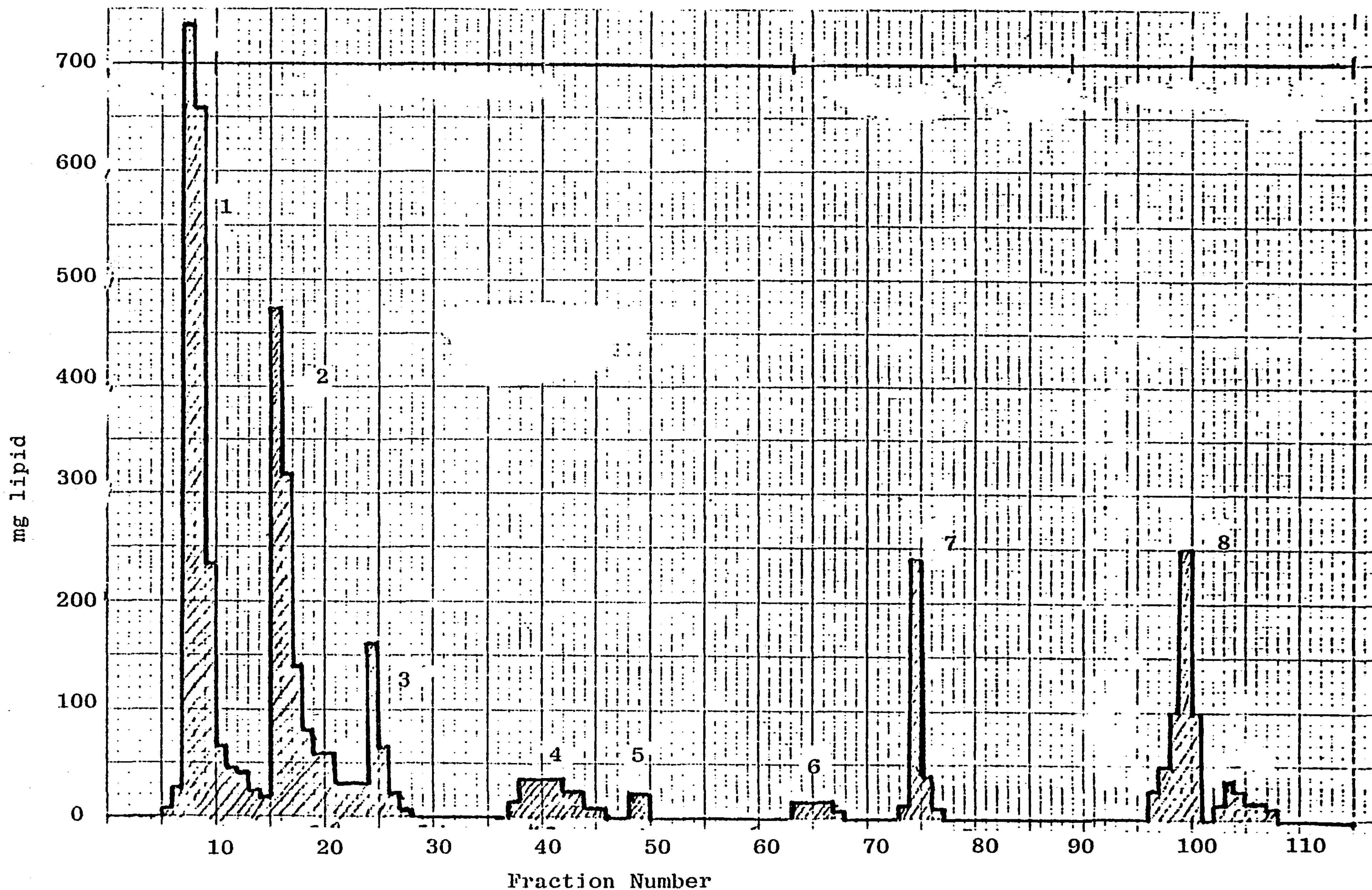

Figure 9. Chromatographic separation of the individual lipid classes of the mother whitetip reef shark liver oil.

Table 8

Fractionation of liver oil (4659 mg) from the mother whitetip reef shark into lipid classes by column chromatography on Florisil

Fraction #	Squalene + Cholesterol ester (mg)	Triglycerides (mg)	Sterols (mg)	Diglycerides (mg)	Monoglycerides (mg)	Phospholipids (mg)	Total	%
1-14	297	1562	-	-	-	-	1859	40.3
15-23	-	1232	-	-	-	-	1482	32.1
24-27	-	250	-	-	-	-		
37-45	-	-	19*	202	-	-		
48-49	-	-	-	46	-	-	248	5.4
63-66	-	-	-	----- 59	----	-	360	7.8
73-76	-	-	-	---- 301	----	-		
96-100	-	-	-	-	-	525	651	14.1
102-107	-	-	-	-	-	126		
	297	3044	19	608		651	4619	100
% in lipid	6.37%	65.3%	0.42%	13.1%		14%		
% in the liver (92.9% lipid)	5.92%	60.7%	0.39%	12.1%		13%		

*The value is taken from Table 4.

Table 9

Fractionation of liver oil (5442 mg) from the embryo whitetip reef shark into lipid classes by column chromatography on Florisil

Fraction #	Squalene + Cholesterol ester (mg)	Triglycerides (mg)	Sterols (mg)	Diglycerides (mg)	Monoglycerides (mg)	Phospholipids (mg)	Total	%
1-15	89	2154	-	-	-	-	2243	42.1
16-23	-	1200	-	-	-	-	1521	28.5
24-30	-	321	-	-	-	-		
37-54	-	-	37*	504	-	-	605	11.4
55-58	-	-	-	64	-	-		
72-82	-	-	-	-----84------		-	418	7.8
91-95	-	-	-	----334------		-		
107-111	-	-	-	-	-	300	540	10.1
115-124	-	-	-	-	-	240		
	89	3675	37	986		540	5327	100
% in lipid	1.64%	67.5%	0.69%	18.1%		9.9%		
% in the liver	1.47%	60.6%	0.62%	16.2%		8.9%		

(Table 4) the lipid composition of the mother and the embryo shark liver oils could be obtained. The results are shown in Figures 9 and 10, and Tables 8 and 9. Triglycerides were found to be the major components, 65.3% in the mother and 67.5% in the embryo shark livers. The total mono- plus di-glycerides are 13.1% for the mother liver and 18.1% for the embryo shark liver. Total phospholipids obtained by this method are 14% for the mother shark liver and 9.9% for the embryo liver oils. For comparison, 14.3% phospholipids were obtained from the mother and 11.8% from the embryo shark liver oils, respectively, as determined by thin layer chromatography (Table 5).

Individual Phospholipids

Quantitative analysis of phospholipids was carried out by thin layer chromatography (30) combined with colorimetric phosphorus assay (24) using a standard calibration curve (Figure 7). Individual phospholipids were identified, on a thin layer chromatoplate, using specific color spray reagents (27) described in the experimental section. The results, shown in Figure 11, indicate that six phospholipid spots appeared on the tlc of both the mother and the embryo shark liver oils. The results are shown in Tables 10 and 11. Both mother and embryo shark liver oils contain about the same amounts of cardiolipin, phosphatidylethanolamine and sphingolipids. The mother liver oil

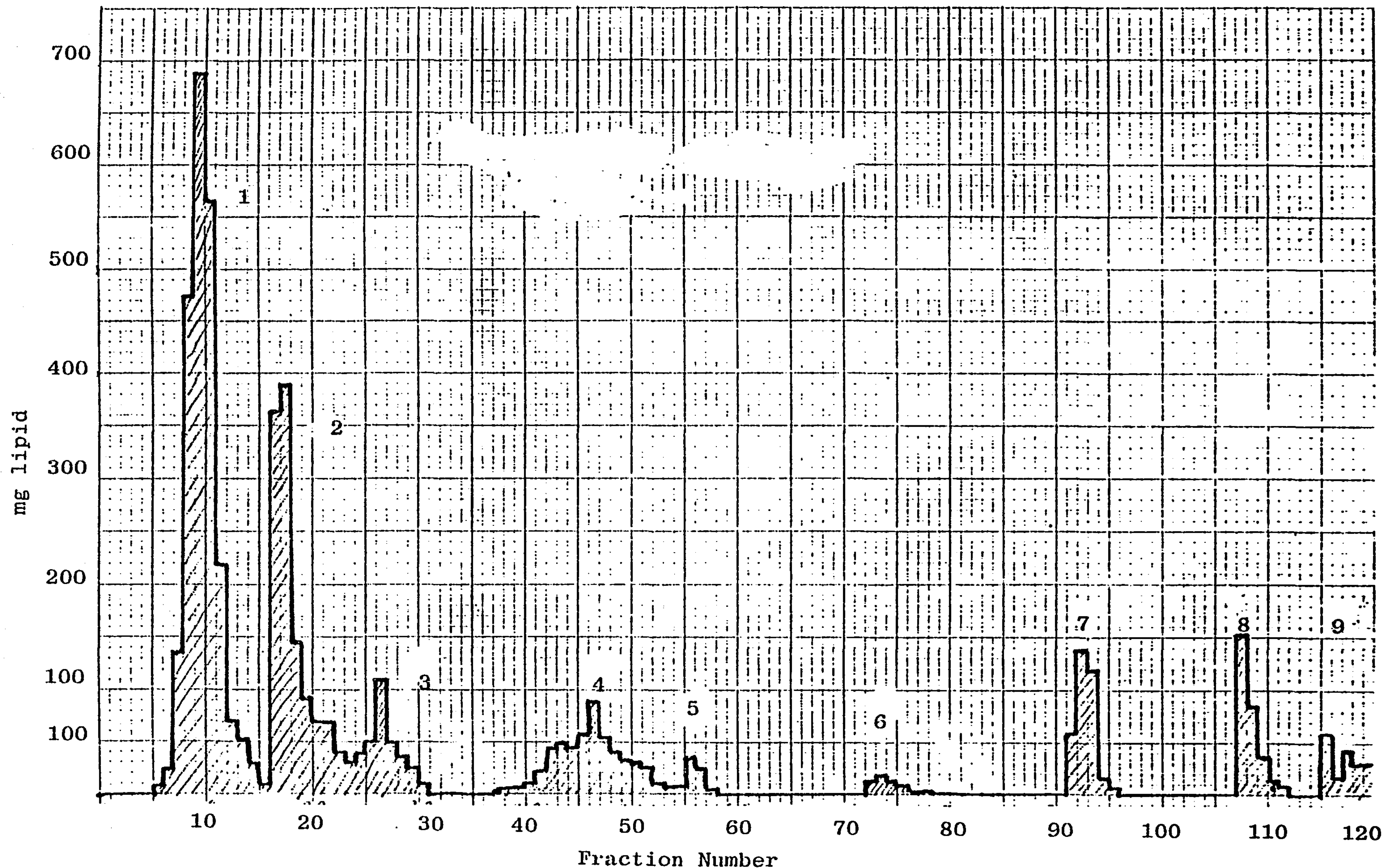

Figure 10. Chromatographic separation of the individual lipid classes of the embryo whitetip reef shark liver oil.

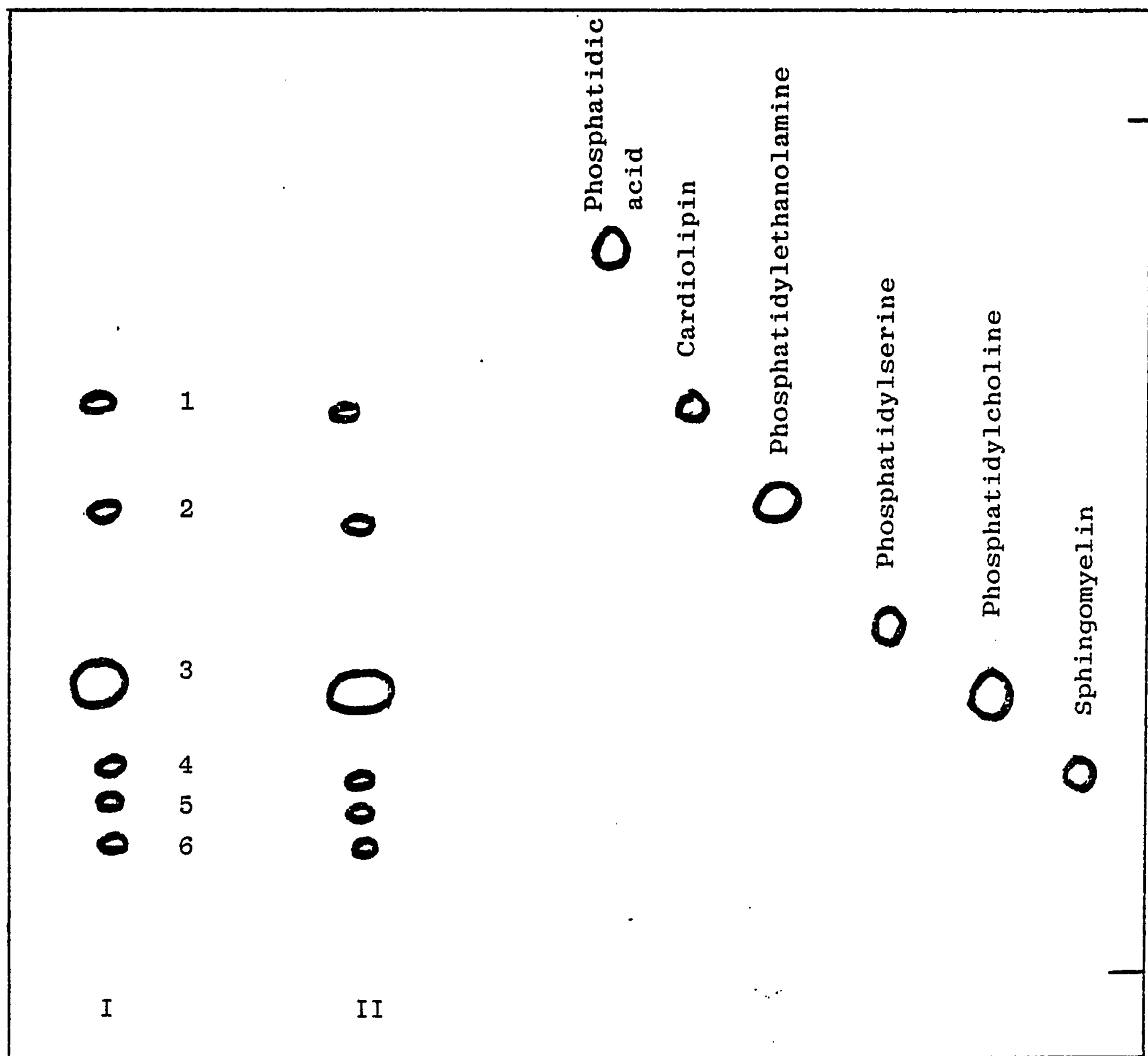

Figure 11. Thin layer chromatography (0.5 mm thick, silica gel H, chloroform-methanol-water (65:25:4) as eluting solvent) of the mother (I) and the embryo (II) whitetip reef shark liver oils. Identification of the spots was done by comparing their R_fs with those of the reference standard phospholipids and by using specific spray reagents (described in the experimental section): 1, cardiolipin, R_f 0.63; 2, phosphatidylethanolamine, R_f 0.52; 3, phosphatidylcholine, R_f 0.33; 4, sphingolipids, R_f 0.22; 5 and 6, unidentified phospholipids, R_f 0.18 and 0.15, respectively.

Table 10

Composition of phospholipids of the mother whitetip reef shark (*T. obesus*) liver. Individual phospholipids were separated by tlc and calculated in terms of P content determined spectrophotometrically as described in the experimental section

Phospholipid*	R_f**	µg P***	% P	
			in phospholipids****	in the oil***
Cardiolipin	0.63	46	1.15	0.16
Phosphatidyl-ethanolamine	0.52	10	0.25	0.04
Phosphatidyl-choline	0.33	88	2.20	0.31
Sphingolipids	0.22	42	1.05	0.15
Unidentified	0.18	50	1.25	0.18
phospholipids	0.15	60	1.50	0.21

*Individual phospholipids were identified as described in the experimental section.

**tlc: 0.5 mm thick of Silica Gel H, and chloroform-methanol-H_2O (65:25:4, v/v) as eluting solvent.

***µg of P in 28 mg shark liver oil, determined by spectrophotometry.

****Calculations based on the values in Table 5.

Table 11

Composition of phospholipids of the embryo whitetip reef shark (*T. obesus*) liver. Individual phospholipids were separated by tlc and calculated in terms of P content determined spectrophotometrically as described in the experimental section

Phospholipid*	R_f**	µg P***	% P	
			in phospholipids****	in the oil***
Cardiolipin	0.63	44	1.24	0.15
Phosphatidyl-ethanol amine	0.52	18	0.25	0.06
Phosphatidyl-choline	0.33	76	2.14	0.25
Sphingolipids	0.22	37	1.04	0.12
Unidentified phospholipids	0.18 0.15	51 16	1.44 0.45	0.17 0.05

*Individual phospholipids were identified as described in the experimental section.

**tlc: 0.5 mm thick of Silica Gel H, and chloroform-methanol-H_2O (65:25:4, v/v) as eluting solvent.

***µg P in 30 mg embryo shark liver oil, determined by spectrophotometry.

****Calculations based on the values in Table 5.

contained less phosphatidylcholine than that of the embryo liver oil. Phosphatidic acid and phosphatidylserine were not found in either of the liver oils.

B. Comparative Study of Liver Lipids Isolated From Five Different Species of Sharks

Six liver samples were obtained from five different species of shark: two livers from whitetip reef shark (*Triaenodon obesus*), one male and one female, one each from grey reef shark (*Carcharhinus amblyrhinchos*), silvertip shark (*C. albimarginatus*), galapagos shark (*C. galapagensis*) and tiger shark (*Galeocerdo cuvieri*). Quantitative assays of total lipids, squalene, cholesterol, iodine value, phosphorus, phospholipid and neutral lipid were carried out by the methods described in the experimental section.

Total Lipid

Table 1 shows that the lipid content of the livers of different species of sharks are significantly different. The lowest, 58.1%, was obtained from the tiger shark and the highest, 92.9%, from the female whitetip reef shark and 92.6% from the grey reef shark. The male whitetip reef shark liver contains about the same amount of lipid, 73.3%, as does the galapagos shark, 72.8%, while that of the silvertip shark contains 87.6%

lipid. The densities varied from 0.810 g/ml for male whitetip reef shark liver oil to 0.895 g/ml for the silvertip shark liver oil. Table 1 shows that in general, the lower densities are obtained from the lower percentage of the shark liver oil. This can be understood if one considers the function of liver oil in the buoyancy mechanism, i.e., the lower density is required for the lower content of shark liver oil to give neutral buoyancy. The higher content of the liver oil balances the higher oil density. A similar situation was reported in deep-sea shark liver oils studied by Corner et al. (7) where the high content of liver oils (between 80 and 86%) showed the relatively higher oil densities (between 0.88 and 9.91).

Squalene

Table 3 shows that, except for the tiger shark, the squalene contents for all species of adult shark are less than 0.5% of the liver oil. The tiger shark has the highest content of squalene, 1.01% of the liver oil or 0.59% of the liver. The silvertip shark contains the least squalene, about 0.28% of the oil or 0.25% of the liver. Obviously all sharks under my investigation belong to the group of sharks that contain insignificant amounts of squalene, similar to the group of sharks studied by Heller et al. (6). Thus, they are not like the kind of

sharks that contain large amounts of squalene, such as *Dalatias licha* (contained 70% squalene in the oil) (6), *Centrophorus uyato* (contained 90% squalene in the oil) (6) or the deep-sea shark liver studied by Corner *et al.* (7) (contained 42% squalene in the oil). Therefore, it is unlikely that the main function of squalene, in the shark liver under my investigation, is to give buoyancy. It is clear, however, that their liver oils (balanced by their densities) are involved in the buoyancy mechanism.

Cholesterol

Table 4 shows that the total liver cholesterol in five different species of shark varies from 3.07% (g cholesterol/100 g liver), for tiger shark (*G. cuvieri*), to 6.52%, for female whitetip reef shark (*T. obesus*). Silvertip shark (*C. albimarginatus*), male whitetip reef shark (*T. obesus*) and grey reef shark (*C. amblyrhinchos*) contain 3.80%, 5.59% and 5.97% total cholesterol, respectively. The highest contents of free cholesterol are obtained from the galapagos shark (*C. galapagensis* (g free cholesterol/ 100 g liver), 0.51%, and the tiger shark (*G. cuvieri*), 0.50%. The free cholesterol contents of grey reef shark, male whitetip reef shark and silvertip shark are about equal, *i.e.*, 0.32%, 0.33% and 0.39%, respectively.

If we compare free cholesterol content with that

of total cholesterol, the highest value, 16.29%, is obtained for the tiger shark liver and the lowest, 5.36%, for the grey reef shark liver. Male and female whitetip reef shark livers show about the same values, i.e., 5.90% and 5.98%, respectively. Galapagos shark liver contains 7.89% and silvertip shark 10.26%. For comparison, the total cholesterol content of the livers of deep-sea sharks studied by Shimma _et al_. (13) and basking shark studied by Gershbein _et al_. (9) ranged between 3.3 and 5.9% (or between 33 mg and 50 mg/g) of the total oil. The total cholesterol of the shark liver oils under my investigation (ranged between 4.34% and 8.87%) are higher than those of Shimma _et al_. and Gersheim _et al_. Their sharks lived in the relatively cooler water temperature than do sharks of Enewetak (shark liver oils under my investigation). This would correlate with the observation of Musacchia and Clark (31) who found that the increase of environmental temperature increased the liver cholesterol of the arctic sculpin, _Myxocephalus quadricornis_.

Iodine Value

The galapagos shark (_C. galapagensis_) liver oil shows the highest iodine value, 109 (shown in Table 1), and the tiger shark (_G. cuvieri_) shows the lowest value, 50. The oil from the two whitetip reef sharks (_T. obesus_),

female and male, show about the same iodine values, i.e., 74 and 75, respectively. The lower iodine values are 65, for the grey reef shark (C. amblyrhinchos) and 53, for the silvertip shark (C. albimarginatus).

These values are relatively lower compared to those of shark oils from Ceborrhinus maximus, iodine values 119-139 (35) or codliver oil, Gadus morhua, iodine value 165 (36).

Phosphorus, Phospholipids and Neutral Lipids

As shown in Table 5, all five adult shark liver oils, except for the male shark liver (T. obesus), contain about the same amount of P, 0.8%. In terms of their P content in the liver, however, the value increases from 0.47% for G. cuvieri, to 0.48% for the male whitetip reef shark, 0.58% for the galapagos shark, 0.68% for the silvertip shark, to 0.77% for the grey reef shark and the female whitetip reef shark.

The values for phospholipid are 11% to 14% of the total oils or 9% to 13% of (g phospholipid/100 g liver) the livers. The tiger shark liver oil (G. cuvieri) has the highest phospholipid content, 14.6%, and the grey reef shark has the lowest, 10.9%. The amount of neutral lipids in total lipids for all six adult shark livers is also about the same, i.e., close to 85%. However, in terms of their percent amount in animal liver, the values

are obviously different. As low as 49.1% was obtained from the tiger shark liver, and as high as 80% from the grey reef shark liver. The amount of 61.3% was obtained from the galapagos shark liver, 63.3% from the male whitetip reef shark, 76.4% for the silvertip shark and 77.5% for the female whitetip reef shark liver. These values are somewhat lower than that of the basking shark (Cetorrhinus maximus) liver oil studied by Lombardi et al. (34), i.e., about 90% neutral lipid in the oil.

Fatty Acid Composition

As shown in Table 7, palmitic and oleic acids are the major fatty acids occurring in all six adult shark liver lipids, and together make up about 75% to 100% of the total fatty acids. C_{19} and C_{21} fatty acids are not found in either male whitetip reef shark, grey reef shark or galapagos shark. More palmitate than oleate was found in female whitetip reef shark (48.3% palmitate, 35.5% oleate), grey reef shark (63.0% palmitate, 36.0% oleate), silvertip shark (48.8% palmitate, 33.8% oleate), and tiger shark (38.5% palmitate, 36.4% oleate). However, less palmitate occurs in male whitetip reef shark (34.5% palmitate, 65.5% oleate), and galapagos (29.9% palmitate, 70.1% oleate). About two to four times more C_{21} than C_{19} fatty acids were found in all livers containing C_{19} and C_{21} fatty acids. Tiger shark liver, which has about the

same amount of palmitic and oleic acids, contains four times as much C_{21} as C_{19}. The silvertip shark liver has twice as much C_{21} fatty acid as C_{19}.

It is not surprising that the major fatty acid constituents are palmitic and oleic acids since in the previous studies of shark liver oil (10,11,12,13,14) palmitic and oleic acids were always found to constitute the largest percentage of the liver oils. The presence of significant amounts of $C_{19:0}$ and $C_{21:0}$ in the female _T. obesus_, _C. albimarginatus_ and _G. cuiveri_ is rather surprising because most of the shark liver oils studied previously (10,12,13,14) contained little or no $C_{19:0}$ and $C_{21:0}$ fatty acids. The highly unsaturated fatty acids, $C_{20:5}$ and $C_{22:6}$, found in the liver oil from sharks or Suruga Bay (12), did not occur in the liver oil of sharks under my investigation. In general, the significant difference is the smaller number of fatty acid constituents in the shark liver oils under my investigation than in the liver oils studied by Shimma _et al_. (12) or Kayama _et al_. (14), the higher percentage of palmitic and oleic acids in the liver oils under my investigation, and the presence of $C_{19:0}$ and $C_{21:0}$ fatty acids in the liver oils under my investigation as a substitute for the $C_{20:5}$ and $C_{22:6}$ fatty acids occurring in the shark liver oils from Suruga Bay (12) and in the so called "kuroko-zame" shark liver

studied by Kayawa, et al. (14). Thus, the degree of unsaturation of fatty acids found in the liver oil of sharks from Enewetak (my shark liver oils), which live in warm water, is lower than those of fatty acids of sharks from Suruga Bay, which live in relatively cooler water temperature. A relationship between the degree of unsaturation of plant and animal lipids and the mean environmental temperature has long been acknowledged (32, 33). In general, it was found that lower environmental temperature was correlated with increased unsaturation of lipids. Caldwell and Vernberg (32) reported that lipids of goldfish gill mitochondria became increasingly unsaturated at low environmental temperatures. The changes were specifically identified with the mitochondrial membrane lipids which play a role in adapting the organism to seasonal fluctuations in environmental temperatures. This would also correlate with the observations of Farkas and Herodek (33), who have shown that the fatty acids of cold water plankton were more unsaturated than those of warm water plankton.

The data presented in this section (Results and Discussion) are based on analyses of single specimens of six species of sharks due to the difficulty in obtaining this type of sample. Therefore, it is not possible to make generalizations on the basis of the available data because of the limited number of samples.

EXPERIMENTAL

Shark Liver Samples

Shark liver samples from five different species of sharks were obtained by Dr. L. S. Ciereszko at the Pacific Marine Biological Laboratory, Enewetak, Marshall Islands. These samples had been collected by Dr. J. Randall in April-May, 1978, as shown in Table 12. Three liver samples were from whitetip reef shark (*Triaenodon obesus*), one each from a mother, one of its embryos and an adult male shark, one sample from the grey reef shark (*Carcharhinus amblyrhinchos*), one from the silvertip shark (*Carcharhinus albimarginatus*), one from the galapagos shark (*Carcharhinus galapagensis*) and one from the tiger shark (*Galeocerdo cuvieri*).

Extraction of Lipids

The shark liver samples were obtained as material wrapped in plastic bags which were freeze dried directly after their collection from the sharks. The samples were cut into small pieces and extracted by chloroform-methanol (1:1, v/v) (300 ml solvent for every 100 g of sample) in a Soxhlet extractor for 4 hr (preliminary experiments showed that 4 hr extraction was long enough to completely extract lipid from the sample). The extracts were evaporated to dryness on a Büchi Rotavap evaporator at

Table 12

Source of shark liver samples: date and place of collection

Shark liver	Place and date of collection
1. Whitetip reef shark (*Triaenodon obesus*), mother of #2, 118.2 cm pcl, 147.7 cm tl 47.5 lbs	Enewetak, 5/6/78
2. Whitetip reef shark (*T. obesus*), embryo, 36 cm pcl	Enewetak, 5/6/78
3. Whitetip reef shark (*T. obesus*), male, 101.7 cm pcl, 128 cm tl, 26 lbs	Enewetak, 4/29/78
4. Grey reef shark (*Carcharhinus amblyrhinchos*), female	Enewetak, 5/5/78
5. Silvertip shark (*Carcharhinus albimarginatus*), female, 165 cm pcl, 50,4 cm tl, 162 lbs	Enewetak, 5/5/78
6. Galapagos shark (*Carcharhinus galapagensis*), female, 183.1 cm pcl, 59.5 cm tl	Enewetak, 5/3/78
7. Tiger shark (*Galeocerdo cuvieri*), female 241 cm pcl, 305.5 cm tl, 357 lbs	Enewetak, 5/1/78

Note: pcl, precaudal length; tl, total length

45°C using a water aspirator and circulating ice water. Benzene was used if necessary to remove water from the extract. A four hr additional extraction with the same solvent was done and the lipid obtained (if any) from this was combined with the first one. The dried lipids were weighed and stored under nitrogen atmosphere in the refrigerator.

The oil density was determined by weighing 1 ml of the oil using a Metler H10 balance. Density is expressed in g/ml.

Isolation and Quantitative Assay of Squalene

One gram of lipid was chromatographed on Florisil (30 g, 100/200 Mesh, Floridin Co., in a 2.5 cm diameter column) with hexane as the eluting solvent. One hundred ml eluate was collected (in the preliminary experiment, it was found that squalene was eluted completely from the column by 100 ml hexane and was identified as one spot when tested by tlc: R_f 0.62, 0.25 mm Silica Gel H, hexane as eluting solvent). Squalene was determined spectrophotometrically by the method of Rothblat *et al*. (20). One ml of the eluate was transferred into a 15 ml screw-capped test tube and was evaporated to dryness under a nitrogen atmosphere. One ml of the concentrated H_2SO_4 was added and the mixture was heated at 70°C for 5 min. A half milliliter of formaldehyde was added, the tube was

capped, shaken and heated on the boiling water bath for 10 min. The mixture was diluted with glacial acetic acid to 10 ml volume, and shaken. The optical density of the solution was read, against the reagent blank, at 400 mμ using a Spectronic 20 spectrophotometer. The amount of squalene was obtained from a standard calibration curve made by using 0.1 to 0.6 mg reference standard squalene (Eastman Organic Co.), shown in Figure 1. In another experiment, the same 100 ml eluate was evaporated to dryness. The dried residue was weighed using a Mettler H10 balance (Max 160 g, d = 0.1 mg), as purified squalene. Squalene was identified by tlc (20 x 20 cm plate, 0.25 mm thick of Silica Gel H, and hexane as eluting solvent) prepared by the method of Stahl (21).

Squalene was identified by tlc (0.25 mm thick, Silica Gel H, hexane as eluting solvent, and by comparing its R_f value with that of the squalene standard), ir and nmr spectrophotometries. The ir spectrum was determined on a Perkin-Elmer 298 IR Spectrophotometer (0.5 abscissa expansion, 4 min scan time, and sodium chloride disks as sample holder). The nmr Spectrum was determined on a T-60 Nuclear Magnetic Resonance Spectrometer (50 rps spinning rate, 250 sec sweep time, 500 Hz sweep width, 0.025 RF power level, and chloroform-d_1 containing 1% TMS as solvent).

To establish the accuracy and applicability of

the squalene assay procedure, the recovery of squalene added to the shark liver lipid was investigated. The varying amounts (0 to 81 mg) of squalene were added to 800 mg shark liver lipid. After column chromatography on Florisil, the squalene content was assayed spectrophotometrically and gravimetrically as above.

Cholesterol Assays

Quantitative determinations of total and free cholesterol were carried out by the spectrophotometric method of Courchaine (22).

Total Cholesterol - Twenty mg of the liver oil was transferred to a 15 ml conical centrifuge tube. Six ml glacial acetic acid and 4 ml ferric chloride solution were added to the tube, and shaken. The mixture was allowed to cool for 10 min and centrifuged to get a clear purple solution. The optical density of the solution was read at 550 mμ, against the reagent blank, using a Spectronic 20 spectrophotometer. The cholesterol content in the lipid was calculated by using the calibrated standard curve of cholesterol shown in Figure 6.

Free Cholesterol - Twenty mg of the liver oil in a 15 ml conical centrifuge tube was mixed with 1 ml of acetone-95% ethanol (1:1, v/v) and 1 ml of 1% digitonin solution (1 g of digitonin in 50 ml of 95% ethanol, diluted to

100 ml with water). The mixture was centrifuged at 3000 rpm for 5 min. The supernatant was discarded and the precipitate was allowed to drain for 5 min. Residue was suspended in 4 ml acetone, mixed and centrifuged as above. The supernatant was discarded and the digitonide precipitate was dried under nitrogen. The digitonide was dissolved in 6 ml glacial acetic acid, mixed with 4 ml ferric chloride reagent and centrifuged to get a clear solution. Optical density was read against the reagent blank (containing 1 ml of 1% digitonin solution). The cholesterol content was calculated by using the calibrated standard curve of cholesterol shown in Figure 6.

Iodine Value

Iodine value of the lipids was determined by the method of Yasuda (23). Forty to 50 mg lipids were dissolved in chloroform to make 50 ml volume. Five ml was transferred to a 50 ml glass-stoppered Erlenmeyer flask. Five ml 0.5 N pyridine dibromide solution (Dam's reagent) was added, mixed and the mixture was allowed to stand at room temperature in the dark for 15 min. A half milliliter of 10% KI solution, 0.5 ml water and a few drops of 1% starch indicator solution were added to the flask. The liberated iodine was titrated with standard 0.02 N sodium thiosulfate solution. Iodine value was calculated using the equation,

$$I = \frac{(a-b)}{c} \times \frac{1.27}{5}$$

where I = iodine value, a = blank titration, b = sample titration, c = weight of lipid in sample (in grams).

Determination of Total Phosphorus

Total phosphorus of the lipids was determined spectrophotometrically by the method of Allen (24). Forty mg of the lipids was dissolved in chloroform to 100 ml volume. One ml of the lipid solution was transferred to a 25 ml marked-test tube (or Kjehldahl digestion tube) and the solution was evaporated to dryness under a stream of air. Two ml of perchloric acid and a glass bead were added to the tube. The sample was digested by heating on a digestion rack until the digest was clear and colorless. The digest was allowed to cool at room temperature and diluted to the 12.5 ml mark with water and mixed. Two ml of Amidol reagent (0.5 g of 2,4-diaminophenol dichloride in 50 ml of 20% sodium bisulfite solution) and 1 ml of molybdate solution (4.2 g of ammonium molybdate in 50 ml of water) were added with shaking. The molybdenum blue color was allowed to develop for 20 min. The solution was diluted to the 25 ml mark with water and mixed. The optical density was read at λ680 mμ against a reagent blank using a Spectronic 20 spectrophotometer. The phosphorus content

of the lipids was obtained by using a standard curve (5 μg to 60 μg P) as shown in Figure 7.

Analysis of Fatty Acid Composition

Fatty acid methyl esters were prepared from the lipids by the method of Morrison and Smith (25). Boron fluoride methanol reagent (140 g of BF_3 per liter of methanol, Eastman Kodak Co.) was mixed, in a tube, with the dried shark liver oils, under nitrogen, in the proportion 1 ml reagent per 4-16 mg of lipid. The tube was screwcapped, heated in a boiling water bath for 10 min, cooled, and opened. The esters were extracted by adding 2 volumes of petroleum ether, shaken and centrifuged until both layers were clear. The petroleum ether layer, containing methyl esters, was saved for fatty acid determination by gas chromatography using a Hewlett-Packard 5985 GC/MS System. GC conditions: column, 1/8" 5% SE-30; temperature, 170°C; time, 2 min; flowrate, 28.8 ml/min; attenuation, 9; chart speed, 0.5. Fatty acid methyl esters derived from shark liver oils were identified by comparing their chromatographic retention times with those of the reference standard methyl esters (Supelco, Inc.), shown in Table 6: straight chain fatty acids of carbon number C_8 (caprylate) to C_{24} (nervonate), including monounsaturated fatty acid methyl esters of palmitoleate, oleate, eicosenoate, heneicosenoate, and nervonate, diunsaturated and triun-

saturated fatty acids of C_{18} (linoleate and linolenate). The percentage of fatty acids was obtained from the areas under the peaks on the gas chromatogram.

Separation, Identification and Quantitative Determination of Individual Lipid Classes

Individual lipid classes were separated from shark liver oil by column chromatography on Florisil (40 g, 100/200 Mesh, Floridin Co., column diameter 2.5 cm, flow rate 10 ml per min) using the method of Carroll (26). The oil was separated into classes of hydrocarbons, cholesterol esters, triglycerides, free sterols, diglycerides, monoglycerides and phospholipids by collecting 10 ml fractions of the eluate from the column by using eluting solvent as follows: 1, 100 ml hexane; 2, 100 ml 5% ether in hexane; 3, 150 ml 15% ether in hexane; 4, 150 ml 25% ether in hexane; 5, 150 ml 50% ether in hexane; 6, 150 ml 2% methanol in ether; 7, 150 ml 4% glacial acetic acid in ether; 8, 100 ml chloroform; and 9, 100 ml chloroform-methanol (1:1, v/v). Each fraction was tested by tlc (20 x 20 cm plate, 0.25 mm thick, Silica Gel G from J. T. Baker Co.) using the following solvent systems: hexane for hydrocarbons, cholesterol esters and triglycerides; hexane-ethyl ether-acetic acid (90:10:1, v/v) for triglycerides; chloroform for triglycerides, sterols, diglycerides, and monoglycerides; chloroform-methanol (9:1, v/v) for monoglycerides and

cardiolipins; and chloroform-methanol-water (65:25:4, v/v) for ceramides, cerebrosides and phospholipids. The reference compounds for identification of lipid classes and individual phospholipids on tlc were obtained from Supelco, Inc. (except squalene, from Eastman Organic Chem.). They were: squalene, cholesterol palmitate, triolein, 2-dipalmitin, 1-monopalmitin, stearoyl ceramide, monogalactosyl-stearoyl cerebroside, phosphatidic acid, cardiolipin, phosphatidylethanolamine, phosphatidylserine, phosphatidylcholine, sphingomyelin, and sulfatide. The tlc spots were observed using uv light, iodine vapor, and specific spray reagents (27) as follows: sterols and sterol esters by antimony(III) chloride-acetic acid reagent (20 g of antimony(III) chloride in a mixture of 20 ml acetic acid and 60 ml chloroform), glycolipids by diphenylamine reagent (a mixture of 20 ml of 10% ethanolic diphenylamine, 100 ml of 36% hydrochloric acid and 80 ml glacial acetic acid), aminolipids by ninhydrin reagent (0.2 g ninhydrin in 100 ml ethanol), phospholipids by Dittmer-Lester reagent modified by Ryu and MacCoss (28) (a mixture of solution I and solution II: solution I, 8 g of molybdic anhydride in 100 ml 70% sulfuric acid; solution II, 0.4 g of powdered molybdenum in 100 ml of solution I).

The distance of migration of lipid spots was measured and compared with the R_f values of standard lipids. For quantitative determination, all fractions showing the

same lipid spots were combined. The solvent was evaporated using a Büchi Rotavap evaporator and evaporation under nitrogen, and dried lipids were weighed. The percentage of individual lipid class was calculated by the equation,

$$IL = \frac{a}{b} \times 100$$

where a = weight of individual lipids (in mg), b = weight of shark liver oil (in mg) used in the chromatography, and IL = the percentage of individual lipids.

Determination of Neutral Lipids

Neutral lipids were separated from the polar lipids (mostly phospholipids) by thin layer chromatography according to the method of Lowenstein (29). Samples of 20-40 mg lipids were applied to a thin layer chromatographic plate (20 x 20 cm plate, Silica Gel H, 0.5 mm thick). Acetone-petroleum ether (1:3) was used as eluting solvent for neutral lipids. The neutral lipid spots were detected by comparing their R_f values with those of reference standard neutral lipids describing in the previous section. Repeated elution with the same solvent system was done to quantitate the elution procedure. The chromatographic plate was dried at room temperature for 30 min. The lipid spots were scraped from the plate and eluted with 20 ml chloroform. The solvent was evaporated by a Büchi Rotavap evaporator, as above, and the lipids were weighed as total neutral

lipids. The non-migrating spot was also scraped from the plate and was treated as above, except that the scraped material was eluted by 20 ml chloroform-methanol (1:1, v/v). The lipid from this spot was weighed as phospholipids.

Separation, Identification and Quantitative Determination of Individual Phospholipids

Individual phospholipids of shark liver oil were determined quantitatively by the combination of thin layer chromatography and spectrophotometric method of phosphorus described by Lowenstein (30). Twenty to 40 mg of oil was applied to a thin layer chromatographic plate (20 x 20 cm, Silica Gel H, 0.5 mm thick). The non-phospholipid components were washed away by elution with acetone-petroleum ether (1:3, v/v). Phospholipids were eluted by chloroform-methanol-water (65:25:4, v/v). Phospholipids were identified by comparing their R_f values with those of the reference standard compounds and by using specific spray reagents: Modified Dittmer-Lester reagent (28) for phospholipid in general; Dragendorff's reagent (a mixture of 20 ml water, 6 ml 6 N HCl, 2 ml stock solution, and 6 ml of 6 N NaOH. Stock solution: 8 g bismuth nitrate in 20 ml of 25% NHO_3 added to 20 g KI with 1 ml 6 N HCl and 5 ml water, and the volume of the solution made up to 100 ml with water) for phosphatidylcholine; p-Quinone reagent (0.5 g of

p-benzoquinone in a mixture of 10 ml pyridine and 40 ml n-butanol) for phosphatidylethanolamine; and modified Dittmer-Lester reagent (28) for cardiolipin, phosphatidic acid and spingolipids.

After the development of the thin layer plate, the plate was dried at room temperature for 30 min. The phospholipid spots were scraped from the plate, eluted with 10 ml chloroform-methanol (1:1, v/v) and the solutions were treated as previously described to get the individual phospholipids. Determinations of phosphorus content from the individual phospholipids were carried out by the method described previously.

SUMMARY

Comparative study of lipids of whitetip reef shark livers derived from an unborn young with that of its mother indicated that both samples contained about the same total oil, *i.e.*, about 90% of the dry weight. The squalene contents in both liver oils were less than 1%; the embryo liver oil contained about twice as much as that of the mother liver oil. The total cholesterol content of the mother liver oil (7.02% was about 5 times as much as that of the embryo liver oil (1.5%) but the free cholesterol content of the embryo (0.69%) was higher than that of the mother liver oil (0.42%).

Relatively small differences were found between the two liver oil samples in terms of their total phosphorus content (0.8%), total phospholipids (10-14%), total neutral lipids (77%), and their phospholipids (cardiolipin, phosphatidylethanolamine, phosphatidylcholine, and sphingolipids). Fractionation of the shark liver oils indicated that in both liver oils, about 65% of the neutral lipids were triglycerides. Unsaturation of the oils, shown by their iodine values and fatty acid compositions indicated that the mother liver oil had a higher percentage of the same unsaturated fatty acids than that of the embryo. Palmitic acid and oleic acid were the major fatty acids in both liver oils. Together they made up about 80-85%. Palmitic acid alone made up about 50% in both liver oils.

Comparative study of liver oil of single specimens of five different species of sharks (female whitetip reef shark, male whitetip reef reef shark, grey reef shark, silvertip shark, galapagos shark and tiger shark) indicated that the total oil contents varied from 58% (g oil/100 g liver), in tiger shark, to 93%, in female whitetip reef shark and in grey reef shark. The squalene content of the liver oils was between 0.29% to 0.39% except that of the tiger shark was as high as 0.95%. Total cholesterol content of the oils varied from 5.28%, in tiger shark, to 7.02%, in whitetip reef shark, and free cholesterol varied from 0.35%, in grey reef shark, to 0.70%, in galapagos shark. Total phosphorus was nearly the same for all liver oils, i.e., about 0.8% of the liver oils. The phospholipid content of the oils varied from 10.88%, in grey reef shark, to 14.61%, in tiger shark. The total liver neutral lipid in the oils was about the same for all samples, i.e., 85%. Palmitic and oleic acids, together, made up about 85% to 100% of the fatty acid in all samples. Female whitetip reef shark, grey reef shark and silvertip shark contained more palmitic acid than oleic acid. Male whitetip reef shark and grey reef shark contained less palmitic acid than oleic acid, and tiger shark contained about an equal percentage of both palmitic and oleic acids. Three of six liver oils (male whitetip reef shark, grey reef shark and galapagos shark) did not contain fatty acid with a carbon number greater than C_{18}.

The data are based on analyses of single specimens of six species of sharks due to the difficulty in obtaining this type of material. It is not possible to make sound generalizations on the basis of the available data because of the limited number of samples.

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CHAPTER II

COMPARATIVE BIOCHEMICAL STUDY OF SOME GORGONIANS

INTRODUCTION

The gorgonians are coelenterates of the Class *Anthozoa*, Subclass *Octocorallia*, Order *Gorgonacea*. They are prominent members of the sessile shallow water coral reef community in the Caribbean area (1,2). Bergmann and Lester called attention to coral reefs as vast accumulators of materials that may be considered as potential precursors of petroleum.

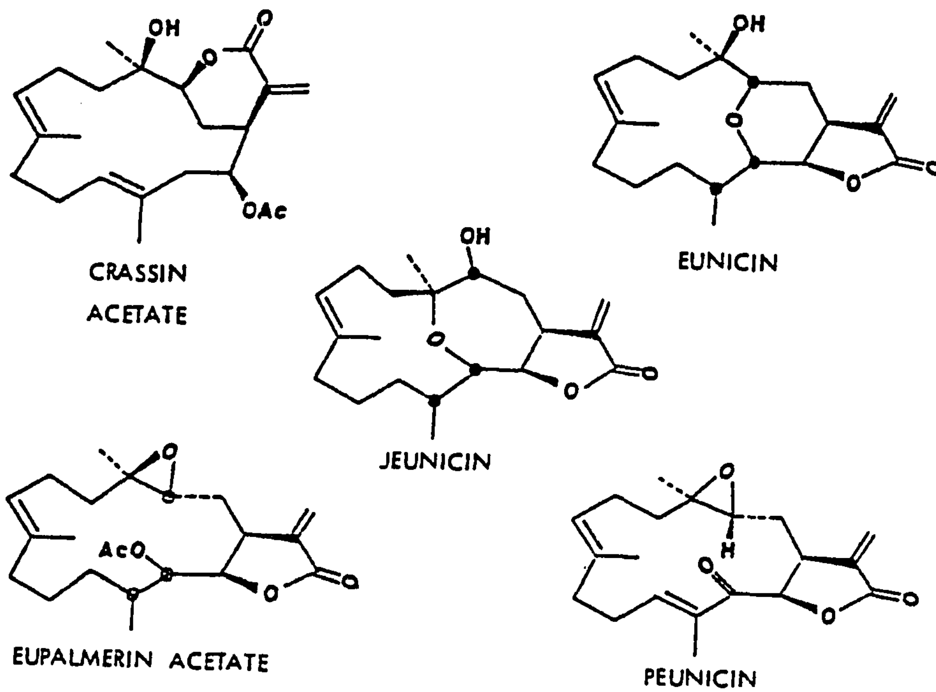
The biosynthetic versatility of reef coelenterates is indicated by the growing list of novel compounds isolated from the gorgonians: prostaglandins; sterols and seco-sterols with unusual side chains; a family of butenolides containing lactone rings joined by a polymethylene chain; a variety of sesquiterpene hydrocarbons; a series of diterpenes, including cembranolides; and taurobetaine and its analogs.

Gorgonians have few predators, and they are successful in the severe competition for space on solid substrate in coral reef areas. They compete successfully

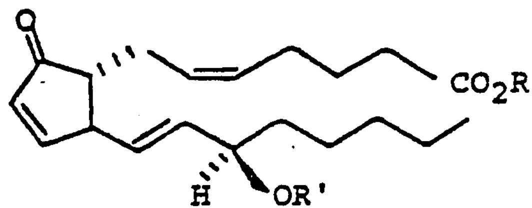
against other marine organisms in part due to the secretion of defense substances such as, the slimy secretion of polysaccharide sulfate from *Pseudopterogorgia americana* (1,3), (6% polysaccharide sulfate in the dried animal), cembrane derivatives (4,5), and other gorgonian cembranolides (6) such as crassin acetate from *Pseudoplexaura crassa* (7,8). The dried gorgonian cortex contained about 1.5% crassin acetate (8). Other cembranolides found in the gorgonians are: eunicin from *Eunicea mammosa* (Lamouroux); jeunicin from *E. mammosa* obtained from Jamaica; eupalmerin acetate from *E. succinea* (Pallas) from Puerto Rico; and peunicin from *E. succinea*, var. *plantiginea* collected off Panama. Two new prostaglandins 15-epi PGA_2 and its diester, accounted for 0.2% and 1.3% of the dry weight of *Plexaura homomalla* (Esper) (9,10), respectively. These prostaglandins were extracted from the ground gorgonian cortex with hexane.

Gorgonians have been found to contain aliphatic, naphthenic, and aromatic hydrocarbons (11). The naphthenes include mono-, di-, tri-, tetra-, penta-, and hexa-cyclic compounds including steranes. Diterpenoid lactones, such as crassin acetate (7,11), are toxic to some protozoa and inhibit the development of some larval animals.

Weinheimer *et al.* (13,14) reported that 95% of the hydrocarbons isolated from *Pseudopterogorgia americana* was accounted for by the four compounds: (+)-9-aristolene



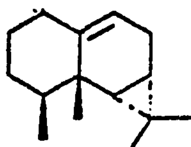
Reference (6).



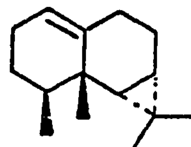
15-epi-PGA_2: R,R' = H

Diester of 15-epi-PGA_2: R = Me,R' = Ac

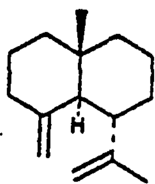
Reference (10)



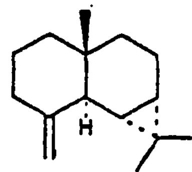
(+)-9-aristolene



(-)1(10)-aristolene



(-)-β -gorgonene



(+)-γ -maaliene

Reference (13).

(8%), (-)-1(10)-aristolene (25%), (+)-γ-maaliene (8%), and (+)-β-gorgonene (55%).

Plexaura _flexuosa_ studied by Ciereszko _et al_. (15) was shown to contain 3% lipid, with 27% unsaponifiable material and 13.5% sterols. The sterols included one C_{27}, two C_{28} and two C_{29} (mono- and di-unsaturated sterols), and a C_{30} sterol identified as gorgosterol ($C_{30}H_{50}O$). _P_. _flexuosa_ was the first marine invertebrate (16,17) found to contain octadecyl alcohol ($C_{18}H_{37}$-OH, m.p. 55-56°C) and batyl alcohol ($HOCH_2CHOH$-CH_2O-$(CH_2)_{17}CH_3$, m.p. 68-69°C), accounting for 50% and 15% of the sterol-free nonsaponifiable fraction, respectively. Gross (18) reported that _P_. _flexuosa_ contained fatty esters, fatty acids, and a minute quantity of a sesquiterpene hydrocarbon mixture (0.01% of the dry animal) as well as a mixture of oxygenated compounds. Unlike _Plexaura_ _homomalla_, _P_. _flexuosa_ did not contain prostaglandins.

Esters of fatty acids with long chain alcohols, the wax esters, occur in many marine animals, including the Class _Actinozoa_ (19), sea anemones (Order _Actinaria_), stony corals (Order _Madreporaria_), zoanthids (Order _Zoantharia_) and gorgonians (Order _Gorgonaria_). Possible functions of wax esters of marine organisms include service as reserve energy stores, as buoyancy agents, and as structural elements. In general, the biosynthesis of wax ester in animals can be pictured as shown on the following page.

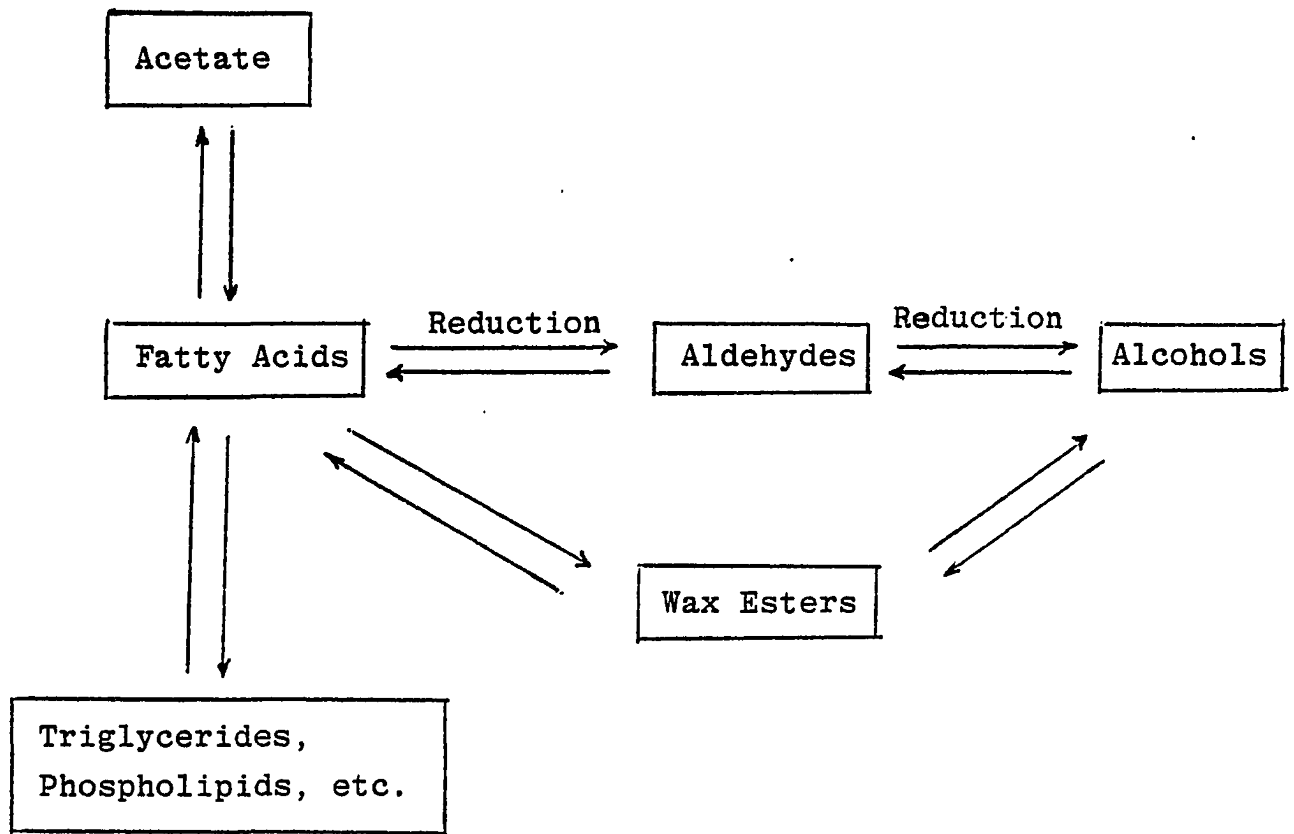


Figure 12. The biosynthetic pathway of wax esters.

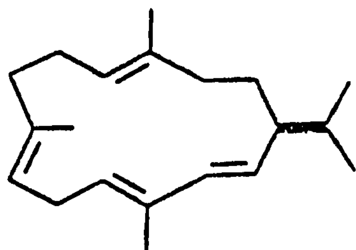
It is known (19) that in most animals the net balance of the reactions is toward the hydrolysis of wax esters and the oxidative catabolism of the alcohols. In most marine organisms, however, the balance is reversed. There is a net synthesis of alcohols, and these in turn are esterified with fatty acids to wax esters, which accumulate. The wax ester, cetyl palmitate, has been found in significant amounts in Xiphogorgia sp. (16,17), Pseudopterogorgia americana (20,21), Goniastrea retiformis (22), Briarium asbestinum (23), Plexaurella nutans (23), Eunicea grandis (23) and Plexaura crassa (23). The eggs of P. americana (Gmelin) from La Parguera, Puerto Rico (20,21) contained 75% lipid (dry weight basis), consisting of 72% cetyl palmitate, 7.5% glycerides, 2.7% saturated branched hydrocarbons, and 6% sterols with cholesterol as the major component. Benson and Muscatine (22) found 80% cetyl palmitate in the lipid extract of Goniastrea retiformis (Lamarck) coral. Small amounts of C_{30} (5%) and C_{34} (1%) wax esters were also found. They suggested (22) that wax-containing coral mucus was ingested by reef fishes, indicating one route by which the energy rich products of coral metabolism is transferred to the reef fish population.

Lee and Hirota (23) reported that wax esters were found as major components of the lipids of deep-water tropical copepods from the central south Pacific. The percentage of wax esters in the lipid varied widely, from

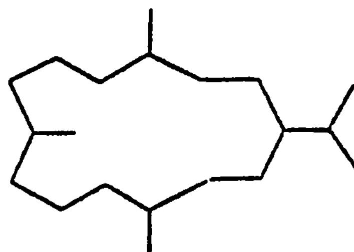
only trace amounts to about 70%, depending upon the type (genus and species) of copepods and the latitude of their habitat in the sea. The widespread use of wax esters as a fuel reserve has been found thus far only in marine organisms and is concentrated in certain zones of the marine environment (23). Wax ester is known as an important lipid component for most bathypelagic organisms. Large quantities of wax esters are stored by species which represent the most abundant groups of marine animals (Annelida, Arthropoda, Chaetognatha, Chordata, Coelenterate, and Mollusca). In the pelagic fauna, however, only the deep-living, temperate and polar zooplankton and nekton store wax esters.

Diterpenoids possessing the cembrane ring system have been found to occur in diverse natural sources (24): in cembrane-A, in a tree Commiphora mukul, in India (25,26). a Siberian spurce (27,28) in Russia, in termites in Australia (29), and in a Pacific soft coral (30); isocembrane from a Russian pine tree (31); and casbene from castor beans (32,33).

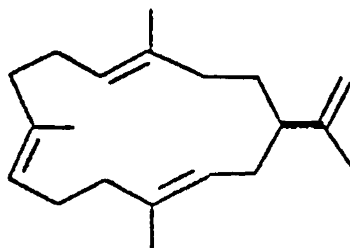
Three diterpenoid compounds, $C_{20}H_{34}O_3$ (labelled compound B-1, 0.32% of the dry weight), $C_{20}H_{32}O_3$ (labelled B-2, small amounts), and $C_{20}H_{36}O_3$ (labelled B-3, 0.37%) were isolated from an undescribed gorgonian of the genus Plexaura (24), collected off the island of Bonaire in the Netherlands Antilles (Plexaura sp. "REM"). B-1, B-2 and B-3 are closely related compounds. They all possess 20



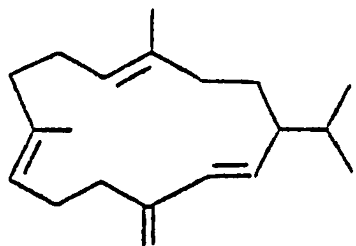
Cembrene



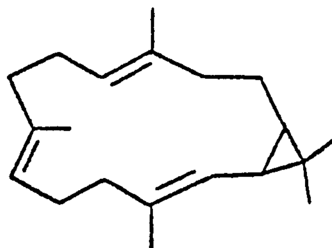
Cembrane



Cembrene-A



Isocembrene



Casbene

carbon and 3 oxygen atoms, 3 secondary methyl groups, an isopropyl group, and a ketone group. Compound B-1 is a monohydroxyketone and B-3 a dihydroxyketone. The structure and absolute configuration of compound B-1 has been determined and named, plexaurolone (38).

The study of natural products from the coral reefs has now become more attractive to chemists and biochemists because of the recent development of techniques for the isolation, identification and determination of properties and structural features of complex molecules. The promising prospect of the biochemistry of coral reefs brightens with the discovery of an ever increasing number of novel compounds of potential value as drugs or as tools for pharmacological and biochemical research.

One of the main reasons for doing this research was my idea that the field of coral reefs, particularly the chemistry of coral reefs, is one of the most important sciences that should be understood by the people living in a country having abundant resources for it. The Indonesian archipelago is known to be one of the richest coral reef areas of the world (34). In spite of their abundance, however, little if anything is known about the nature of their organic constituents. As part of our continuing study of the chemistry of gorgonians my research work has been directed toward the investigation of diterpenoid compounds, wax esters, and hydrocarbons from *Pseudopterogorgia americana*

and from species of *Plexaura*; *Plexaura Sp* "*REM*" St. Croix; *Plexaura* "*REM*" San Cristobal; *Plexaura kukenthali*; *Plexaura homomalla*; and *Plexaura flexuosa*.

RESULTS AND DISCUSSION

Cetyl Palmitate From Eggs of *Pseudopterogorgia americana*

Eggs of *P. americana*, 10.466 g, were obtained from Dr. L. S. Ciereszko collected at Cayo Enrique, Puerto Rico, on February 2, 1978. Extraction of the sample with hexane using a Soxhlet extractor yielded 6.426 g dried lipid (equal to 61.4% lipid in the dry sample). Urea inclusion (35,36) was used to isolate cetyl palmitate from the lipid, yielding 4.166 g solid (equal to 64.8% wax ester in the lipid). A sample of 1.245 g of this solid was chromatographed on Florisil using hexane-ether (9:1, v/v) as eluting solvent. Fractions 5 to 20 (150 ml eluate) were collected, combined, and evaporated to dryness, yielding 855 mg solid (equal to 68.7% cetyl palmitate in the wax ester, or 44.5% in the lipid, or 27.3% in the dried eggs). Gas chromatography and gas chromatography/mass spectrometry showed one major component with a molecular weight of 480, as shown in Figure 15 (mass spectrum). It was identified as cetyl palmitate after purification by tlc, ir (Figures 13 and 16) and nmr (Figures 14 and 17). The one minor component with molecular weight 508 was not identified.

Hydrocarbons from Eggs of *P. americana*

Lipids were recovered, from the filtrate of the urea inclusion compound, by liquid-liquid extraction with

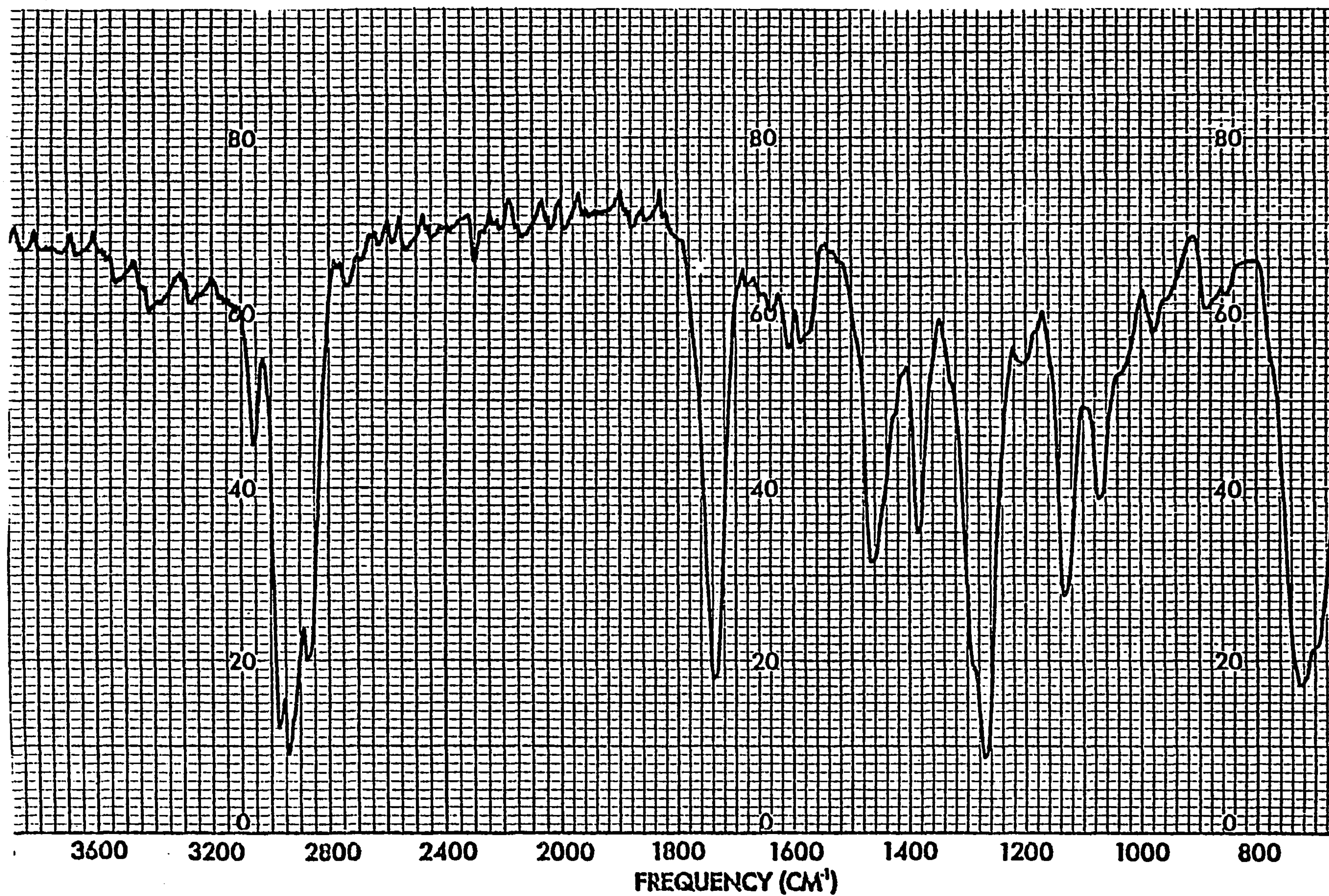


Figure 13. The ir spectrum of cetyl palmitate isolated from *Pseudopterogorgia americana*.

60 MHz, T-60 NMR Spectrometer

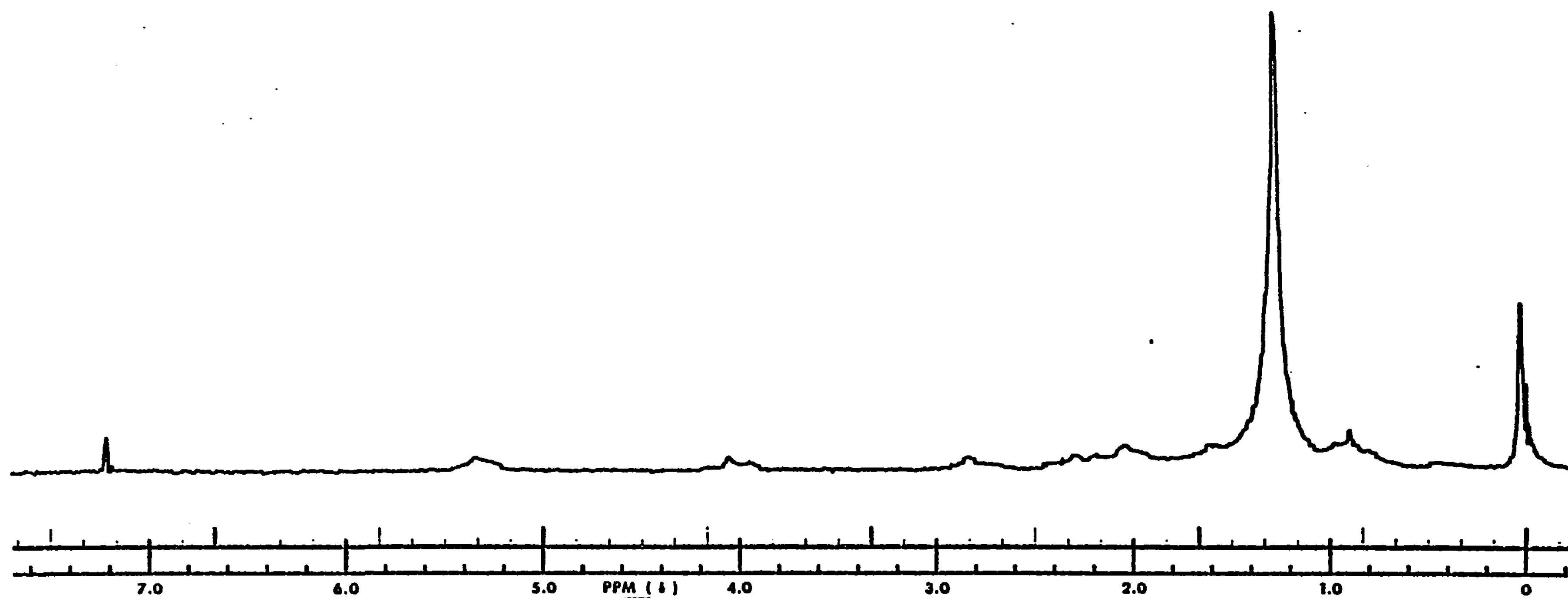


Figure 14. The nmr spectrum of cetyl palmitate isolated from Pseudopterogorgia americana.

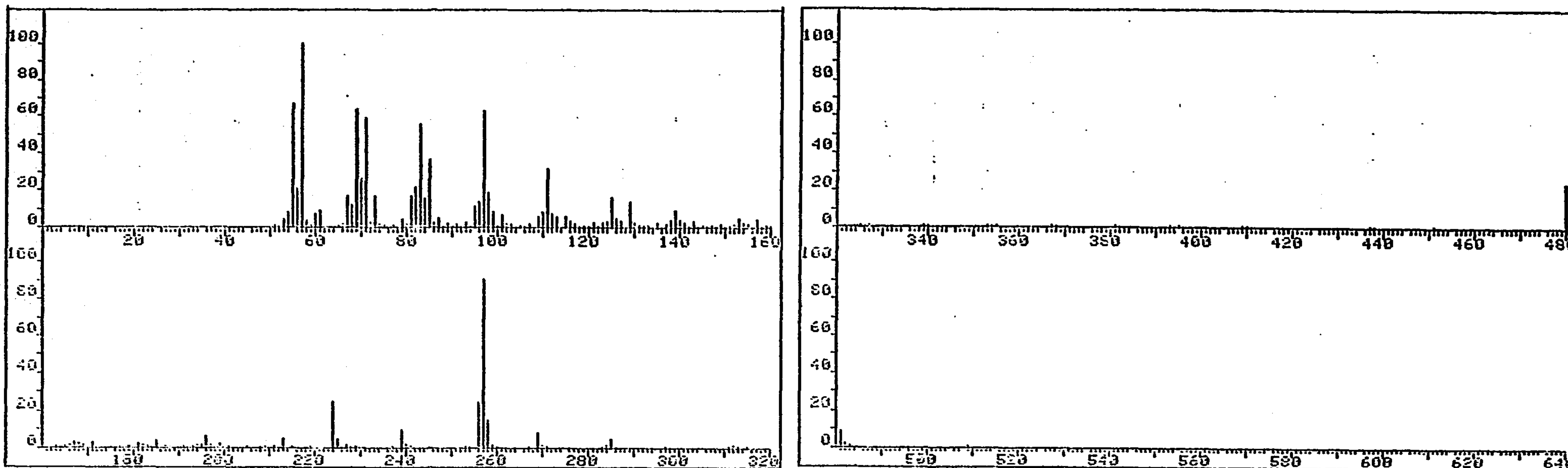


Figure 15. The mass spectrum of cetyl palmitate isolated from Pseudopterogorgia americana.

FILE 5692 SPECTRUM 169 220 PEAKS RT=26.12 MIN BASE PEAK= 10173
LARGEST 182

MASS	%	MASS	%	MASS	%	MASS	%	MASS	%	MASS	%	MASS	%
51.2	.3	80.2	1.4	108.2	.4	136.2	.3	166.2	1.4	201.2	.5	267.2	.2
52.2	.4	81.1	17.5	109.2	5.8	137.1	1.6	167.2	3.0	209.4	.3	269.3	7.6
53.1	4.2	82.1	21.7	110.3	8.1	138.2	4.3	168.3	1.9	213.3	4.7	270.4	1.3
54.2	8.6	83.2	55.1	111.2	31.4	139.2	8.8	169.3	1.0	214.3	.7	271.3	.2
55.1	67.0	84.2	15.9	112.2	7.5	140.2	4.2	171.3	3.2	215.3	.3	284.3	1.0
56.2	20.6	85.1	36.1	113.2	5.8	141.2	2.6	172.2	.8	219.3	.8	285.4	4.4
57.1	100.0	86.2	2.7	114.2	1.0	142.2	.3	173.3	.4	220.3	.3	286.4	.8
58.2	4.5	87.1	5.1	115.2	5.5	143.2	3.0	177.2	.4	222.4	.4	297.4	.8
59.2	1.0	88.1	.7	116.2	3.9	144.1	.6	179.2	.3	224.4	24.5	311.4	.3
60.2	7.3	89.2	1.8	117.2	1.5	145.1	.5	180.3	.7	225.4	4.9	312.4	1.0
61.1	8.7	91.2	1.3	118.2	.2	147.2	.3	181.3	1.9	226.4	.5	313.4	.3
62.1	.3	92.0	.3	119.3	.4	148.2	.2	182.3	.9	227.4	1.4	315.2	.7
65.1	1.2	93.1	2.6	121.2	2.3	149.2	1.5	183.3	.5	228.4	.3	325.4	.2
66.1	1.3	94.1	1.3	122.3	.4	150.2	.3	185.3	3.6	229.3	.5	326.4	.2
67.2	16.9	95.2	11.5	123.2	2.4	151.2	.7	186.2	.5	236.3	.7	353.5	.9
68.1	12.2	96.2	13.9	124.2	3.6	152.2	2.0	187.3	.4	237.4	.8	354.5	.2
69.1	63.5	97.2	62.8	125.2	15.9	153.2	4.9	191.3	.2	238.4	.5	351.5	.4
70.1	26.4	98.2	18.7	126.2	5.2	154.2	2.4	192.2	.3	239.4	9.7	353.2	.2
71.2	58.8	99.2	8.6	127.3	3.0	155.2	1.7	193.2	.2	240.4	2.0	355.4	.2
72.2	3.5	100.2	1.2	128.1	.4	156.3	.3	194.3	1.0	241.4	.5	409.5	.4
73.1	16.7	101.2	6.2	129.2	13.8	157.2	4.0	195.3	1.4	252.3	.2	437.6	.4
74.1	1.8	102.2	1.3	130.2	2.3	158.1	.8	196.3	6.4	254.3	.2	480.6	23.9
75.1	.3	103.2	1.7	131.1	.7	159.3	.6	197.3	1.4	256.3	25.0	481.6	8.8
77.1	1.0	105.2	.7	133.1	.4	161.2	.2	198.1	.2	257.3	90.7	482.6	1.8
78.0	.4	106.2	.2	134.1	.2	163.2	1.1	199.2	1.8	258.3	15.4	483.6	.2
79.2	4.0	107.2	2.0	135.1	2.5	165.2	.4	200.2	.5	259.3	1.5	508.7	.5

LAST 35

MASS	%	MASS	%	MASS	%	MASS	%	MASS	%	MASS	%	MASS	%
254.4	.1	297.4	.8	298.4	.2	311.4	.3	312.4	1.0	313.4	.3	315.2	.7
316.2	.2	325.4	.2	326.4	.2	327.2	.2	339.4	.2	340.4	.2	353.5	.9
354.5	.2	355.3	.1	367.5	.2	368.4	.2	381.5	.4	382.5	.1	393.2	.2
394.2	.1	395.4	.2	396.3	.1	409.5	.4	410.4	.2	437.6	.4	438.6	.2
451.3	.2	480.6	23.9	481.6	8.8	482.6	1.8	483.6	.2	508.7	.5	509.6	.1

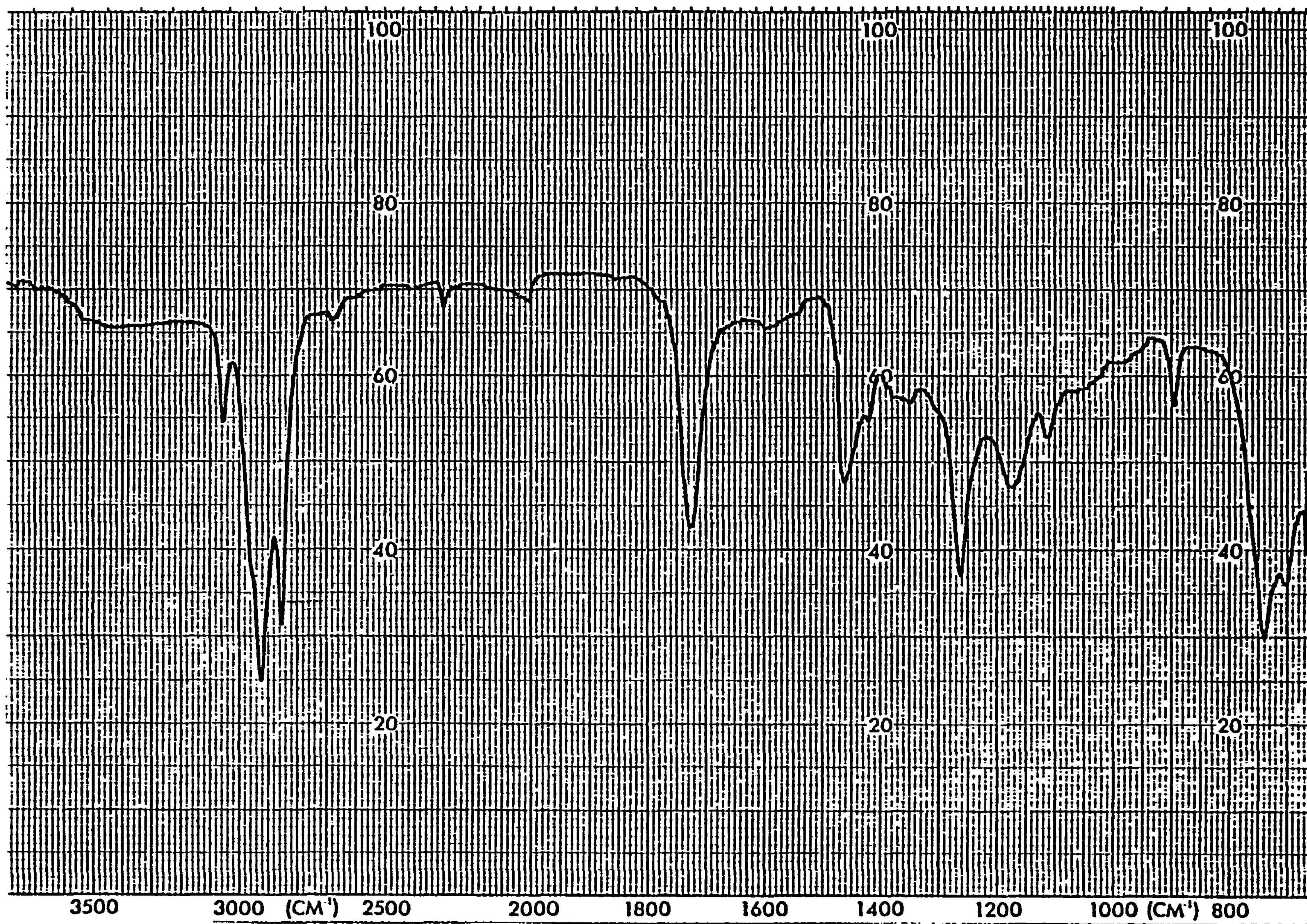
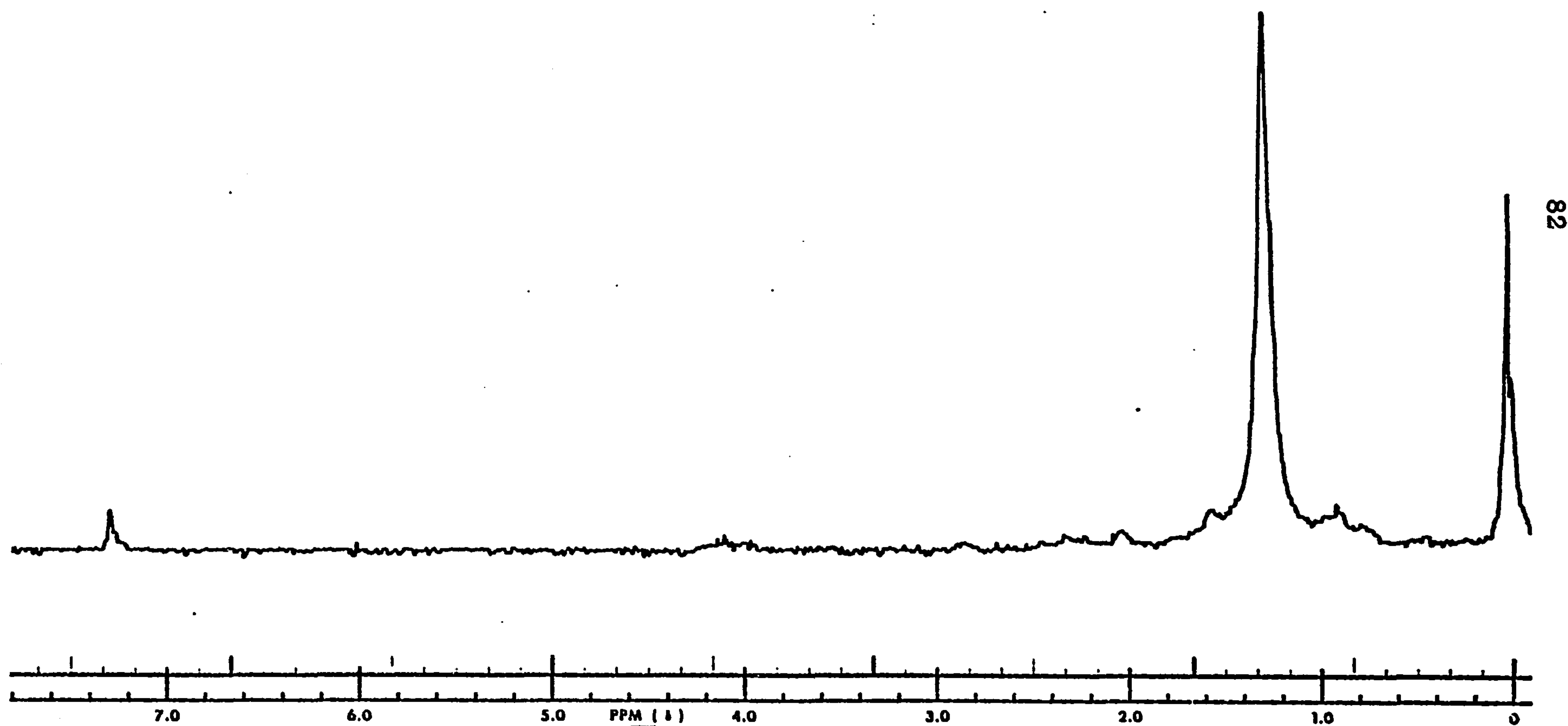


Figure 16. The ir spectrum of reference standard cetyl palmitate.

60 MHz, T-60 NMR Spectrometer



<u>Figure 17</u>. The nmr spectrum of reference standard cetyl palmitate

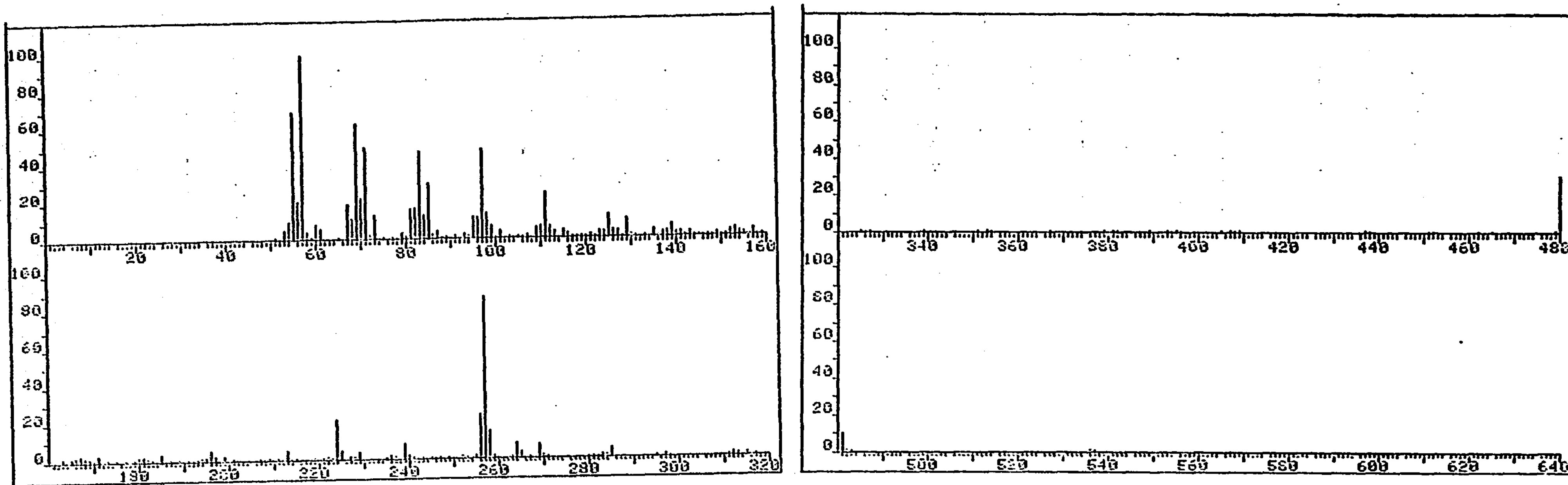


Figure 18. The mass spectrum of reference standard cetyl palmitate.

MASS	%	MASS	%	MASS	%	MASS	%	MASS	%	MASS	%	MASS	%
51.1	.6	83.2	47.9	113.2	3.5	143.1	2.5	179.3	.6	221.3	.7	269.3	6.9
52.2	.4	84.2	13.5	114.2	1.0	144.1	.5	180.2	2.0	222.3	1.5	270.4	1.6
53.2	4.8	85.1	29.7	115.2	4.3	145.1	.5	181.3	2.2	223.4	.6	280.3	.5
54.2	10.1	86.2	2.6	116.2	2.2	147.2	.7	182.3	1.1	224.4	21.0	283.3	1.7
55.1	69.2	87.1	4.6	117.2	1.3	148.2	.7	183.3	.6	225.4	4.5	284.3	1.3
56.2	21.0	88.1	.7	119.2	.6	149.2	1.7	185.3	3.7	226.3	.7	285.4	4.4
57.1	100.0	89.1	1.0	121.3	2.0	150.2	.5	186.3	.7	227.4	1.3	286.4	.8
58.2	4.6	91.1	1.8	122.2	.7	151.2	1.1	187.2	.5	228.3	1.2	297.3	1.3
59.1	1.0	93.1	2.4	123.2	3.7	152.2	2.8	189.2	.4	229.3	3.7	298.3	.4
60.2	6.2	94.1	1.5	124.2	3.2	153.2	3.9	192.2	.6	236.3	2.4	312.4	1.7
61.1	5.9	95.2	11.6	125.2	11.8	154.2	2.2	193.2	.7	237.4	1.4	313.4	1.1
65.0	1.3	96.2	11.1	126.2	3.9	155.2	1.9	194.3	1.5	238.4	.8	314.3	.4
66.0	1.1	97.2	48.4	127.2	3.4	157.2	3.7	195.3	1.3	239.4	8.3	315.2	1.3
67.2	18.9	98.2	14.2	128.2	.5	158.2	1.0	196.3	5.2	240.3	1.8	316.2	.5
68.1	11.2	99.2	6.5	129.1	10.1	159.3	.4	197.2	1.7	241.3	.5	339.4	.4
69.1	62.8	100.2	1.0	130.2	1.3	163.2	1.2	199.2	1.7	250.4	.5	353.5	1.0
70.1	22.7	101.1	4.5	131.1	.6	164.2	.6	200.2	.7	252.4	1.2	381.5	.6
71.2	50.0	102.2	.8	133.1	.9	165.2	1.2	207.1	.5	253.2	.9	393.2	.6
72.2	2.6	103.2	1.0	134.1	.8	166.3	1.8	208.3	.5	255.3	1.1	395.4	.6
73.1	13.5	105.1	.9	135.2	4.0	167.3	2.5	209.2	.5	256.3	24.2	409.4	.6
74.1	1.0	107.2	1.8	136.1	.5	168.2	1.8	211.2	.6	257.3	86.9	437.5	.5
77.0	1.2	108.2	.9	137.1	2.7	169.3	1.1	213.2	4.5	258.3	14.7	480.6	30.7
79.2	3.9	109.2	6.0	138.2	3.2	171.3	2.8	214.3	.9	259.3	1.3	481.6	10.1
80.2	1.8	110.2	6.7	139.2	6.5	172.2	.7	215.3	.5	264.3	7.3	482.5	1.6
81.1	16.2	111.2	23.9	140.2	2.4	173.3	.4	219.3	.8	265.4	3.2	534.6	.5
82.1	17.5	112.2	6.2	141.2	2.2	177.2	.5	220.3	.8	267.3	.4	536.6	1.2

LAST 35

MASS	%	MASS	%	MASS	%	MASS	%	MASS	%	MASS	%	MASS	%
326.4	.3	327.1	.2	339.4	.4	351.4	.2	353.5	1.0	354.5	.2	357.5	.3
374.2	.2	379.4	.3	381.5	.6	382.6	.2	389.1	.2	393.2	.6	394.3	.2
395.4	.6	396.4	.2	405.2	.2	407.5	.2	408.1	.3	409.4	.6	437.5	.5
450.6	.3	451.2	.4	451.8	.2	452.4	.3	462.5	.2	465.2	.2	478.7	.2
480.6	30.7	481.6	10.1	482.5	1.6	483.6	.3	534.6	.5	536.6	1.2	537.6	.3

ether as described in the experimental section, and dried, yielding 1.2 g lipid. Hydrocarbon (120 mg) was obtained from fractions 1-9 of the eluate (100 ml) derived from the lipid chromatographed on Florisil using hexane as eluting solvent. Tlc showed two major fractions showing spots with R_f 0.6 (hydrocarbon I) and R_f 0.4 (hydrocarbon II), respectively. The two fractions were separated, from each other, by rechromatography as described in the experimental section, yielding 45 mg of hydrocarbon I (37.5% (g hydrocarbon I/100 g total hydrocarbon) and 60 mg of hydrocarbon II (50%). The ir spectra of both hydrocarbons (Figures 19 and 22) show strong C-H stretching around 3000 cm^{-1}, C-H (vinyl) stretching to the left of 3000 cm^{-1}, characteristic CH_2 absorption at 1460 cm^{-1}, absorptions at 1400 cm^{-1}, 1380 cm^{-1} and 890 cm^{-1} (consistent with exo-methylene group proton), and C=C stretching at 1660 cm^{-1}. The nmr spectra of both hydrocarbons show methylene proton absorption at δ1.2, methyl terminal absorption at δ0.95, and olefinic proton absorption at δ4.3 (for hydrocarbon I) and δ4.70 (for hydrocarbon II), as shown in Figures 19 and 23. Mass spectrometry suggested a molecular weight of 214 for hydrocarbon I (Figure 21) and a molecular weight of 216 for hydrocarbon II (Figure 24).

Diterpenoids from Plexaura samples

Lipids were extracted from the samples with hexane

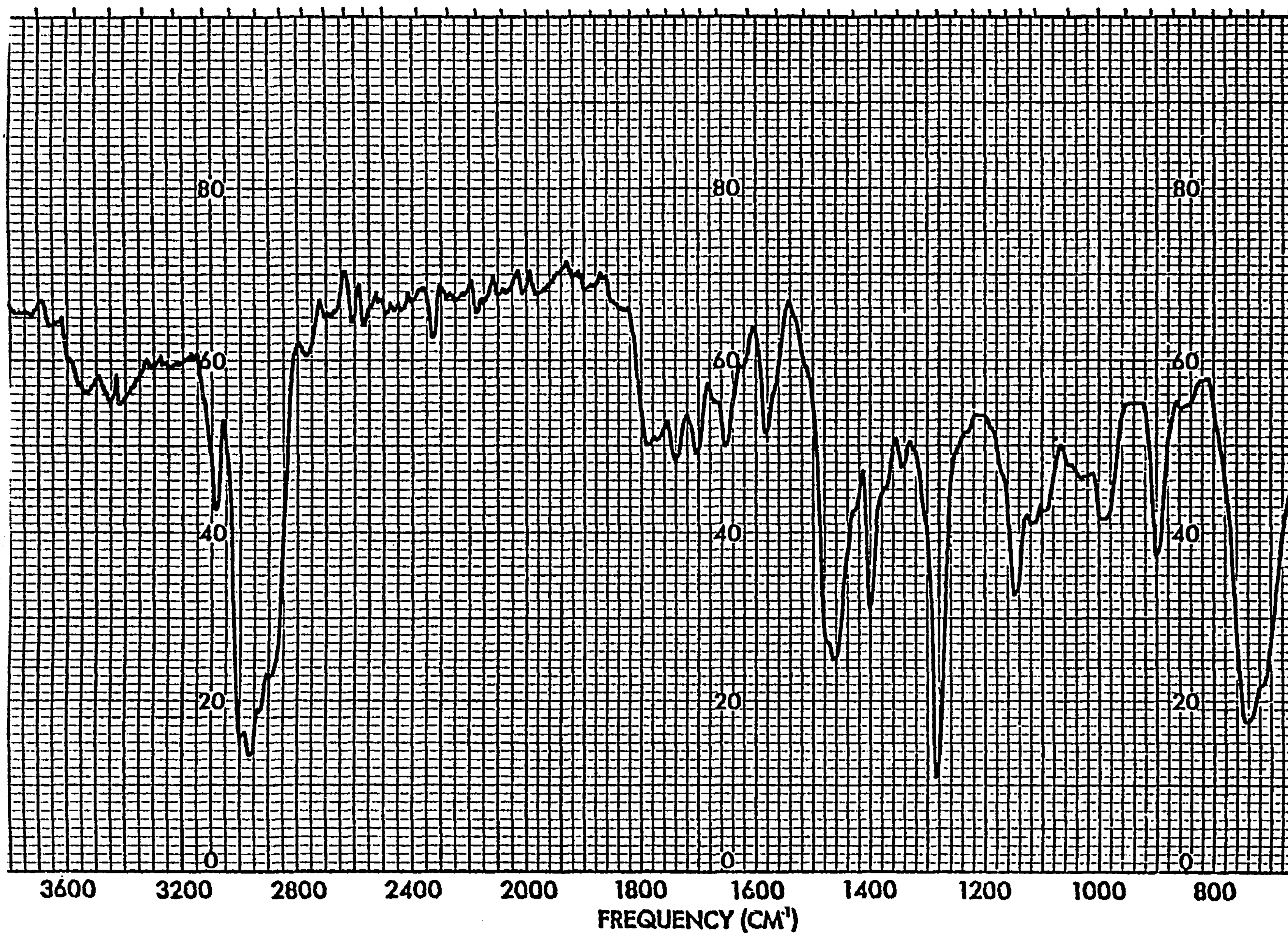


Figure 19. The ir spectrum of hydrocarbon I isolated from Pseudopterogorgia americana.

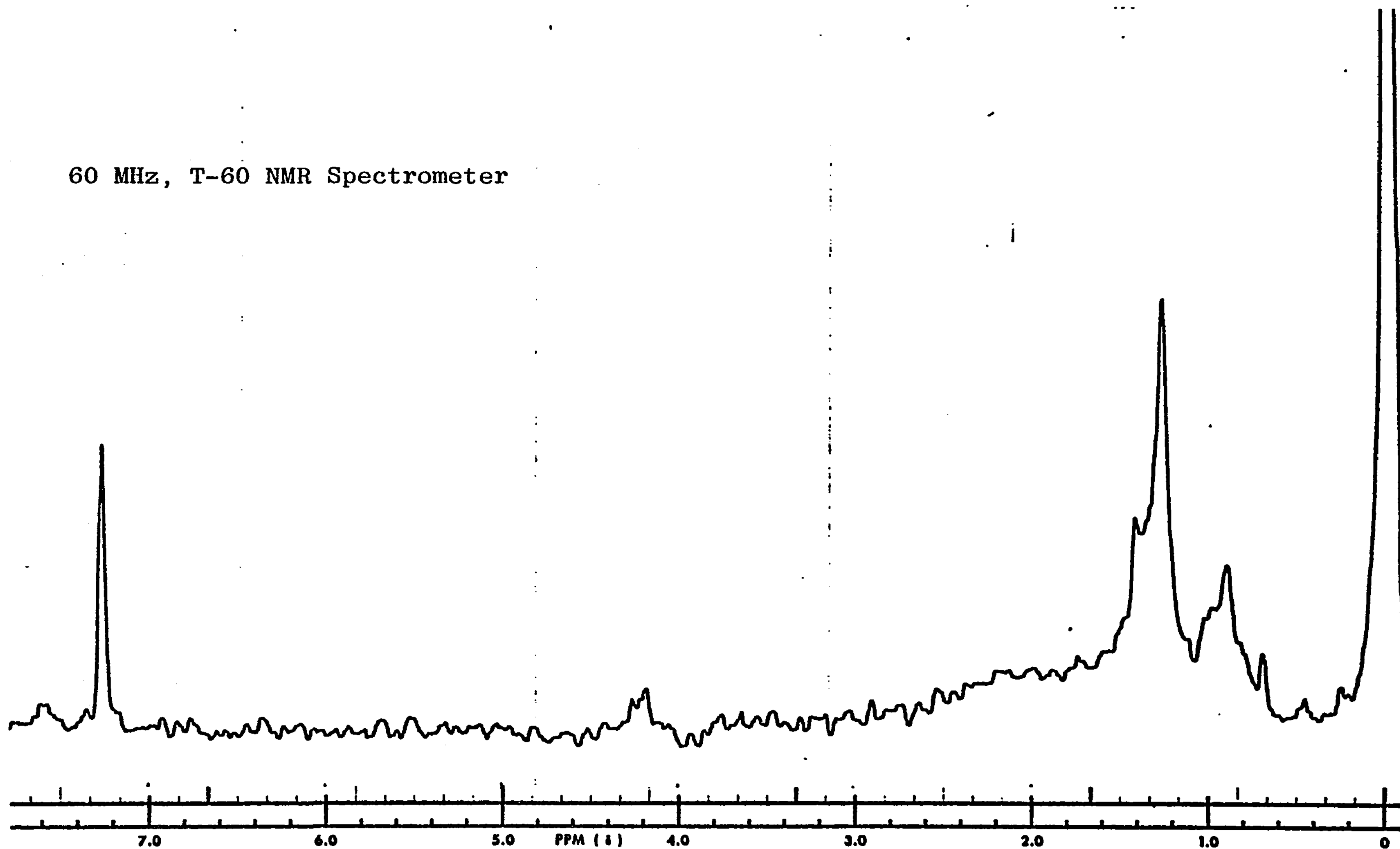


Figure 20. The nmr spectrum of hydrocarbon I isolated from *Pseudopterogorgia americana*.

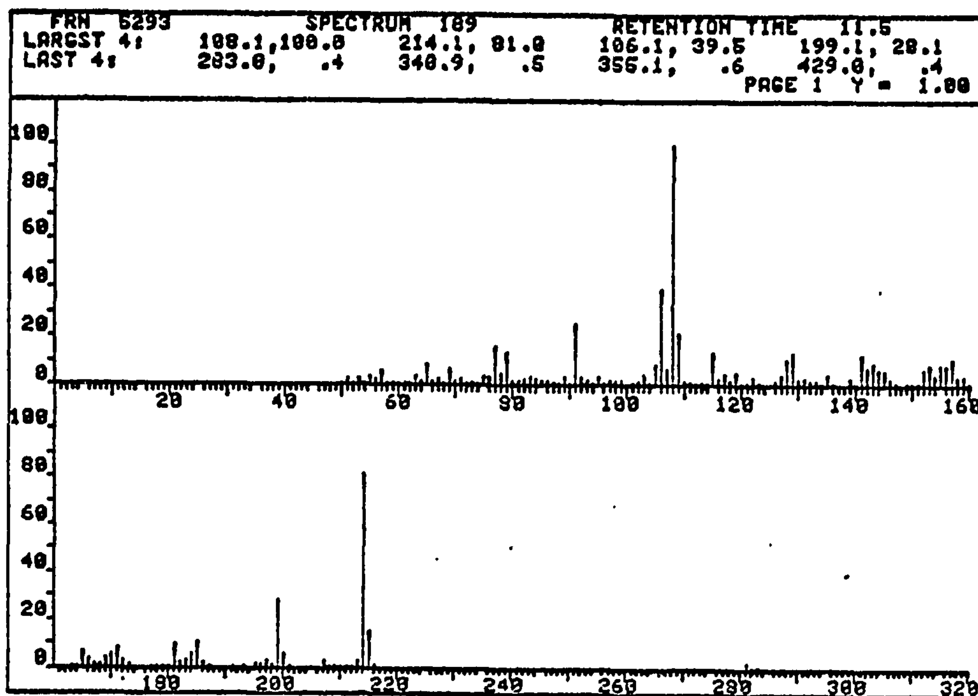


Figure 21. The mass spectrum of hydrocarbon I isolated from *Pseudopterogorgia americana*.

FILE 5293 SPECTRUM 189 152 PEAKS RT=11.48 MIN BASE PEAK= 2648
LARGEST 152

MASS	%	MASS	%	MASS	%	MASS	%	MASS	%	MASS	%	MASS	%
50.0	.9	76.1	3.0	103.1	4.0	131.1	2.3	160.1	.8	196.1	1.5		
51.1	2.3	77.0	15.1	104.2	1.0	132.1	1.5	163.1	.5	197.1	2.6		
52.1	1.2	78.1	4.8	105.1	8.6	132.9	1.5	164.1	.6	198.2	1.6		
53.1	2.4	79.1	13.5	106.1	39.5	134.1	.6	165.1	6.2	199.1	28.1		
54.2	.6	80.1	2.0	107.1	6.6	135.1	4.2	166.1	3.7	200.0	5.1		
55.1	2.8	81.1	1.6	108.1	100.0	136.0	.6	167.1	1.6	200.9	.7		
56.2	2.0	82.1	2.2	109.1	21.1	139.0	2.5	168.1	1.2	207.0	3.3		
57.1	5.8	83.1	3.1	110.1	2.0	140.0	.9	169.1	3.9	208.1	.8		
58.1	.5	84.1	2.3	111.2	.6	141.1	12.4	170.1	5.1	209.0	.9		
59.7	.5	85.1	1.9	112.1	.4	142.1	7.6	171.1	7.6	210.1	.4		
59.9	.5	86.1	1.9	113.1	.9	143.1	8.8	172.1	3.2	211.2	.4		
61.3	.4	87.0	.9	114.0	.6	144.1	6.2	173.1	1.0	212.1	.9		
62.1	.5	88.2	.6	115.1	12.6	145.1	5.6	177.2	.5	213.1	3.4		
63.1	3.2	89.2	3.3	116.1	2.3	146.1	2.4	178.1	.6	214.1	81.0		
64.1	1.7	90.1	.9	117.1	4.6	147.1	.9	179.2	.6	215.2	15.2		
65.1	7.8	91.1	25.3	118.0	1.9	148.9	.7	180.1	.6	216.1	1.6		
66.0	1.5	92.1	3.8	119.1	5.1	149.9	.6	181.1	9.4	281.0	1.7		
67.1	2.1	93.1	1.9	120.1	.6	151.1	1.3	182.1	2.3	282.0	.4		
68.1	.8	94.1	.7	121.1	.9	152.1	6.2	183.1	2.9	283.0	.4		
69.1	6.3	95.1	2.8	122.1	2.9	153.0	8.4	184.1	5.2	340.9	.5		
70.2	1.9	96.1	1.2	123.2	.5	154.0	4.5	185.1	9.8	355.1	.6		
71.1	2.7	97.1	2.0	126.0	1.3	155.1	8.0	186.1	2.0	429.0	.4		
72.0	.6	98.1	1.6	127.2	4.3	156.1	8.5	187.2	.4				
73.1	.9	99.1	1.5	128.1	10.4	157.1	10.8	191.1	.6				
74.1	.7	100.9	.6	129.0	13.3	158.1	2.8	193.0	.5				
75.1	2.9	102.1	2.0	130.0	2.4	159.1	3.4	195.0	1.6				

LAST 35

MASS	%	MASS	%	MASS	%	MASS	%	MASS	%	MASS	%	MASS	%
178.1	.6	179.2	.6	180.1	.6	181.1	9.4	182.1	2.3	183.1	2.9	184.1	5.2
185.1	9.8	186.1	2.0	187.2	.4	191.1	.6	193.0	.5	195.0	1.6	196.1	1.5
197.1	2.6	198.2	1.6	199.1	28.1	200.0	5.1	200.9	.7	207.0	3.3	208.1	.8
209.0	.9	210.1	.4	211.2	.4	212.1	.9	213.1	3.4	214.1	81.0	215.2	15.2
216.1	1.6	281.0	1.7	282.0	.4	283.0	.4	340.9	.5	355.1	.6	429.0	.4

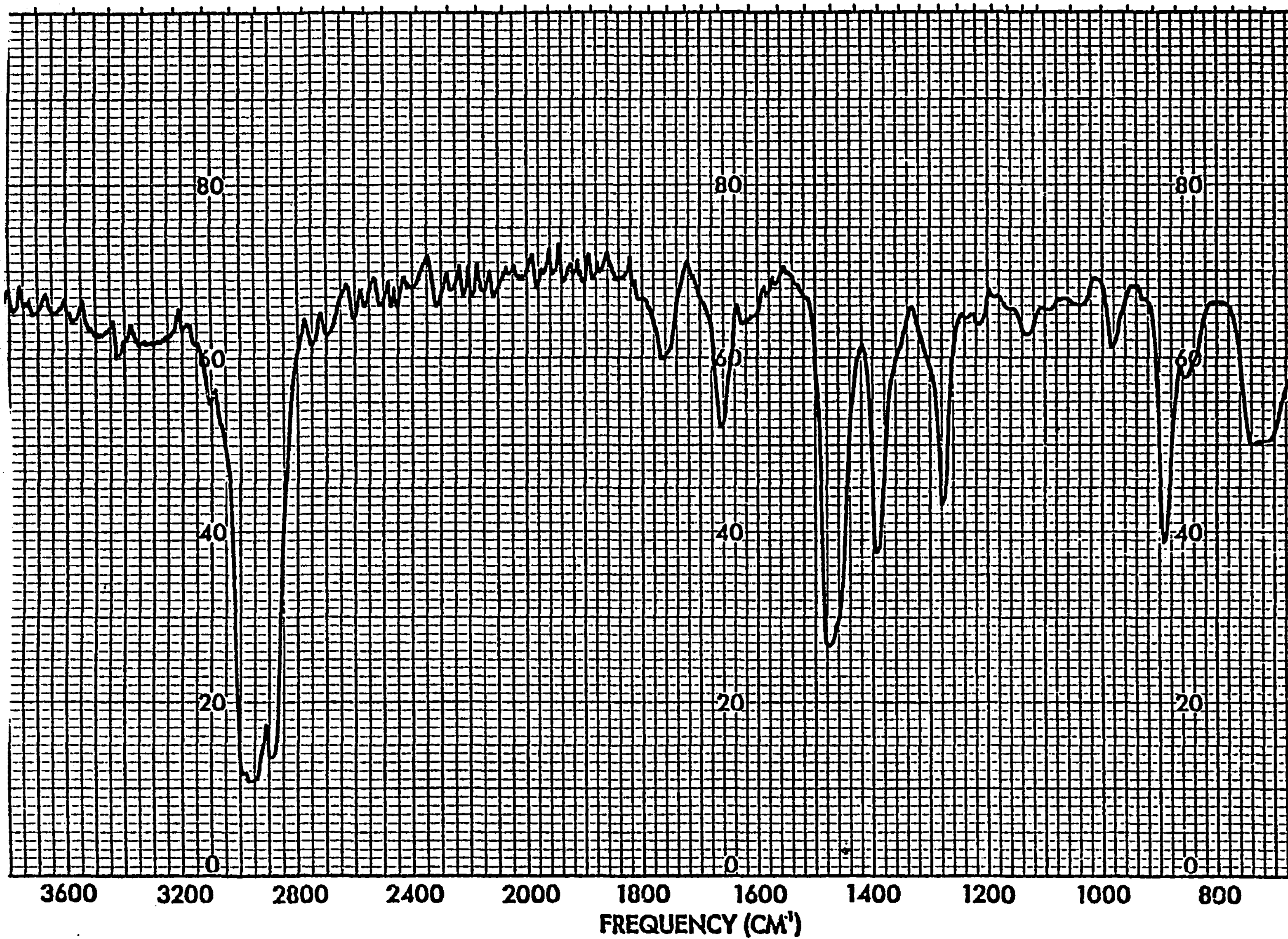


Figure 22. The ir spectrum of hydrocarbon II isolated from *Pseudopterogorgia americana*.

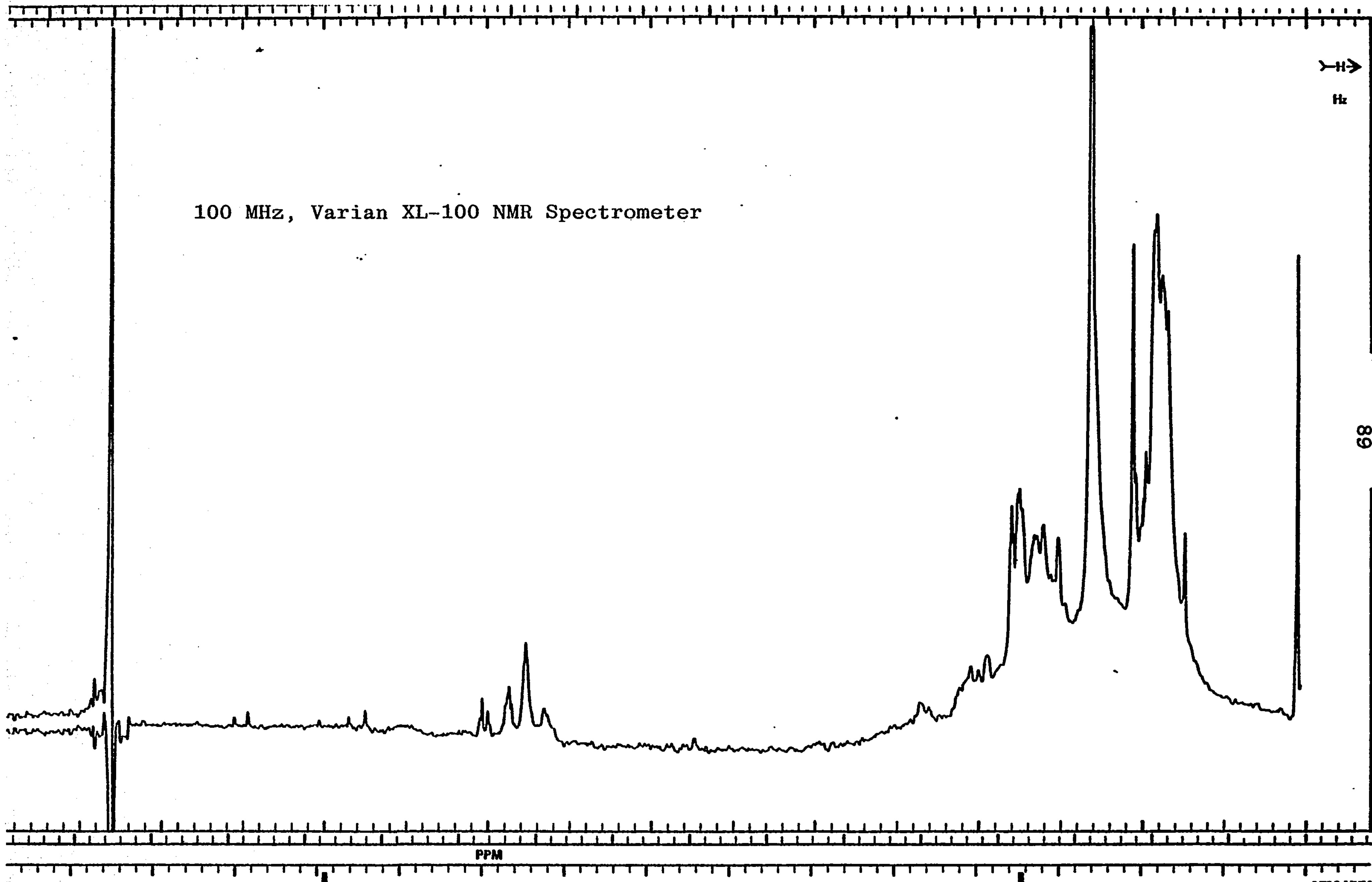


Figure 23. The nmr spectrum of hydrocarbon II isolated from *Pseudopterogorgia americana*.

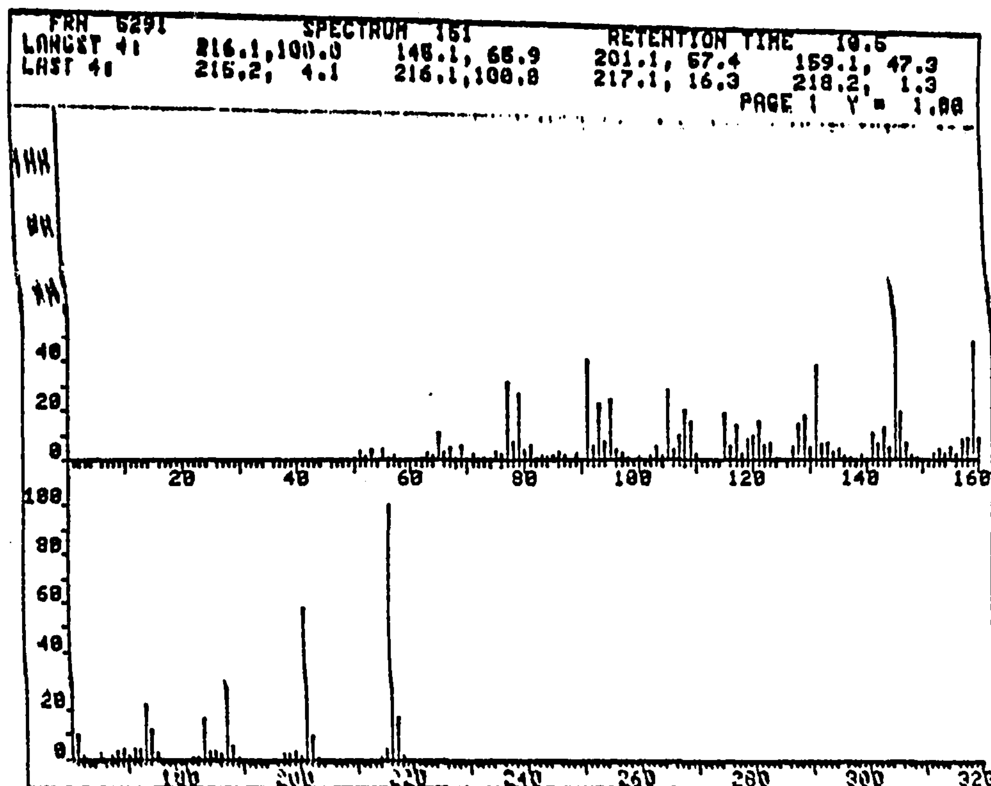


Figure 24. The mass spectrum of hydrocarbon II from *Pseudopterogorgia americana*.

FILE 5291 SPECTRUM 151 125 PEAKS RT=10.47 MIN BASE PEAK= 1295

LARGEST 125

MASS	%	MASS	%	MASS	%	MASS	%	MASS	%	MASS	%	MASS	%
50.1	1.2	81.1	5.6	115.1	10.5	145.1	65.9	181.1	.8				
51.1	3.6	82.1	1.2	116.1	5.8	146.1	19.2	182.1	.9				
52.1	1.9	83.0	1.5	117.1	13.7	147.1	6.6	183.1	15.4				
53.1	4.5	84.1	1.4	118.1	2.3	148.0	1.9	184.1	3.4				
55.2	4.6	85.1	1.6	119.1	8.4	149.0	.8	185.1	2.5				
56.0	1.0	86.1	3.0	120.2	9.6	152.1	2.5	186.1	2.2				
57.1	1.6	87.2	1.9	121.1	15.3	153.0	3.9	187.1	28.7				
61.9	.8	89.1	2.3	122.2	5.6	154.0	2.5	188.1	5.3				
63.1	2.2	91.1	39.8	123.2	6.8	155.1	4.9	189.2	.8				
64.1	1.8	92.1	5.7	124.1	1.0	156.1	2.7	197.1	1.9				
65.0	10.7	93.1	22.6	127.1	4.9	157.1	8.4	198.2	1.9				
66.1	3.0	94.1	7.5	128.1	14.9	158.1	9.1	199.1	2.6				
67.1	5.0	95.1	24.6	129.0	18.1	159.1	47.3	200.1	1.0				
68.0	.8	96.1	4.2	130.0	4.7	160.1	9.0	201.1	57.4				
69.1	5.8	97.1	2.1	131.1	37.6	161.1	9.3	202.1	9.2				
70.0	1.1	98.0	.8	132.1	6.2	162.1	1.0	203.2	.8				
71.0	2.2	100.6	1.5	133.0	6.6	165.1	1.9	214.1	1.3				
72.1	1.2	102.0	1.9	134.1	3.2	167.2	1.1	215.2	4.1				
73.0	.8	103.1	5.2	135.1	4.3	168.1	3.0	216.1	100.0				
74.0	1.1	104.1	2.0	136.1	1.5	169.1	3.6	217.1	16.3				
75.1	3.8	105.1	28.1	137.1	.8	170.2	1.5	218.2	1.3				
76.1	2.3	106.1	4.3	139.1	1.6	171.1	4.2						
77.0	31.7	107.2	9.7	141.0	10.4	172.1	4.2						
78.1	7.4	108.1	20.2	142.0	6.4	173.1	21.0						
79.1	26.9	109.1	15.4	143.1	12.4	174.2	11.4						
80.1	4.3	110.1	2.7	144.1	5.1	175.1	2.0						

LAST 35

MASS	%	MASS	%	MASS	%	MASS	%	MASS	%	MASS	%	MASS	%
159.1	47.3	160.1	9.0	161.1	9.3	162.1	1.0	165.1	1.9	167.2	1.1	168.1	3.0
169.1	3.6	170.2	1.5	171.1	4.2	172.1	4.2	173.1	21.0	174.2	11.4	175.1	2.0
181.1	.8	182.1	.9	183.1	15.4	184.1	3.4	185.1	2.5	186.1	2.2	187.1	28.7
188.1	5.3	189.2	.8	197.1	1.9	198.2	1.9	199.1	2.6	200.1	1.0	201.1	57.4
202.1	9.2	203.2	.8	214.1	1.3	215.2	4.1	216.1	100.0	217.1	16.3	218.2	1.3

using a Continuous Percolator-Extractor (37) as described in the experimental section. Lipid contents, shown in Table 13, vary widely from 1.6% for P. flexuosa to 8.8% for P. homomalla. White crystals of diterpenoids were isolated from the extract by the method described by R. A. Gross (24). Table 14 shows that 6.235 g (equal to 0.61%) of yellow crystals were obtained from extract of P. REM St. Croix, 674 mg (equal to 0.09%) from P. REM San Cristobal, and none from the other three species of Plexaura. Column chromatography of the crystals from P. REM St. Croix gave one minor fraction and two major fractions, fraction I (51.64%, m.p. 109-111°C) and fraction II (37.64%, m.p. 120-121°C), in Table 14. The ir spectrum of diterpenoid I (Figure 25) showed a broad peak of O-H stretching at 3500-3400 cm^{-1}, C-H (vinyl) stretching to the left of 3000 cm^{-1}, strong C-H stretching around 3000 cm^{-1}, absorptions at 1720 cm^{-1} for C=O, at 1640 cm^{-1} for C=C, at 1460 cm^{-1} for methylene, at 1375 cm^{-1} for methyl, at 1275 cm^{-1}, and at 890 cm^{-1} for exomethylene group. The nmr spectrum (Figure 26) showed signals for methyl groups centered at δ1.02, a vinylic methyl at δ1.70, secondary alcohol, -C$\underline{H}$(OH)-, at δ3.40 and exomethylene group at δ4.70. The molecular weight, indicated by its mass spectrum (Figure 27), was 304. The ir and nmr spectra of this diterpenoid I were similar to a compound, labelled B-1 (Figure 28), already under investigation in this laboratory by Dr. R. A. Gross

Table 13

Lipid contents of *Plexaura* samples

Plexaura*	Dry weight sample (g)	Lipid content	
		g	%**
1. *P. REM* St. Croix	1025	39.67	3.87
2. *P. REM* San Cristobel	715	38.82	5.43
3. *P. kukenthali*	295	22.74	7.71
4. *P. homomalla*	100	8.85	8.85
5. *P. flexuosa*	62	1.02	1.64

*All *Plexaura* samples, except *P. REM* St. Croix, were collected at Cayo San Cristobal, Puerto Rico (see Table 18).

**gram total lipid/100 g dry weight animal.

Table 14

Diterpenoid fractions isolated from Plexaura samples

Plexaura	Diterpenoids								
	Total crystalline fraction			Diterpenoid I**			Diterpenoid II**		
	mg	%* in the sample	%* in the lipid	mg	% in total diter-penoids	% in the sample*	mg	% in total diter-penoids	% in the sample*
P. "REM", St. Croix	6235	0.61	15.72	3150	51.64	0.31	2296	37.64	0.23
P. "REM", San Cristobal	674	0.09	1.74	370	54.90	0.05	210	31.16	0.03

*Calculations based on the dry weight samples and lipid contents shown in Table 1.

**Diterpenoid I is similar to compound B-1 of P. "REM" Bonaire.
Diterpenoid II is similar to compound B-3 of P. "REM" Bonaire.

% = g diterpenoid I or II/100 g of total diterpenoid crystals.

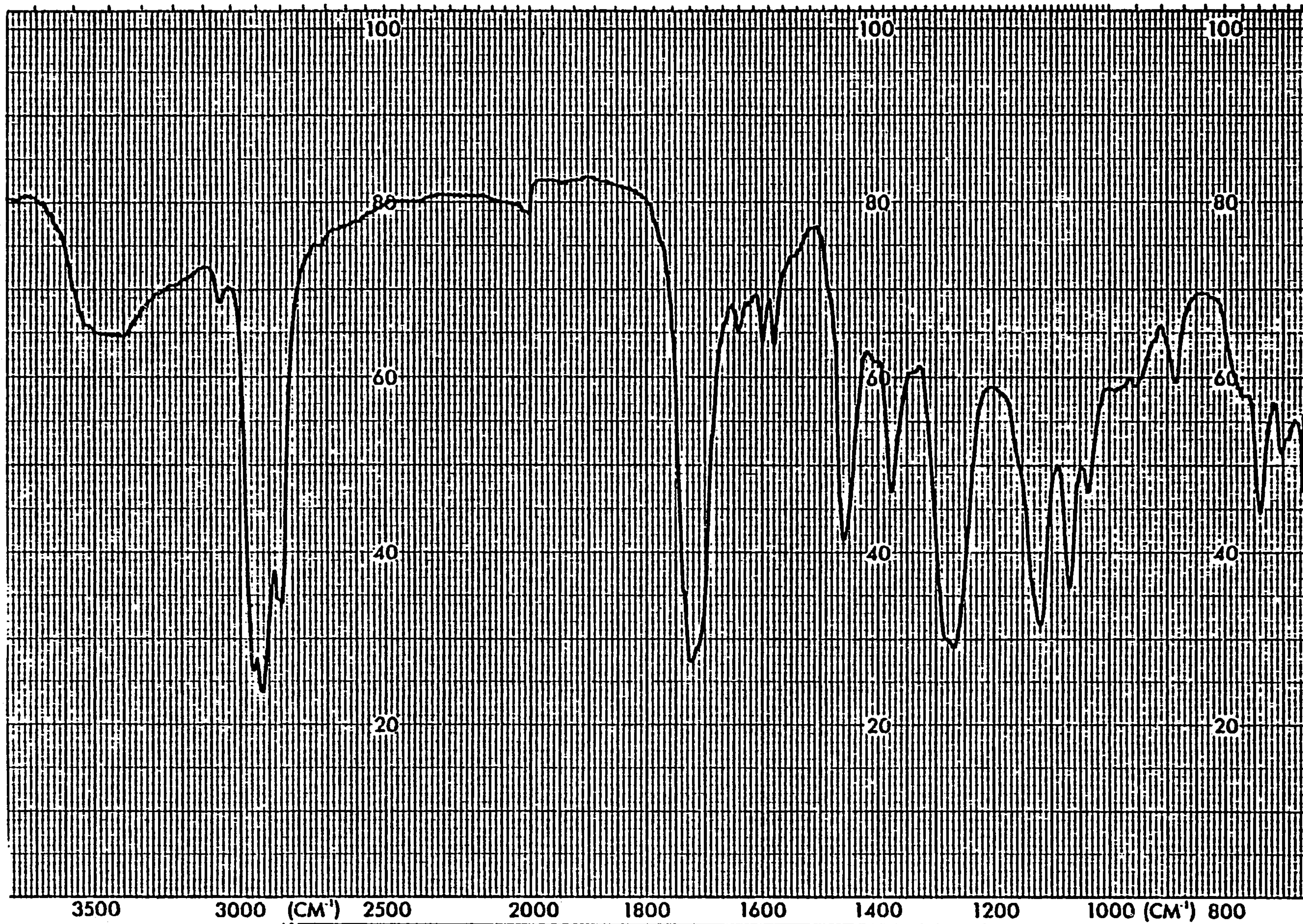


Figure 25. The ir spectrum of fraction I (diterpenoid I) isolated from Plexaura REM, St. Croix.

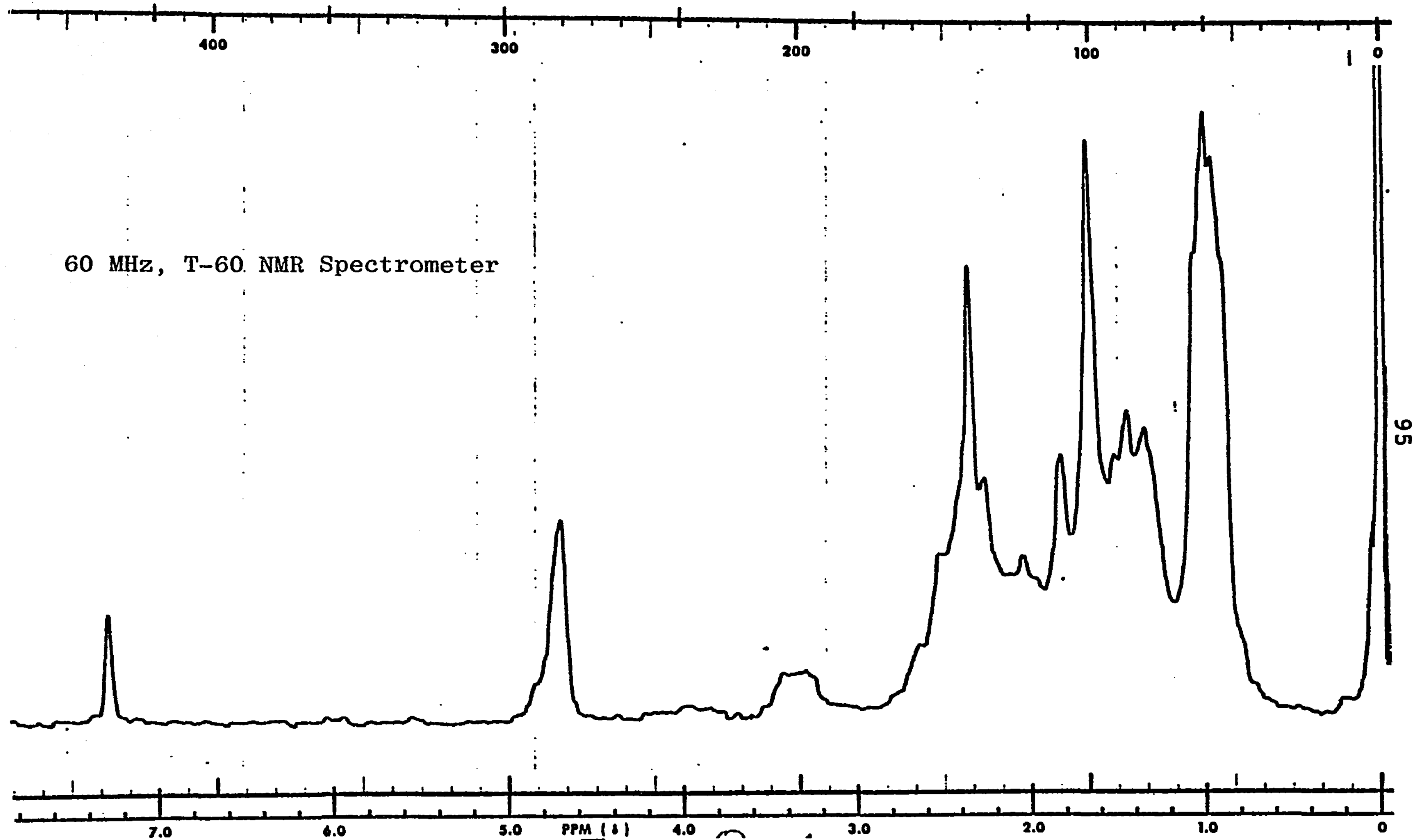


Figure 26. The nmr spectrum of fraction I (diterpenoid I) isolated from Plexaura REM, St. Croix.

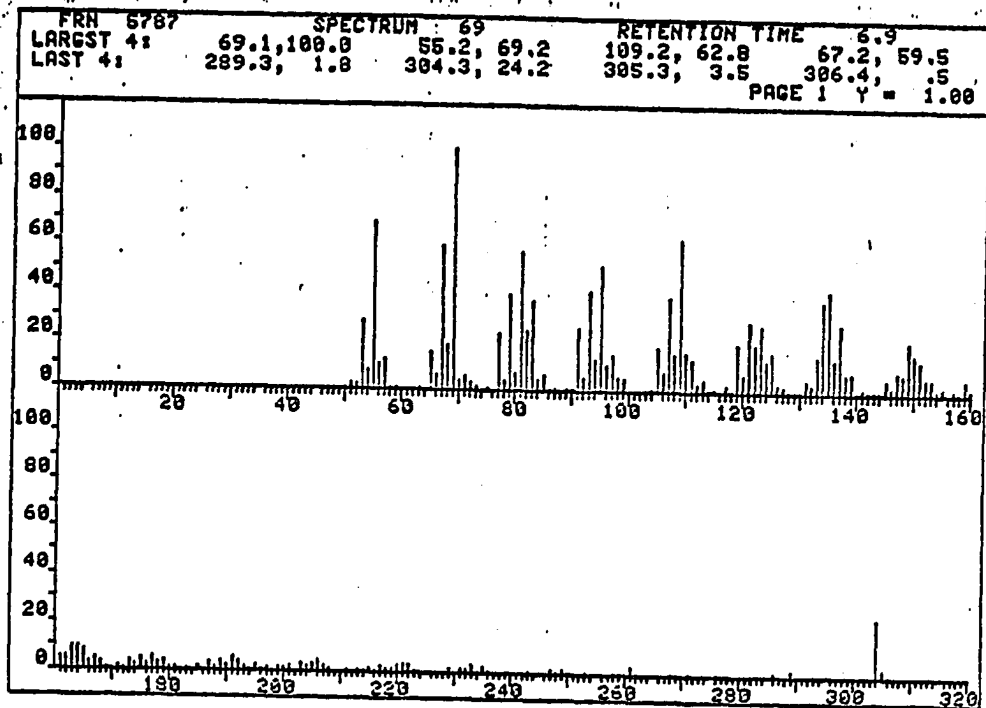


Figure 27. The mass spectrum of fraction I (diterpenoid I) isolated from Plexaura REM, St. Croix.

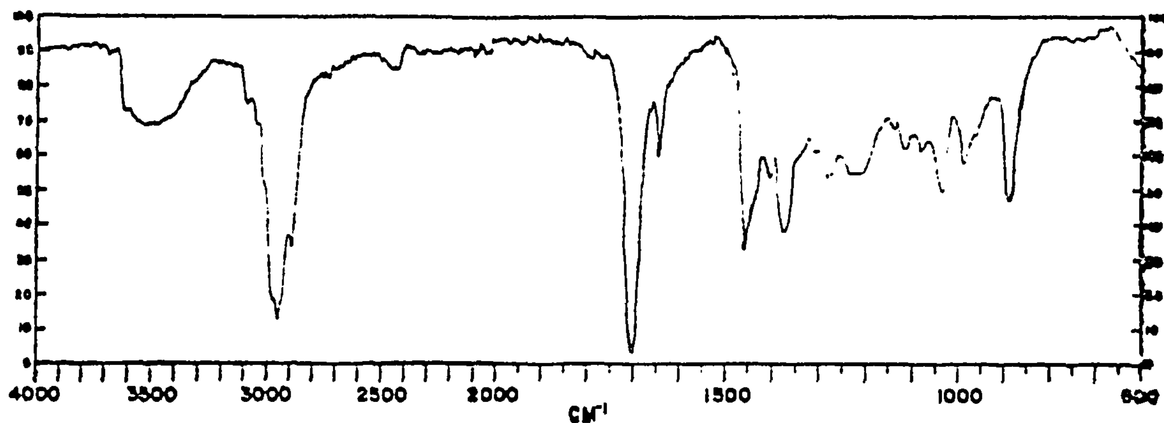
FILE 5787 SPECTRUM: 69 178 PEAKS RT= 6.93 MIN BASE PEAK= 2655

LARGEST 178

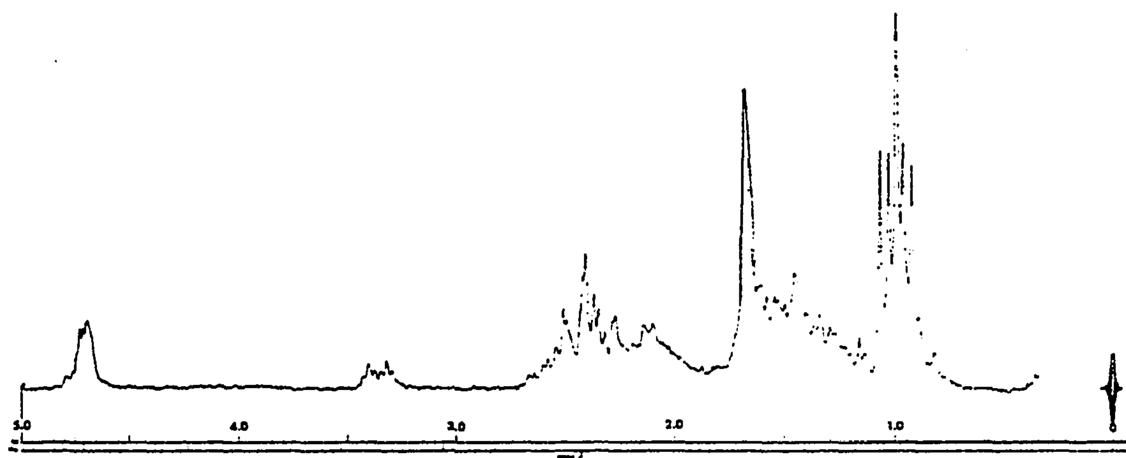
MASS	%	MASS	%	MASS	%	MASS	%	MASS	%	MASS	%
50.1	.4	82.2	25.3	112.2	3.6	139.2	8.6	166.1	3.1	195.2	2.1	231.2	1.0
51.2	2.7	83.2	36.9	113.2	5.1	140.2	.8	167.2	4.4	196.1	.4	232.2	1.4
52.3	2.2	84.2	5.3	114.3	.8	141.0	1.9	168.2	2.5	197.1	.9	233.2	2.4
53.2	28.1	85.2	6.4	115.2	1.2	142.0	.4	169.1	.7	199.2	1.0	234.3	.9
54.2	7.8	86.2	1.0	117.2	2.6	143.1	.9	171.1	1.0	200.1	.9	235.3	2.0
55.2	69.2	87.1	.5	118.2	.9	144.2	.6	172.3	.4	201.2	2.2	236.1	.8
56.2	10.7	89.1	.5	119.2	19.3	145.2	5.4	173.2	4.1	203.2	3.2	243.2	.9
57.2	11.9	90.2	.7	120.2	7.3	146.2	2.7	174.2	2.1	204.0	2.2	247.3	1.7
58.1	1.2	91.2	25.5	121.2	29.1	147.2	9.3	175.2	4.9	205.2	3.3	248.2	.7
59.2	.9	92.2	5.6	122.2	19.5	148.2	8.5	176.2	2.3	206.1	4.6	249.2	1.4
63.1	.4	93.2	40.7	123.3	27.3	149.1	21.0	177.2	5.6	207.2	2.4	253.3	.4
65.1	15.7	94.2	13.2	124.3	12.9	150.2	16.2	178.2	2.4	208.1	1.0	258.3	.5
66.1	6.2	95.2	51.6	125.1	16.4	151.2	13.2	179.3	3.8	210.7	.4	261.2	3.2
67.2	59.5	96.2	10.6	126.3	3.6	152.2	6.5	180.2	1.5	211.1	.5	262.2	.6
68.2	18.6	97.2	14.7	127.2	2.2	153.1	5.6	181.2	1.1	213.2	.8	268.3	.5
69.1	100.0	98.2	5.5	128.2	.7	154.2	1.6	182.2	.8	214.2	.5	271.1	.4
70.2	4.2	99.2	5.2	129.0	.7	155.2	2.5	183.3	.5	215.3	1.4	276.3	.6
71.2	5.5	103.1	1.1	129.8	.5	157.3	1.4	185.1	1.1	216.1	.5	286.1	1.1
72.2	3.5	104.2	.4	131.2	4.6	158.1	1.1	187.3	2.6	217.3	1.9	289.3	1.8
73.1	1.5	105.2	17.7	132.1	3.4	159.2	6.0	188.2	1.6	218.1	1.1	304.3	24.2
75.1	.4	106.2	8.4	133.1	14.5	160.2	1.3	189.3	4.1	219.2	1.5	305.3	3.5
77.1	23.2	107.2	39.0	134.1	37.7	161.2	4.7	190.3	2.0	220.1	2.9	306.4	.5
78.1	4.0	108.2	15.7	135.1	42.0	162.3	5.6	191.2	5.2	221.2	3.2		
79.2	39.8	109.2	62.8	136.1	13.6	163.2	9.2	192.2	3.6	222.2	3.1		
80.2	7.4	110.2	15.9	137.1	28.5	164.2	9.4	193.2	1.7	223.3	.7		
81.1	56.9	111.2	13.3	138.1	8.6	165.2	7.3	194.0	.5	229.2	1.5		

LAST 35

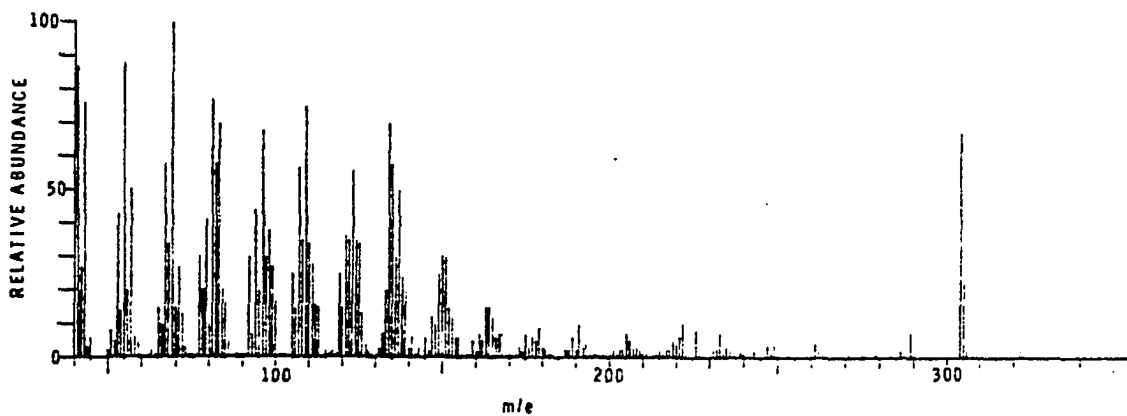
MASS	%	MASS	%	MASS	%	MASS	%	MASS	%	MASS	%
211.1	.5	213.2	.8	214.2	.5	215.3	1.4	216.1	.5	217.3	1.9	218.1	1.1
219.2	1.5	220.1	2.9	221.2	3.2	222.2	3.1	223.3	.7	229.2	1.5	231.2	1.0
232.2	1.4	233.2	2.4	234.3	.9	235.3	2.0	236.1	.8	243.2	.9	247.3	1.7
248.2	.7	249.2	1.4	253.3	.4	258.3	.5	261.2	3.2	262.2	.6	268.3	.5
271.1	.4	276.3	.6	286.1	1.1	289.3	1.8	304.3	24.2	305.3	3.5	306.4	.5



The ir spectrum of compound B-1



The nmr spectrum of compound B-1 (100 MHz, Varian XL-100 Spectrometer).



The mass spectrum of compound B-1

Figure 28. The ir, nmr, and mass spectra of compound B-1 (24)

(24). However, one significant difference is the appearance of relatively stronger peaks at 1120 cm^{-1} and 1270 cm^{-1} in the ir spectrum of diterpenoid I. Its mw, 304, was 18 units less than that of compound B-1. Compound B-1 had been identified as a diterpene containing non-conjugated, unsaturated ketone, with molecular formula $C_{20}H_{34}O_3$. It was suggested that compound B-1 arose in nature from geranyl-geranyl pyrophosphate, the normal diterpenoid precursor (32,33) by a single cyclization, and hence a cembrane skeleton, was suggested as a ring system for B-1. One of the many possible structures for B-1 and its proposed biosynthetic sequence is shown in Figure 29. The possible molecular formula for fraction I (diterpenoid I) is $C_{20}H_{32}O_2$.

The ir spectrum of diterpenoid II (Figure 30) showed a large O-H band around 3500 cm^{-1}, C-H (vinyl) stretching to the left of 3000 cm^{-1}, absorption to the right of 3000 cm^{-1} for C-H, absorptions at 1700 cm^{-1} for carbonyl group, at 1460 cm^{-1} for methylene, at 1370 cm^{-1} for methyl, at 1640 cm^{-1}, and at 1270 cm^{-1}. The nmr spectrum (Figure 31) showed absorptions at δ1.00, δ1.70, δ3.40, and δ4.70. The mass spectrum of diterpenoid II (Figure 32) gave a molecular weight of 306. The characteristics of both ir and nmr spectra of diterpenoid II show similarity to a compound, labelled B-3 (Figure 33), already isolated

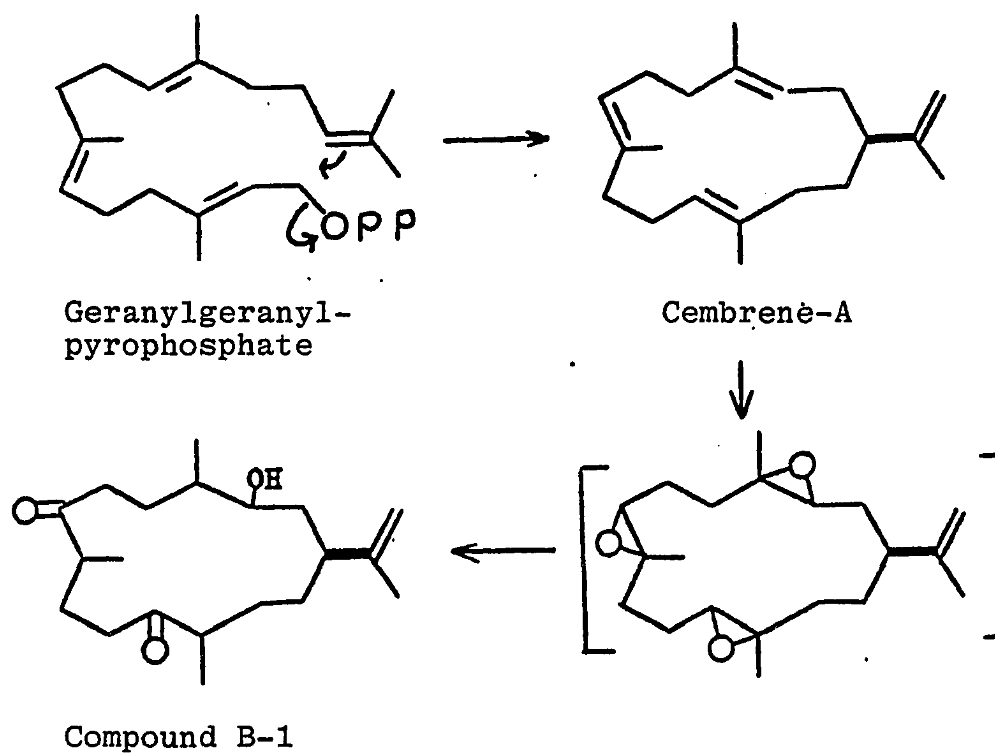
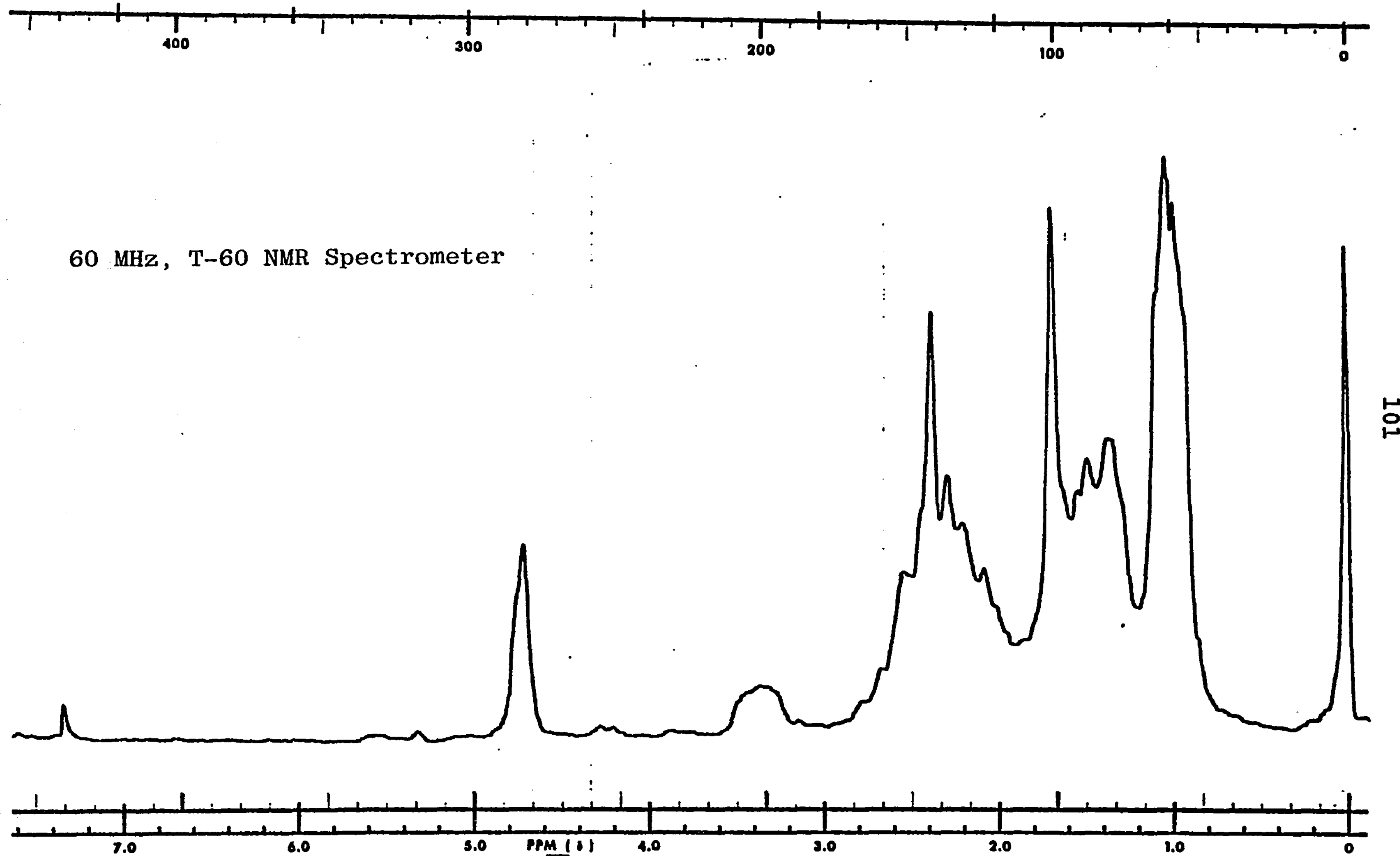


Figure 29. A proposed biosynthetic pathway for compound B-1 (24).

Figure 30. The ir spectrum of fraction II (diterpenoid II) isolated from *Plexaura* REM, St. Croix.



<u>Figure 31</u>. The nmr spectrum of fraction II (diterpenoid II) isolated from <u>Plexaura</u> <u>REM</u>, St. Croix.

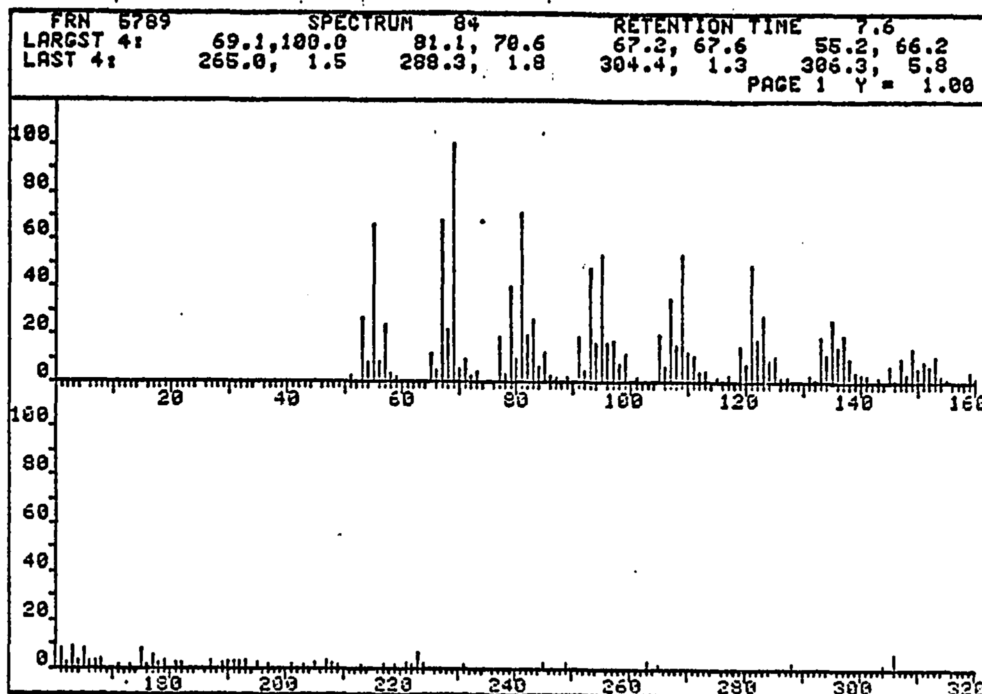


Figure 32. The mass spectrum of fraction II (diterpenoid II) isolated from Plexaura REM, St. Croix.

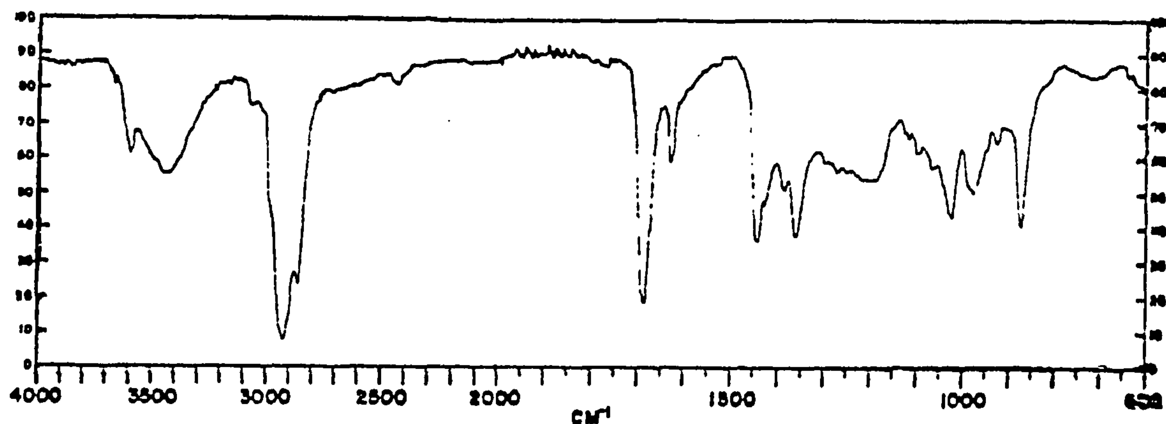
FILE 5789 SPECTRUM 84 132 PEAKS RT= 7.58 MIN BASE PEAK= 970

LARGEST 132

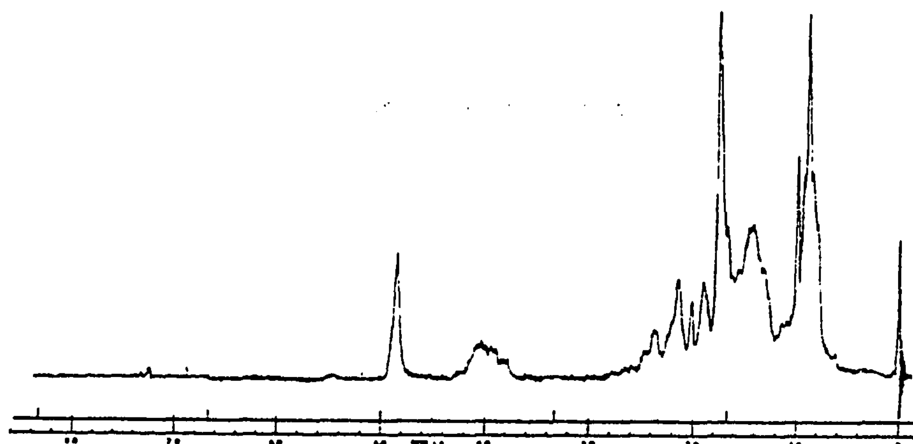
MASS	%	MASS	%	MASS	%	MASS	%	MASS	%	MASS	%	MASS	%
51.1	2.4	86.1	2.6	119.2	14.6	150.2	6.0	189.4	2.4	304.4	1.3		
53.1	26.9	87.3	1.4	120.3	7.4	151.1	8.0	190.2	2.6	306.3	5.8		
54.1	7.8	88.8	1.4	121.2	49.4	152.2	6.5	191.2	2.8				
55.2	66.2	91.2	18.8	122.2	17.8	153.2	10.2	192.1	2.7				
56.2	7.8	92.2	4.8	123.3	27.8	154.2	2.2	193.2	2.8				
57.1	23.6	93.2	47.8	124.3	9.2	155.3	1.2	195.3	2.1				
58.3	3.1	94.1	15.3	125.1	10.4	159.1	4.1	197.3	1.1				
59.1	1.3	95.2	53.3	126.1	1.9	160.2	1.6	200.9	1.0				
65.1	11.6	96.2	15.8	127.2	1.6	161.2	7.9	203.2	1.4				
66.2	5.3	97.1	17.2	131.0	2.6	162.1	1.9	205.3	2.2				
67.2	67.6	98.1	7.1	132.0	1.2	163.2	8.8	207.0	3.0				
68.2	21.9	99.1	11.2	133.2	18.8	164.1	3.4	208.3	2.4				
69.1	100.0	100.1	1.2	134.1	10.9	165.3	7.9	209.0	1.0				
70.0	5.5	101.1	1.3	135.1	25.5	166.2	2.7	213.1	.9				
71.2	9.1	105.2	19.5	136.1	14.9	167.2	2.7	217.0	1.5				
72.2	2.5	106.2	6.4	137.1	19.7	168.2	3.8	219.3	1.0				
73.2	4.2	107.2	34.5	138.2	10.1	171.1	1.1	221.3	1.8				
77.1	19.0	108.2	15.2	139.2	4.2	173.2	1.0	222.3	1.1				
78.2	3.7	109.2	52.8	140.2	2.9	175.2	7.5	223.2	6.3				
79.2	39.8	110.2	12.2	141.1	2.5	176.2	1.6	224.2	2.4				
80.2	9.8	111.2	10.4	143.0	1.3	177.2	5.5	231.3	1.0				
81.1	70.6	112.3	4.2	145.1	6.2	178.2	2.0	245.3	1.8				
82.1	19.7	113.3	3.9	146.1	1.2	179.2	3.1	249.2	2.0				
83.2	26.1	115.2	1.4	147.2	9.9	181.2	2.2	263.2	2.7				
84.2	6.3	116.2	1.1	148.2	3.6	182.2	2.1	265.0	1.5				
85.0	11.8	117.1	2.3	149.2	13.7	187.2	2.9	288.3	1.8				

LAST 35

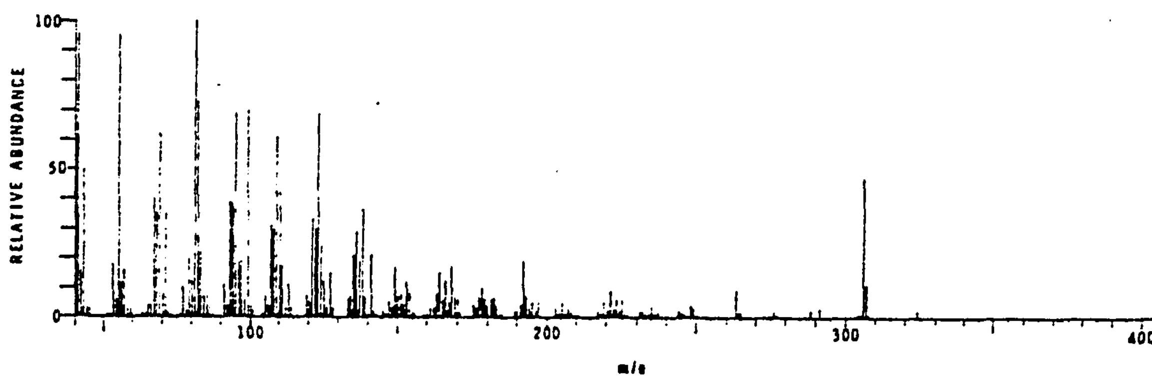
176.2	1.6	177.2	5.5	178.2	2.0	179.2	3.1	181.2	2.2	182.2	2.1	187.2	2.9
189.4	2.4	190.2	2.6	191.2	2.8	192.1	2.7	193.2	2.8	195.3	2.1	197.3	1.1
200.9	1.0	203.2	1.4	205.3	2.2	207.0	3.0	208.3	2.4	209.0	1.0	213.1	.9
217.0	1.5	219.3	1.0	221.3	1.8	222.3	1.1	223.2	6.3	224.2	2.4	231.3	1.0
245.3	1.8	249.2	2.0	263.2	2.7	265.0	1.5	288.3	1.8	304.4	1.3	306.3	5.8



The ir spectrum of compound B-3



The nmr spectrum of compound B-3
(100 MHz, Varian XL-100 Spectrometer).



The mass spectrum of compound B-3

Figure 33. The ir, nmr, and mass spectra of compound B-3 (24).

in this laboratory by Dr. R. A. Gross (24). However, one significant difference is the substitution of the nmr peak at $\delta 4.0$ in compound B-3 by the nmr peak at $\delta 3.4$ in the spectrum of diterpenoid II. The molecular weight of diterpenoid II was 18 units less than that of compound B-3. The mobility of fraction II (R_f 0.53) on tlc (20 x 20 cm plate, 0.25 mm thick of Silica Gel H, 2% methanol in chloroform as eluting solvent) was lower than that of fraction I (R_f 0.83). Compound B-3 was identified as a diterpenoid dihydroxyketone with molecular formula of $C_{20}H_{36}O_3$. The possible molecular formula for fraction II (diterpenoid II) is $C_{20}H_{34}O_2$.

Column chromatography of 674 mg crystals isolated from _P_. _REM_ San Cristobal gave 370 mg of fraction I (equal to 54.9%) and 210 mg of fraction II (equal to 31.16%). The ir spectra of diterpenoids I and II (Figures 34 and 37) showed O-H absorptions around 3500 cm^{-1} (sharp band for diterpenoid I, and broad band for diterpenoid II), C-H (vinyl) stretching to the left of 3000 cm^{-1}, C-H absorptions to the right of 3000 cm^{-1}, carbonyl absorption around 1700 cm^{-1}, methyl absorptions at 1370 cm^{-1}, absorptions at 1040 cm^{-1}, and 900 cm^{-1}. The nmr spectra of diterpenoids I and II (Figures 35 and 38) showed absorptions at $\delta 0.95$, $\delta 1.70$, $\delta 2.30$ and $\delta 4.70$. The mass spectra of diterpenoid I (Figure 36) and diterpenoid II (Figure 39) indicated mw 304 and 306, respectively.

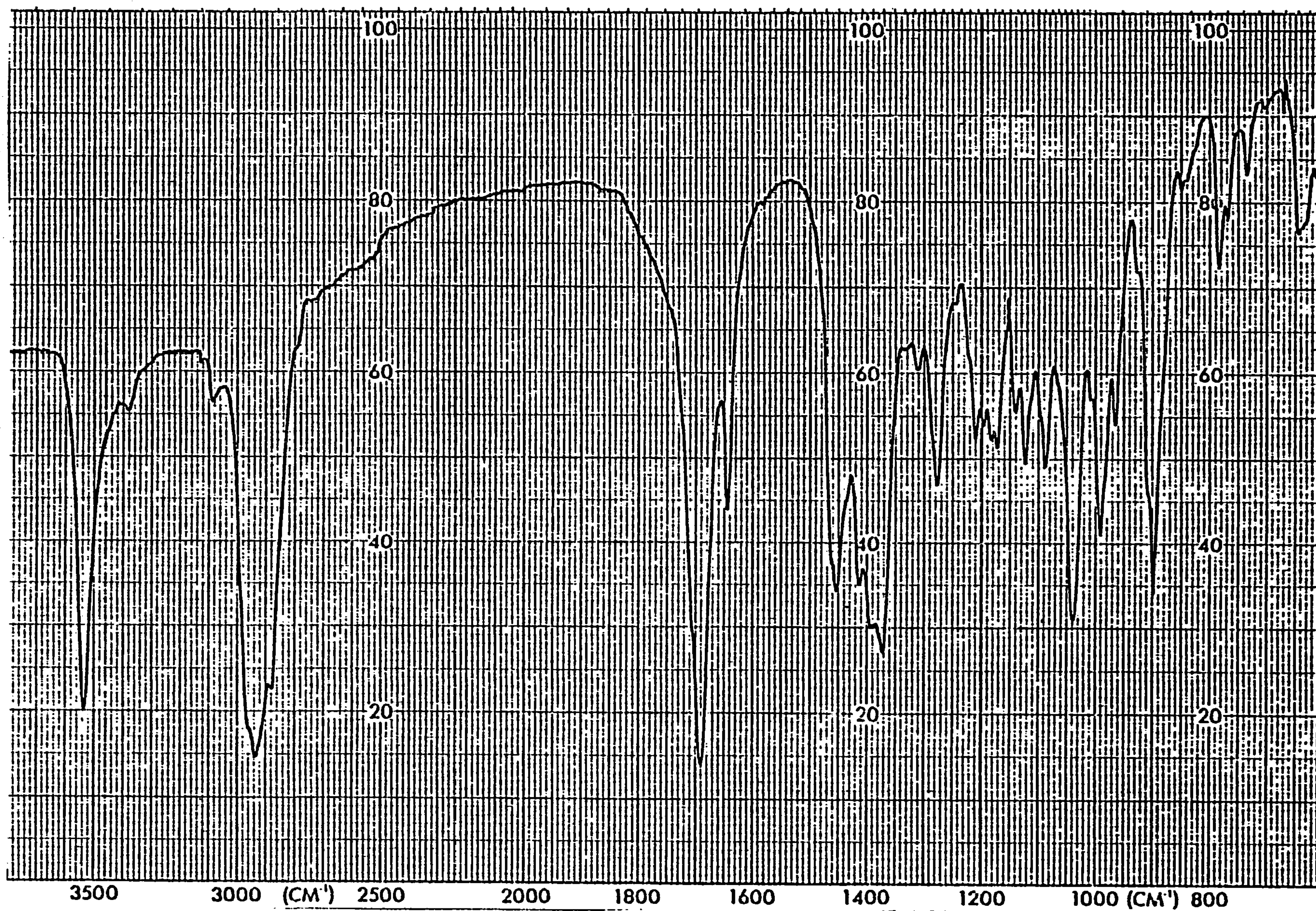


Figure 34. The ir spectrum of fraction I (diterpenoid I) isolated from *Plexaura* REM, San Cristobal.

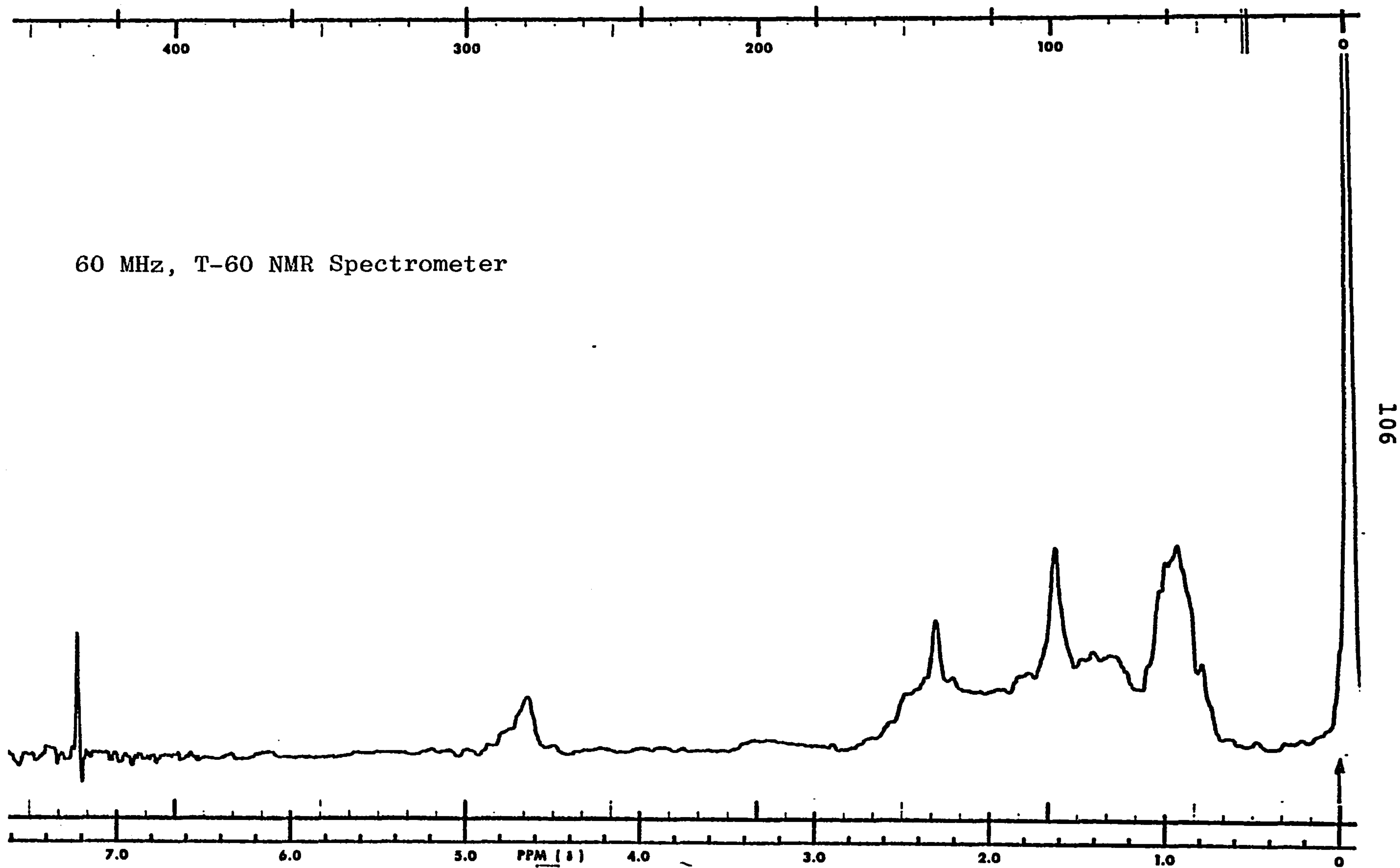


Figure 35. The nmr spectrum of fraction I (diterpenoid I) isolated from *Plexaura* REM, San Cristobal.

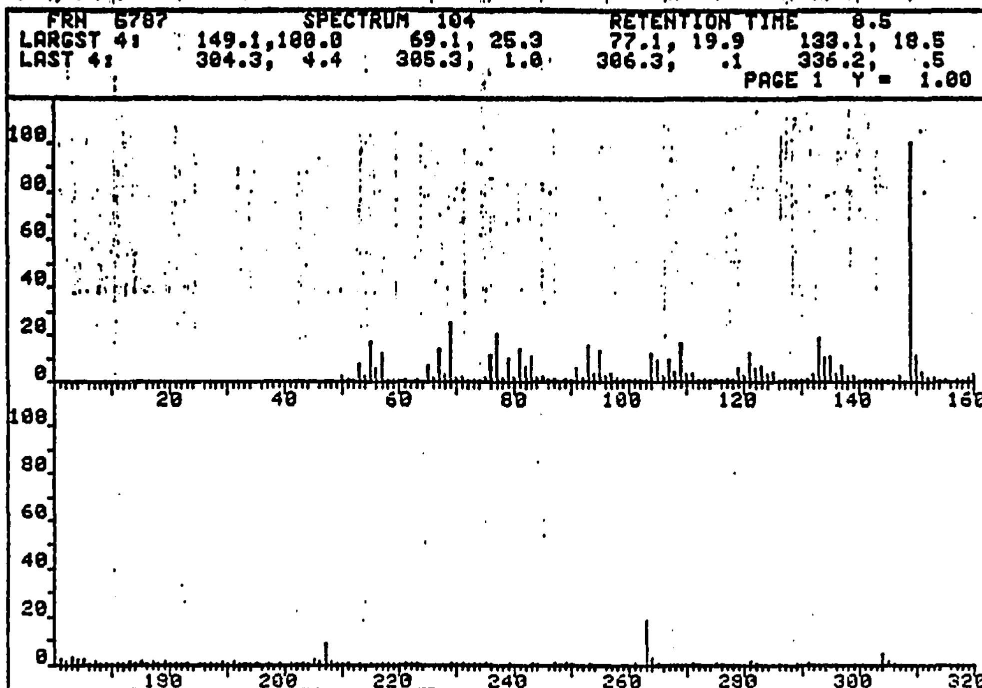


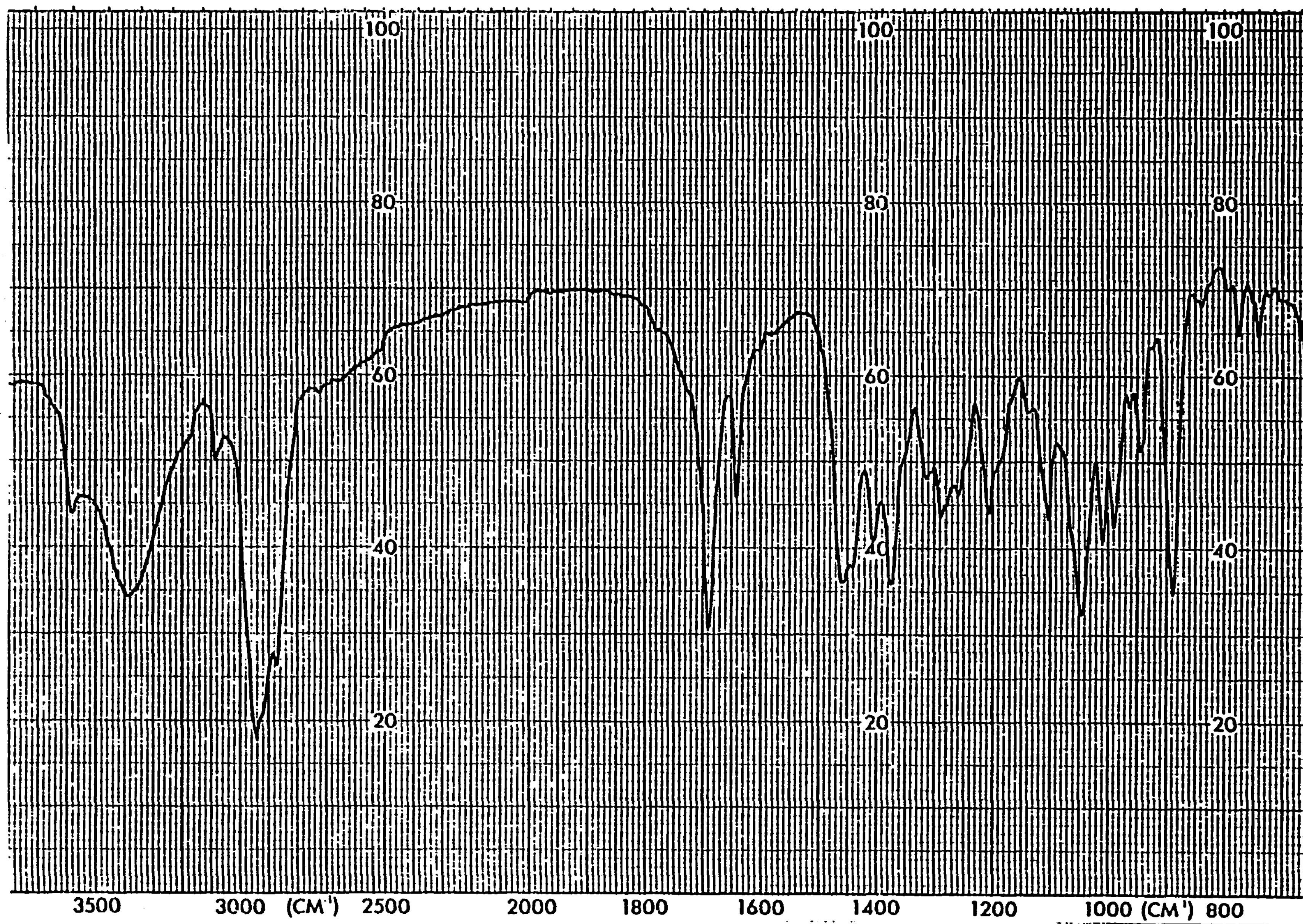
Figure 36. The mass spectrum of fraction I (diterpenoid I) isolated from Plexaura REM, San Cristobal

FILE 5787 SPECTRUM 104 196 PEAKS RT= 8.47 MIN BASE PEAK= 11214
LARGEST 182

MASS	%	MASS	%	MASS	%	MASS	%	MASS	%	MASS	%	MASS	%
50.1	2.4	78.1	1.8	106.2	2.1	132.1	3.8	162.2	1.6	189.2	1.1	221.2	.9
51.1	1.1	79.1	9.5	107.2	9.2	133.1	18.5	163.2	2.6	190.3	.6	222.3	.8
52.3	.8	80.2	1.8	108.2	4.1	134.1	10.6	164.2	2.0	191.3	1.0	230.3	.1
53.1	7.3	81.1	14.2	109.2	15.9	135.1	10.6	165.2	1.8	192.2	.7	231.3	.2
54.1	2.4	82.2	6.5	110.2	3.7	136.2	3.6	166.2	.6	193.2	.6	232.1	.5
55.2	17.3	83.2	10.6	111.2	3.6	137.1	6.5	167.1	1.1	194.1	.1	233.2	.6
56.2	5.8	84.2	1.7	112.2	.8	138.2	2.1	168.2	.4	195.1	.5	234.3	.2
57.2	11.7	85.2	1.4	113.2	1.3	139.2	2.5	169.2	.2	197.1	.2	235.2	.4
58.2	.9	86.2	.3	114.2	.2	140.2	.4	170.3	.1	199.1	.2	236.0	.1
59.2	.5	87.2	.4	115.2	.4	141.2	.6	171.2	.2	201.2	.3	243.2	.2
60.2	.2	90.2	.2	116.2	.1	142.2	.1	172.2	.2	202.4	.2	247.3	.4
61.1	.2	91.2	5.9	117.2	.6	145.2	1.1	173.3	1.0	203.2	.6	248.2	.4
63.1	.3	92.2	1.6	118.2	.3	146.1	.6	174.3	.3	204.2	.7	249.2	.3
65.1	6.2	93.1	15.4	119.2	5.5	147.2	2.4	175.2	1.1	205.2	2.4	250.1	.2
66.1	1.8	94.2	2.8	120.2	2.1	149.1	100.0	176.2	.6	206.3	1.1	261.2	.4
67.2	14.1	95.2	12.6	121.2	12.4	150.1	11.2	177.2	1.2	207.1	8.6	263.2	18.1
68.2	3.4	96.2	2.5	122.2	5.7	151.1	4.4	178.3	.6	209.1	1.2	264.1	3.1
69.1	25.3	97.1	3.5	123.3	6.5	152.2	1.4	179.2	1.1	209.1	.4	265.2	.2
70.1	1.8	98.1	1.9	124.2	2.9	153.2	1.5	180.3	.3	211.3	.1	275.3	.2
71.2	1.4	99.2	1.1	125.2	4.2	154.2	.4	181.1	.3	213.0	.2	281.1	.9
72.1	.7	100.2	.2	126.2	1.0	155.2	.2	182.1	.2	215.2	.2	282.3	.1
73.1	.9	101.0	.2	127.4	.4	157.2	1.1	183.3	.3	216.2	.1	286.3	.2
74.1	.8	102.1	.1	128.2	.2	158.1	.3	185.2	.2	217.3	.3	289.2	.4
75.1	1.5	103.1	1.1	129.0	.4	159.1	1.4	186.1	.2	218.2	.4	304.3	4.4
76.1	11.5	104.2	11.8	130.1	.2	160.1	3.3	187.2	.3	219.2	.5	305.3	1.0
77.1	19.9	105.1	9.4	131.1	.9	161.1	2.2	188.2	.4	220.1	.4	336.2	.5

LAST 35

221.2	.9	222.3	.8	223.1	.1	229.3	.1	230.3	.1	231.3	.2	232.1	.5
233.2	.6	234.3	.2	235.2	.4	236.0	.1	239.2	.1	243.2	.2	244.3	.1
247.3	.4	248.2	.4	249.2	.3	250.1	.2	253.3	.1	261.2	.4	263.2	18.1
264.1	3.1	265.2	.2	271.2	.1	275.3	.2	276.1	.1	281.1	.9	282.3	.1
286.3	.2	289.2	.4	291.3	.1	304.3	4.4	305.3	1.0	306.3	.1	336.2	.5



<u>Figure 37</u>. The ir spectrum of fraction II (diterpenoid II) isolated from <u>Plexaura</u> <u>REM</u>, San Cristobal.

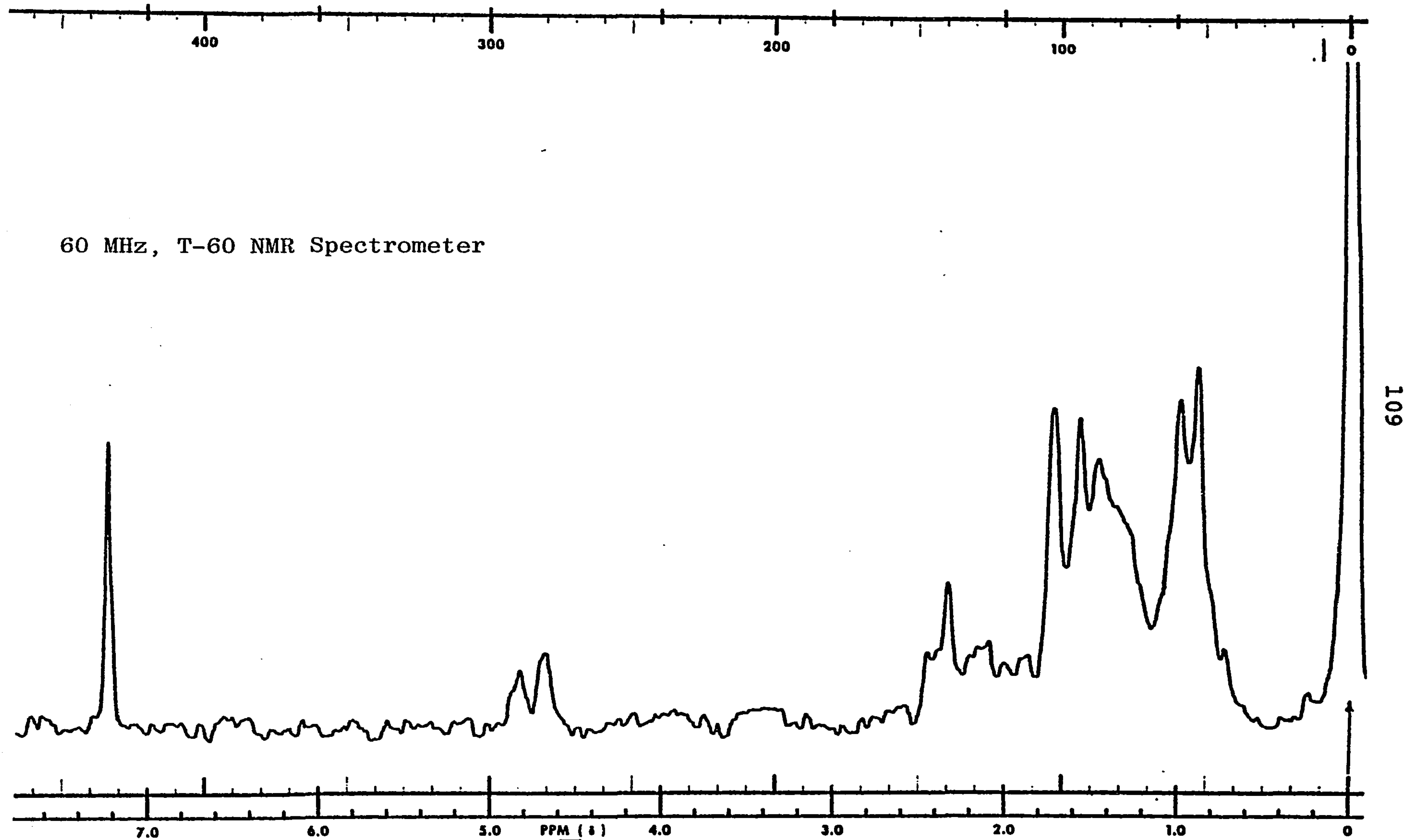


Figure 38. The nmr spectrum of fraction II (diterpenoid II) isolated from *Plexaura* REM, San Cristobal.

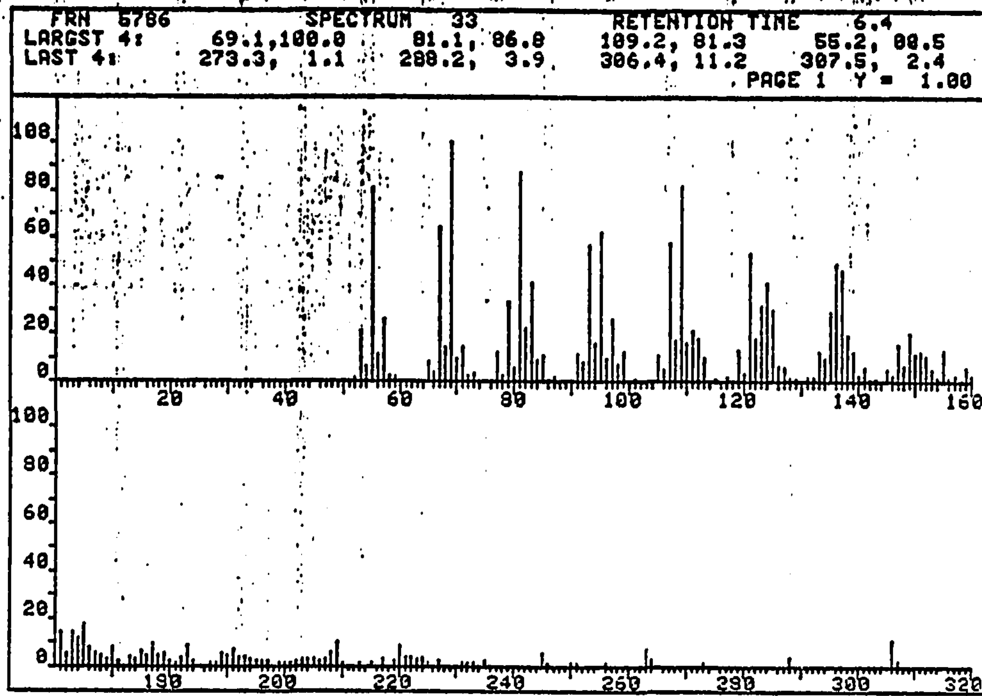


Figure 39. The mass spectrum of fraction II (diterpenoid II) isolated from Plexaura REM, San Cristobal.

FILE 5786 SPECTRUM 33 161 PEAKS RT= 6.42 MIN BASE PEAK= 970

LARGEST 161

MASS	%	MASS	%	MASS	%	MASS	%	MASS	%	MASS	%	MASS	%
50.1	.9	85.2	10.4	121.2	52.8	149.2	20.3	177.2	9.2	206.2	2.0	264.3	2.6
52.1	1.3	86.2	1.1	122.3	18.2	150.2	11.1	178.2	4.9	207.1	3.0	273.3	1.1
53.2	20.9	87.1	1.3	123.2	31.3	151.2	12.2	179.2	5.1	208.1	6.3	288.2	3.9
54.2	6.7	91.2	11.0	124.3	41.1	152.2	10.6	180.2	2.2	209.3	10.0	306.4	11.2
55.2	80.5	92.2	7.8	125.2	29.7	153.2	4.6	181.3	1.5	210.3	1.6	307.5	2.4
56.2	11.5	93.2	56.3	126.3	6.6	154.2	1.6	182.2	4.0	213.1	1.2		
57.2	25.8	94.2	16.2	127.2	5.5	155.1	12.5	183.2	8.9	215.3	1.6		
58.1	2.1	95.2	62.1	128.1	1.5	156.1	1.3	184.1	1.9	217.3	2.6		
59.2	1.9	96.2	9.5	129.1	1.1	157.0	2.1	187.3	1.6	219.3	1.8		
65.1	8.5	97.2	25.5	131.2	1.8	159.3	5.9	188.1	1.4	220.3	8.5		
66.1	4.0	98.1	7.3	132.2	1.8	160.2	2.3	189.2	5.5	221.2	4.2		
67.1	64.7	99.1	12.0	133.2	12.2	161.2	14.0	190.3	4.9	222.3	4.1		
68.2	14.8	101.1	1.0	134.1	9.9	162.3	5.7	191.3	7.1	223.2	2.5		
69.1	100.0	105.1	10.7	135.2	29.4	163.2	14.5	192.2	3.9	224.1	3.8		
70.1	9.6	106.2	4.9	136.2	49.4	164.2	12.0	193.3	4.2	225.2	1.0		
71.2	14.6	107.2	57.5	137.2	46.3	165.2	16.9	194.2	3.5	227.4	1.9		
72.3	2.2	108.2	17.2	138.2	19.7	166.1	7.5	195.3	2.4	231.2	1.6		
72.9	3.2	109.2	81.3	139.2	12.3	167.2	5.2	196.2	1.9	232.3	1.6		
77.1	12.3	110.2	16.0	140.2	2.7	168.2	4.7	197.2	1.8	233.4	1.0		
78.1	2.3	111.2	20.9	141.1	6.0	169.2	2.5	199.2	1.1	235.1	'1.9		
79.2	33.4	112.2	17.8	142.1	1.1	170.2	7.8	200.2	1.3	245.2	5.2		
80.1	6.1	113.1	9.5	143.0	1.2	171.3	2.1	201.2	1.6	246.2	1.3		
81.1	86.8	115.1	1.0	145.2	5.1	173.1	4.2	202.2	1.8	250.3	1.3		
82.1	23.1	117.1	1.4	146.3	2.6	174.1	2.5	203.2	3.4	251.1	1.0		
83.1	41.1	119.2	12.5	147.1	15.3	175.2	6.3	204.1	3.2	256.3	1.1		
84.2	9.1	120.2	3.0	148.2	6.3	176.2	4.5	205.3	2.6	263.2	6.6		

LAST 35

202.2	1.8	203.2	3.4	204.1	3.2	205.3	2.6	206.2	2.0	207.1	3.0	208.1	6.3
209.3	10.0	210.3	1.6	213.1	1.2	215.3	1.6	217.3	2.6	219.3	1.8	220.3	8.5
221.2	4.2	222.3	4.1	223.2	2.5	224.1	3.8	225.2	1.0	227.4	1.9	231.2	1.6
232.3	1.6	233.4	1.0	235.1	1.9	245.2	5.2	246.2	1.3	250.3	1.3	251.1	1.0
256.3	1.1	263.2	6.6	264.3	2.6	273.3	1.1	288.2	3.9	306.4	11.2	307.5	2.4

Comparison of diterpenoid components of Plexaura samples under my investigation with those studied previously, shows that the total diterpenoid content (Table 14) of P. "REM" St. Croix (0.61%) (g diterpenoids/100 g dry weight animal) is about the same as that obtained from P. "REM" Bonaire (0.69%) studied by Gross (24). However, P. "REM" San Cristobal contained much less diterpenoids (0.09%) (Table 14). The percentage of the two diterpenoid fractions in P. "REM" Bonaire (24), B-1 (0.32%) and B-3 (0.37%), were about the same, while in P. "REM" St. Croix, the fraction similar to B-1 is significantly higher (0.31%) than that of the fraction similar to B-3 (0.23%). In P. "REM" San Cristobal, the percentages are 0.05% and 0.03%, for diterpenoids I and II, respectively. It is noteworthy that P. "REM" species collected during warmer seasons of the year (P. "REM" St. Croix in April and P. "REM" Bonaire in June) contain much higher diterpenoid compounds than P. "REM" collected during cooler seasons (P. "REM" San Cristobal in February).

Cetyl Palmitate from Five Plexaura Samples

The lipid solution, separated from the diterpenoid crystals, was subjected to urea adduction (35,36) to isolate the non-branched long chain lipids. P. "REM" St. Croix yielded 410 mg of wax ester (0.04%, from 1.025 kg dry weight sample) (Table 15) when chromatographed on Florisil with hexane-ether (9:1, v/v) as eluting solvent. It was identified as cetyl

palmitate by tlc (R_f 0.8, 0.25 mm thick Silica Gel H, benzene as eluting solvent, or R_f 0.24 when 5% chloroform in hexane was used as eluting solvent), ir (Figure 40) and nmr (Figure 41). *P.* "*REM*" from San Cristobal yielded 800 mg of cetyl palmitate (0.11%, from 715 g sample) (Figures 42 and 43). *P. kukenthali* yielded 485 mg cetyl palmitate (0.16%, from 295 g sample) (Figures 44 and 45). A sample of 100 g *P. homomalla* gave 8.85 g total lipid (Table 13), yielding 125 mg of solid recovered from the urea adduct. Five mg of material (equal to 0.005% of sample) identified as cetyl palmitate, was obtained when the solid was chromatographed on Florisil with hexane-ether (9:1, v/v) as eluting solvent. No cetyl palmitate was obtained from 1.02 g lipids extracted from 62 g dry weight sample of *P. flexuosa* using the same procedure described above. *P. flexuosa* from San Cristobal yielded 1.64% lipid. This value is lower than that of *P. flexuosa* from Bermuda studied earlier by Dr. L. S. Ciereszko (15), 3%, which also contained octadecyl alcohol and butyl alcohol (16,17) and a minute quantity of sesquiterpene hydrocarbon structure (0.01%).

Table 16 compares lipid and cetyl palmitate contents of eggs of *Pseudopterogorgia americana* and *Plexaura* samples under my investigation with those of gorgonians previously studied: the total lipid and the percentage of cetyl palmitate in the lipid, for *Plexaura* samples are much less (1.6-8.8% lipids, <1-2% cetyl palmitate) than

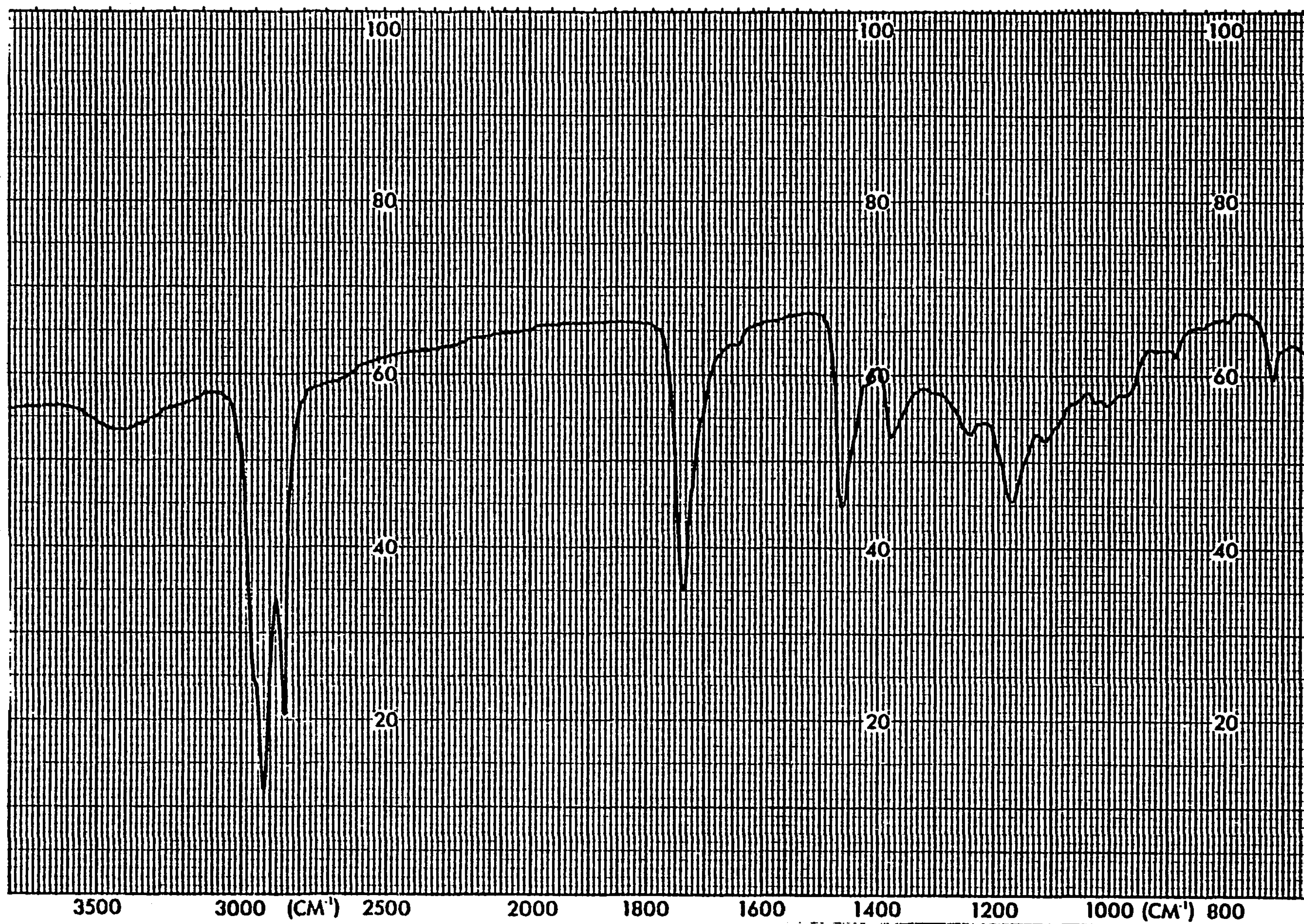
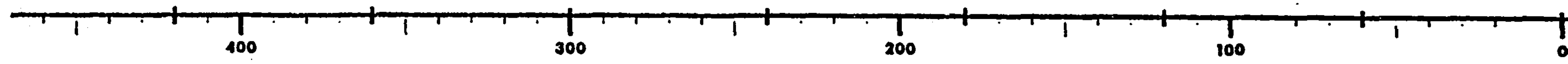


Figure 40. The ir spectrum of cetyl palmitate isolated from Plexaure REM, St. Croix.



60 MHz, T-60 NMR Spectrometer

Figure 41. The nmr spectrum of cetyl palmitate isolated from Plexaura REM, St. Croix.

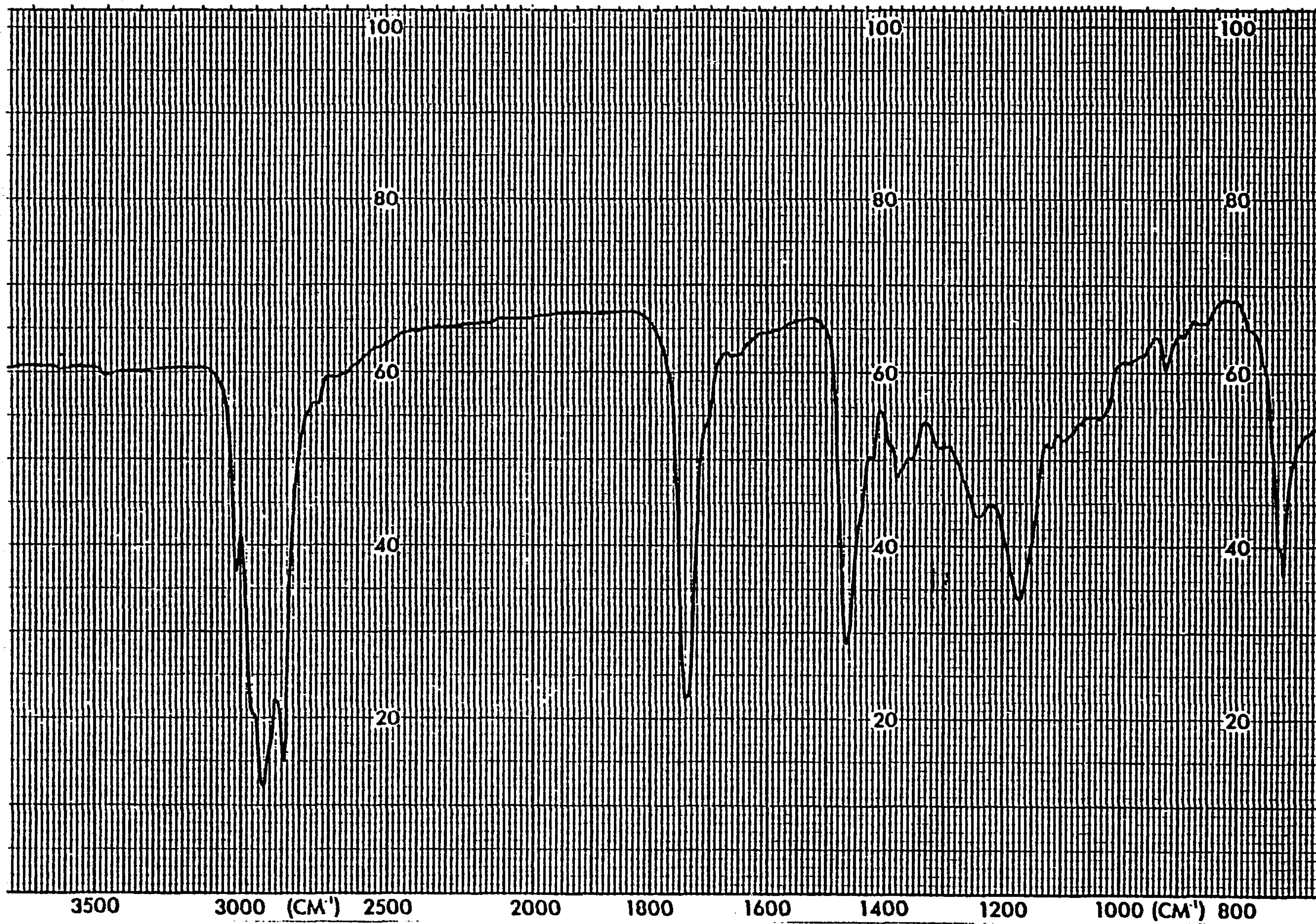


Figure 42. The ir spectrum of cetyl palmitate isolated from Plexaura REM, San Cristobal.

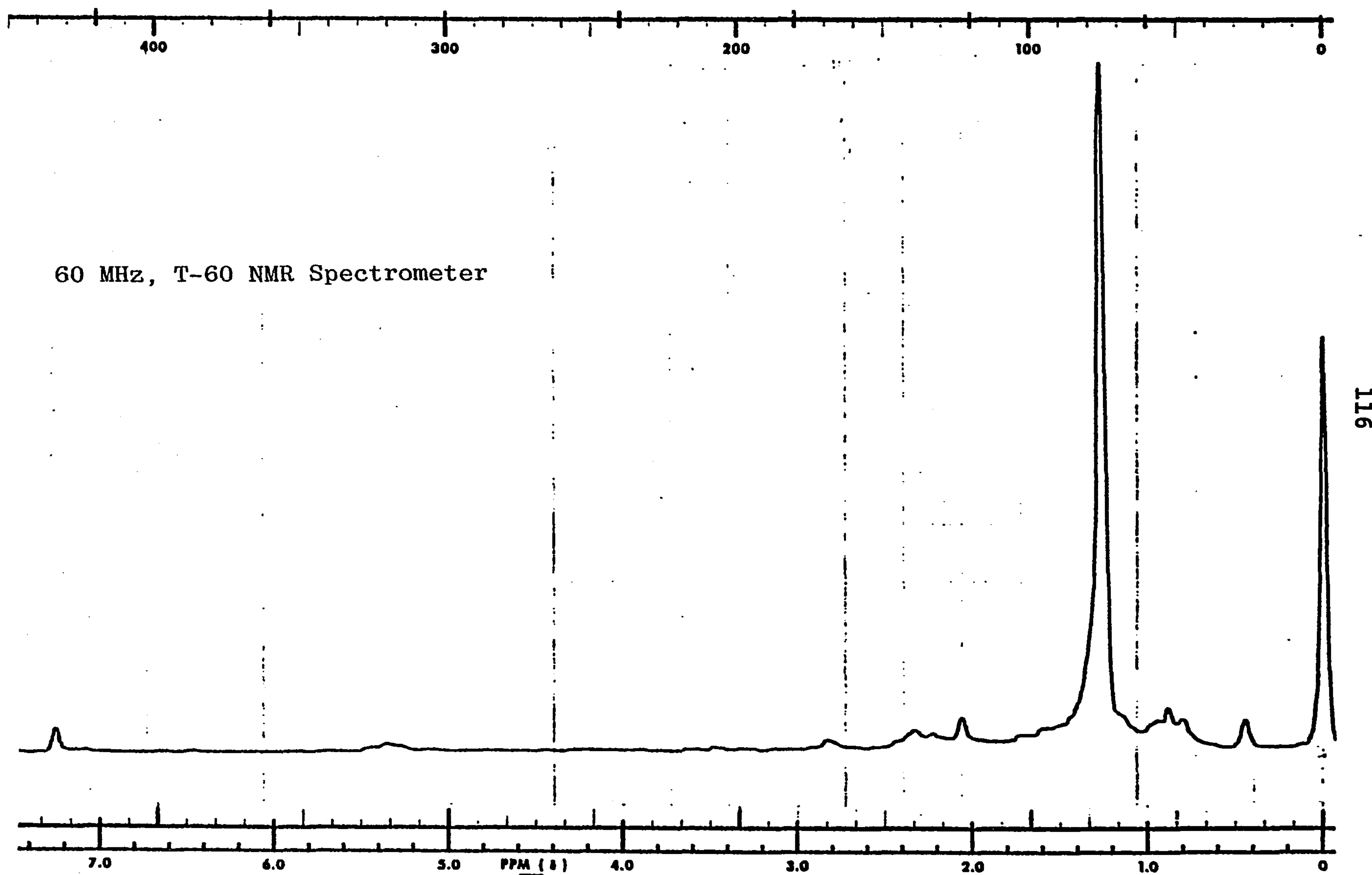
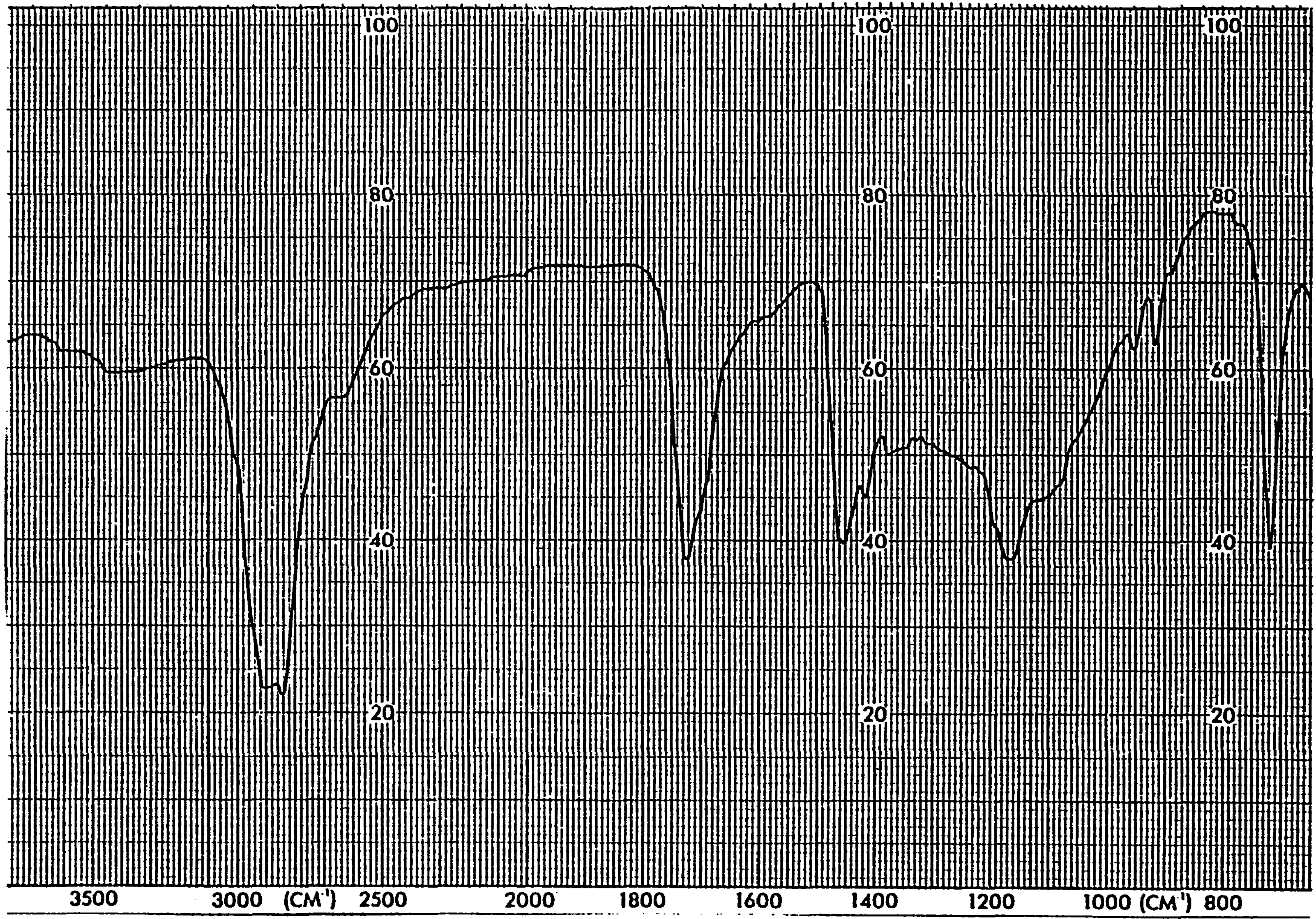


Figure 43. The nmr spectrum of cetyl palmitate isolated from Plexaura REM, San Cristobal.



<u>Figure 44</u>. The ir spectrum of cetyl palmitate isolated from <u>Plexaura</u> <u>kukentali</u>.

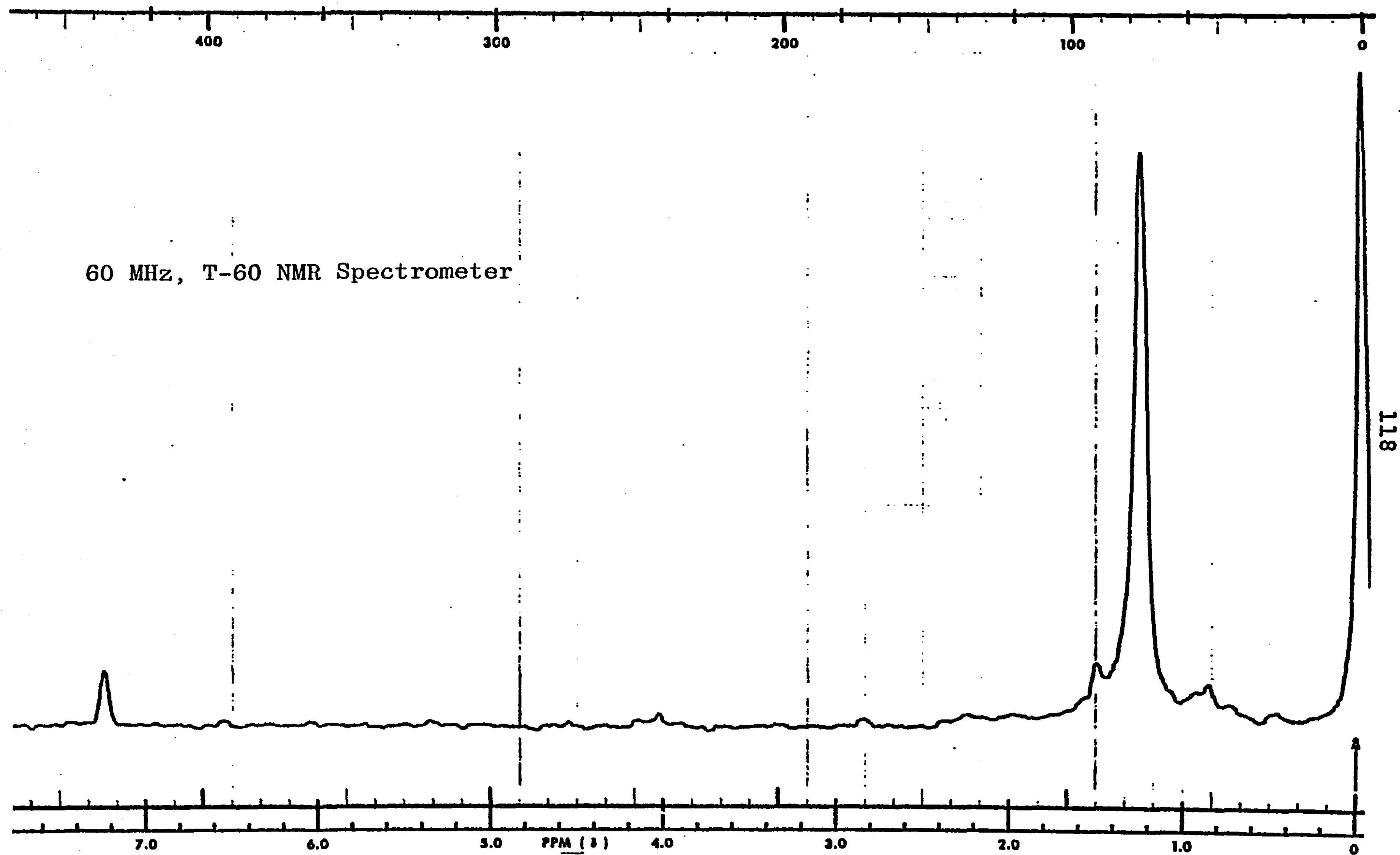


Figure 45. The nmr spectrum of cetyl palmitate isolated from Plexaura kukentali.

Table 15

Cetyl Palmitate from *Plexaura* samples

Plexaura	Cetyl Palmitate		
	mg	in the Sample (%) *	in the lipid (%) **
1. *P.* "*REM*" St. Croix	410	0.04	1.03
2. *P.* "*REM*" San Cristobal	800	0.11	2.11
3. *P. kukenthali*	485	0.16	2.13
4. *P. homomalla*	5	0.005	0.057
5. *P. flexuosa*	-	-	-

*g of cetyl palmitate/100 g of dry weight animal

**g of cetyl palmitate/100 g of total lipids

Table 16

Lipids and Cetyl Palmitate contents of *Pseudopterogorgia americana*, *Plexaura* samples and other gorgonians

Gorgonian	Lipid in the sample (%)**	Cetyl Palmitate (%) in the lipid***	Cetyl Palmitate (%) in the sample****	Reference
Ps. americana (Eggs), Enrique	61.4	44.5	27.3	*
Ps. americana (Eggs), La Parguera	75	72	54	(20,21)
Goniastrea retiformis (Lamk.)		80		(22)
P. "*REM*", St. Croix	3.87	1.03	0.04	*
P. "*REM*", San Cristobal	5.43	2.11	0.11	*
P. kukenthali, San Cristobal	7.71	2.13	0.16	*
P. homomalla, San Cristobal	8.85	0.057	0.005	*
P. homomalla, (Esper)				(9,10)
P. flexuosa, San Cristobal	1.64	-	-	*
P. flexuosa (Lamouroux), Bermuda	3			(15)

*Data from my investigation
**g lipids/100 g dry weight animal
***g cetyl palmitate/100 g total lipids
****g cetyl palmitate/100 g dry weight animal

those for the eggs of Ps. americana (61-75% lipids, 45-80% cetyl palmitate).

Hydrocarbons from Five Plexaura Samples

Hydrocarbons were separated from the urea adduction filtrate by the method described in the experimental section. As shown in Table 17, 50 mg of hydrocarbon were obtained from P. "REM" St. Croix (0.005% of the sample or 0.13% of the lipid), composed of two hydrocarbon fractions, hydrocarbon I (48%) and hydrocarbon II (32%), with R_f 0.79 and 0.59, respectively, when tested by tlc (0.25 mm Silica Gel H, hexane as eluting solvent). Mass spectrometric determination gave molecular weights 204 for hydrocarbon I (Figure 48) and 220 for hydrocarbon II (Figure 51). The ir spectrum of hydrocarbon I (Figure 46) showed C-H stretching around 3000 cm^{-1}, CH_2 bending at 1460 cm^{-1}, CH_3 bending at 1375 cm^{-1}, C-H (vinyl) stretching to the left of 3000 cm^{-1}, C=C stretching at 1660 cm^{-1} and absorption at 890 cm^{-1}. The ir spectrum of hydrocarbon II (Figure 49) showed CH stretching around 3000 cm^{-1}, C=C stretching at 1660 cm^{-1}, CH_2 bending at 1460 cm^{-1}, CH_3 bending at 1375 cm^{-1}, and exomethylene group absorption at 890 cm^{-1}. The nmr spectra of hydrocarbons I and II (Figures 47 and 50) indicated a long methylene chain signal at δ1.27, and a terminal methyl group at δ0.95. The nmr of hydrocarbon I showed an olefinic proton absorption at δ5.3.

The amount of hydrocarbons isolated from the other

Table 17

Component Hydrocarbons from Five Plexaura Samples

Plexaura	Hydrocarbon			Hydrocarbon I (MW 204)		Hydrocarbon II (MW 220)	
	mg	% in the sample*	% in the lipid***	mg	%**	mg	%**
1. P. "REM", St. Croix	50	0.005	0.13	12	48	8	32
2. P. "REM", San Cristobal	298	0.042	7.68				
3. P. kukenthali	10	0.003	0.04				
4. P. homomalla	22	0.022	0.25				
5. P. flexuosa	trace	-	-				

*Calculation based on the amount of samples and the total lipid contents shown in Table 13 (g total hydrocarbons/100 g dry weight animal).

**From 25 mg hydrocarbon, separated into hydrocarbons I and II by tlc (g hydrocarbon I or II/100 g total hydrocarbons).

***g total hydrocarbons/100 g total lipids

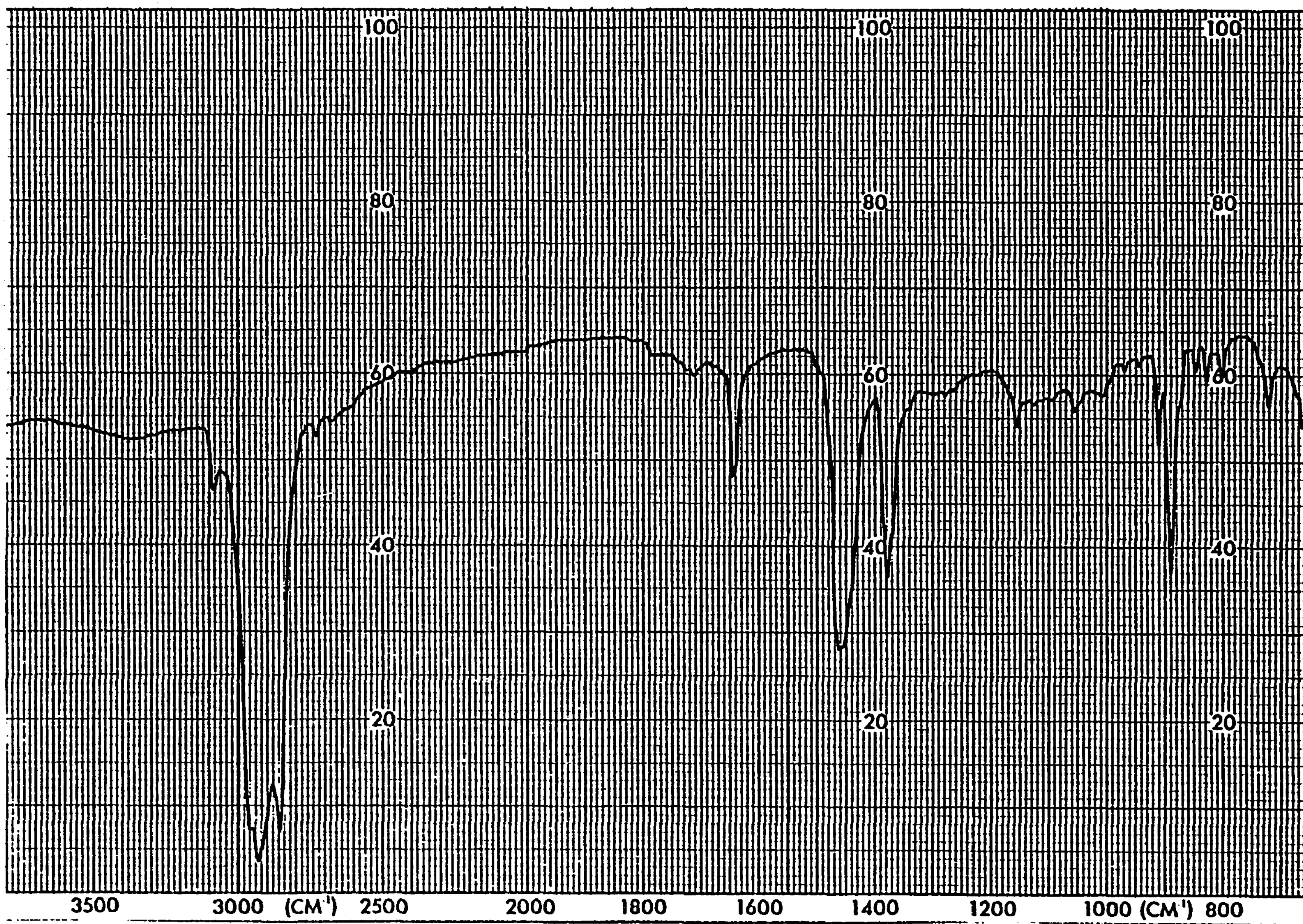


Figure 46. The ir spectrum of hydrocarbon I isolated from Plexaura REM, St. Croix.

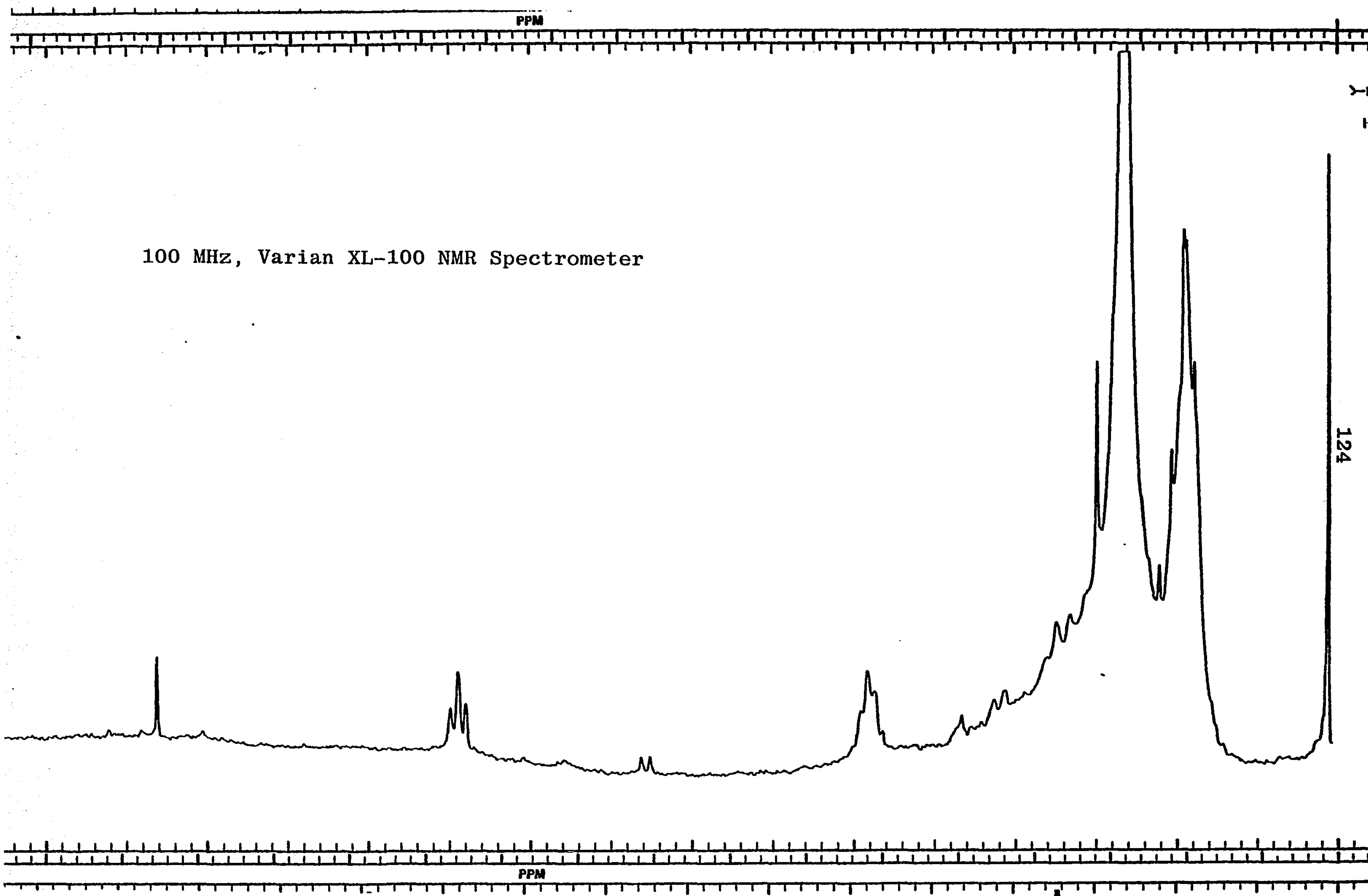


Figure 47. The nmr spectrum of hydrocarbon I isolated from *Plexaura* "REM", St. Croix

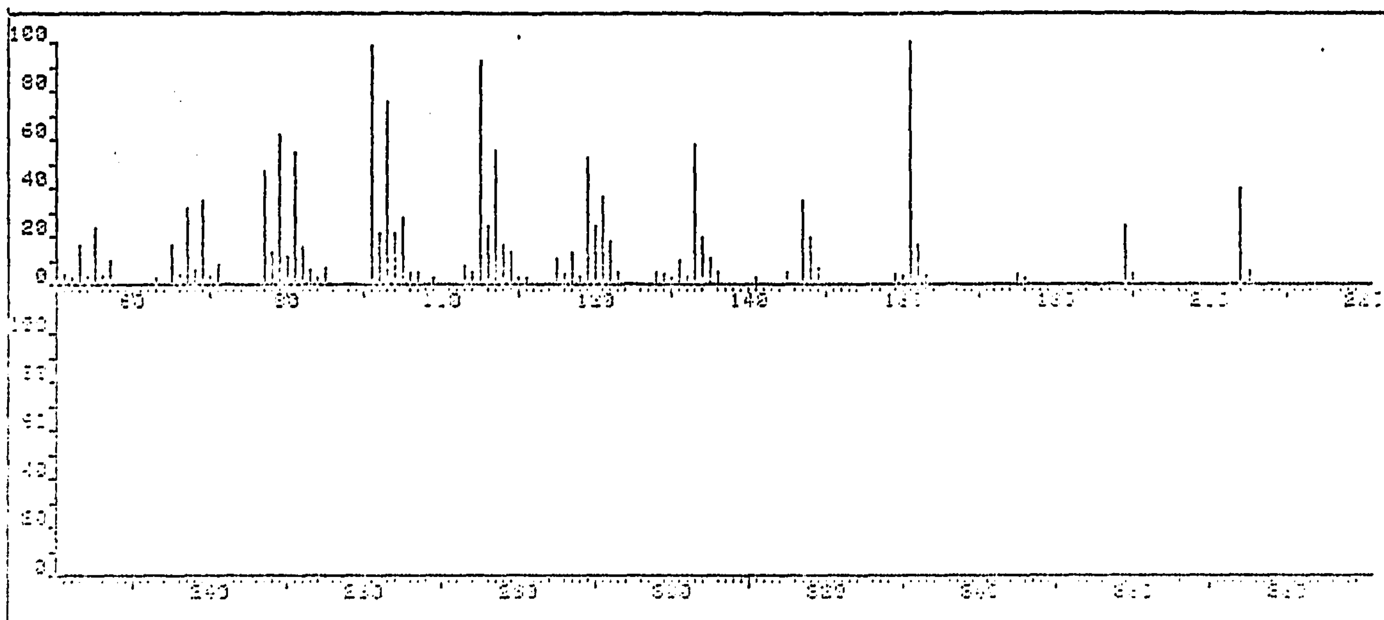


Figure 48. The mass spectrum of hydrocarbon I isolated from *Plexaura* REM, St. Croix.

MASS	%	MASS	%		
51.0	4.3	93.1	75.4	130.2	2.5
52.0	2.8	94.1	20.8	131.1	10.2
53.1	16.5	95.1	27.4	132.1	3.6
54.1	3.3	96.2	5.1	133.1	58.1
55.1	23.6	97.2	5.3	134.1	19.3
56.1	3.3	99.2	2.8	135.1	10.9
57.1	9.6	103.1	7.9	136.1	5.3
63.0	2.8	104.0	5.1	141.2	2.8
65.1	16.5	105.1	92.6	145.0	4.8
66.0	4.3	106.1	24.6	147.1	35.0
67.1	32.0	107.1	55.6	148.1	19.3
68.2	5.8	108.1	16.2	149.1	6.6
69.2	35.0	109.1	13.5	159.0	4.3
70.1	3.3	110.1	2.5	160.1	3.6
71.1	8.6	111.1	3.0	161.1	100.0
77.1	45.7	115.1	11.2	162.1	16.2
78.1	13.2	116.1	4.5	163.1	3.3
79.1	62.2	117.1	13.5	175.1	4.3
80.1	11.7	118.1	3.8	171.2	2.8
81.1	54.5	119.1	52.5	189.2	24.4
82.1	15.7	120.1	24.6	190.2	4.3
83.2	6.3	121.1	36.0	204.2	39.3
84.0	2.5	122.1	10.3	205.3	6.3
85.1	4.5	123.1	5.6		
91.1	69.0	125.1	5.5		
92.1	21.1	127.1	4.3		

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111.1	3.0	115.1	11.2	116.1	4.6	117.1	13.5	118.1	3.8	119.1	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	122.1	10.3	123.1	5.5	125.1	5.5	129.1	4.3	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	3.3	133.1	58.1	134.1	19.3	135.1	10.9	136.1	5.3	[illegible]	[illegible]	[illegible]	[illegible]
147.1	35.0	[illegible]	19.3	[illegible]	6.6	159.0	4.3	160.1	3.6	161.1	[illegible]	[illegible]	[illegible]
163.1	3.3	175.1	4.3	176.2	2.8	189.2	24.4	190.2	4.3	204.1	[illegible]	[illegible]	[illegible]

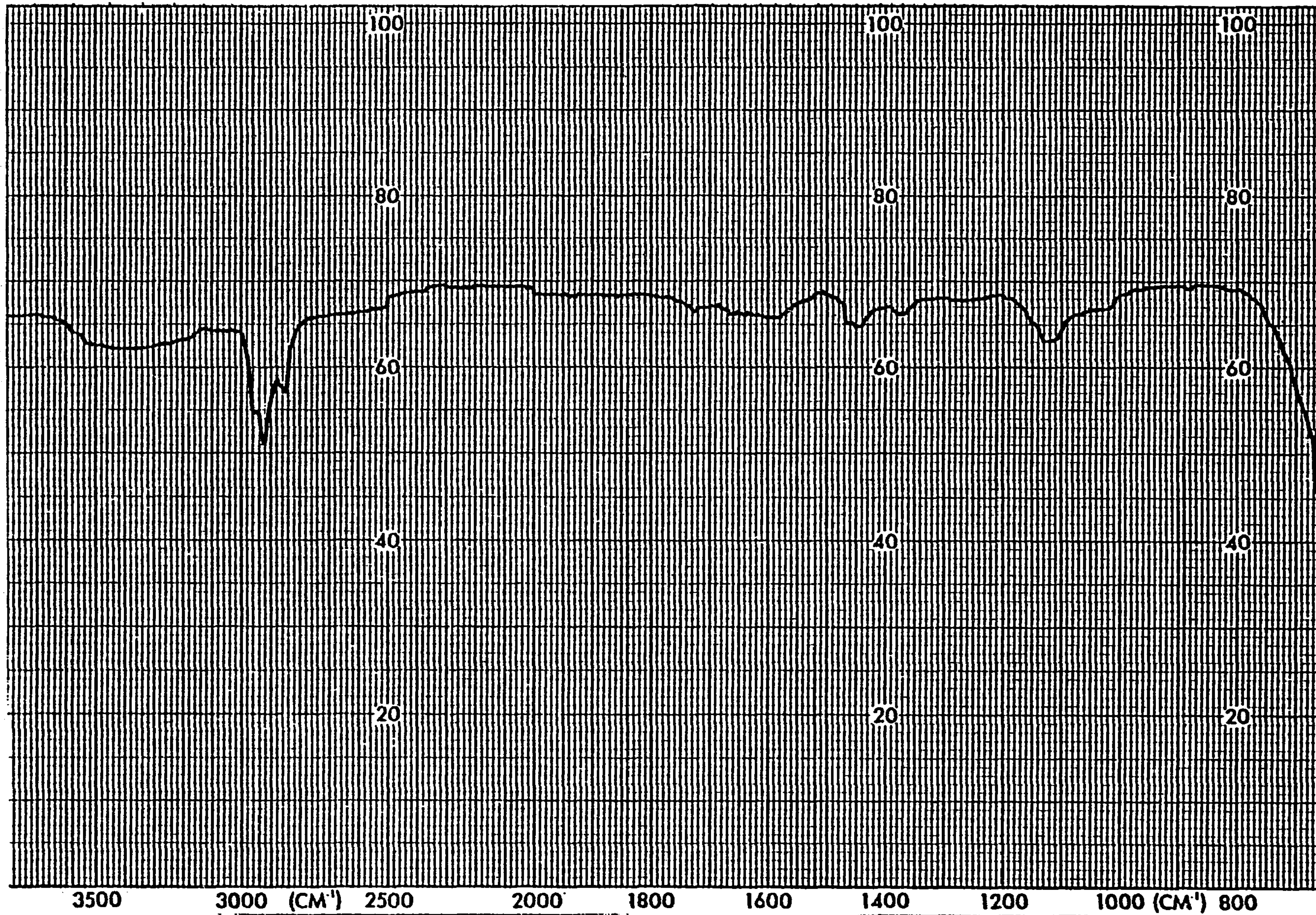


Figure 49. The ir spectrum of hydrocarbon II isolated from Plexaura REM, St. Croix.

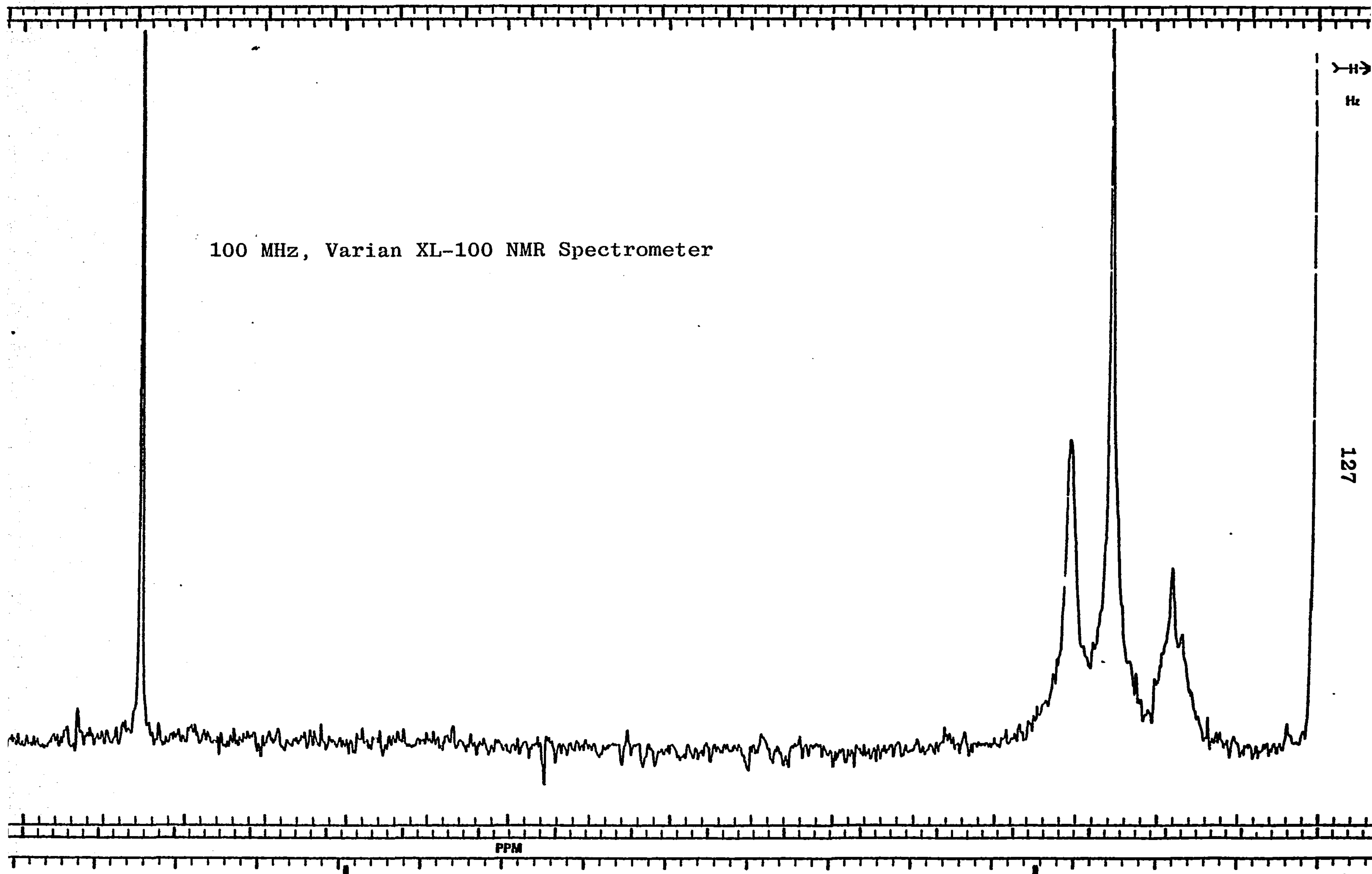


Figure 50. The nmr spectrum of hydrocarbon II isolated from Plexaura "REM", St. Croix

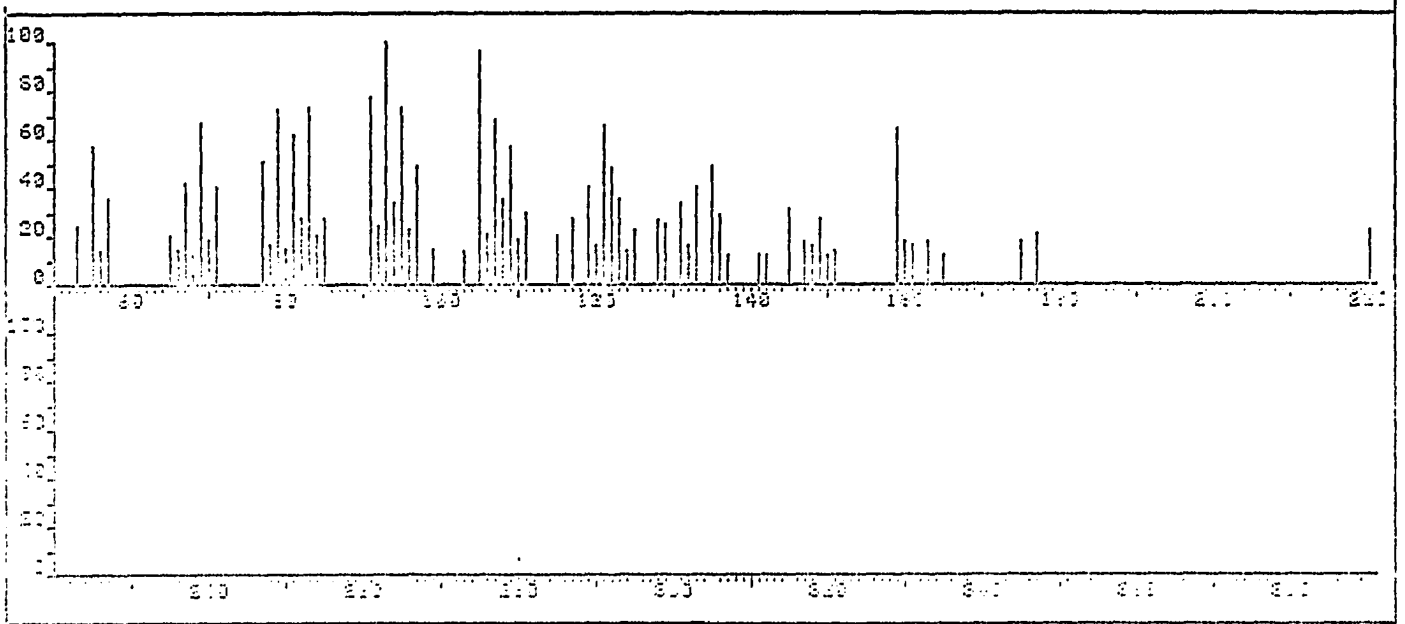


Figure 51. The mass spectrum of hydrocarbon II isolated from Plexaura REM, St. Croix.

MASS	%	MASS	%		
53.1	24.1	97.1	49.4	137.0	12.7
55.1	57.9	99.1	15.2	141.0	12.7
56.1	13.9	103.0	13.9	142.0	12.7
57.1	35.4	105.2	95.2	145.1	31.6
65.2	20.3	106.1	21.5	147.1	17.7
66.1	15.2	107.1	68.4	148.1	16.5
67.2	41.8	108.1	35.4	149.1	27.8
68.0	12.7	109.1	57.0	150.1	12.7
69.2	67.1	110.0	19.0	151.1	13.9
70.1	19.0	111.1	30.4	159.1	64.6
71.1	40.5	115.1	29.3	160.1	17.7
77.1	50.6	117.3	27.8	161.0	16.5
78.1	15.5	119.0	40.5	163.1	17.7
79.1	72.2	120.1	16.5	165.1	12.7
80.1	15.2	121.1	65.0	175.0	17.7
81.2	[illegible]	122.1	48.1	177.1	21.5
[illegible]	[illegible]	[illegible]	28.4	[illegible]	22.8
[illegible]	[illegible]	[illegible]	13.9		
[illegible]	[illegible]	[illegible]	22.8		
[illegible]	27.3	[illegible]	26.5		
[illegible]	[illegible]	[illegible]	15.3		
[illegible]	[illegible]	131.1	34.2		
[illegible]	[illegible]	[illegible]	16.8		
[illegible]	[illegible]	[illegible]	43.8		
[illegible]	[illegible]	[illegible]	49.4		
[illegible]	22.8	[illegible]	29.1		

species of Plexaura are (Table 17): 298 mg from 715 g P. REM San Cristobal (0.042% of sample or 7.68% of lipid); 10 mg from 295 g P. kukenthali (0.003% of sample or 0.04% of lipid); and 22 mg from 100 g P. homomalla (0.022% of sample or 0.25% of lipid). The tlc and mass spectrophotometric determinations indicated that all of the hydrocarbons from the three Plexaura samples showed one component with a molecular weight 204 (Figures 54, 57 and 60).

The ir spectrum of hydrocarbon from P. REM San Cristobal (Figure 52) showed strong C-H stretching to the right of 3000 cm^{-1}, C-H vinyl stretching to the left of 3000 cm^{-1}, CH_2 absorption at 1460 cm^{-1}, CH_3 absorption at 1375 cm^{-1}, C=C stretching at 1640 cm^{-1} and absorption at 895 cm^{-1}. The nmr spectral absorptions (Figure 53) indicated the presence of a long methylene chain, δ1.27, terminal methyl groups, δ0.95, and an olefinic proton, δ4.7.

The ir spectrum of hydrocarbon from P. kukenthali (Figure 55) showed C-H stretching around 3000 cm^{-1}, C=C absorption at 1640 cm^{-1}, CH_2 and CH_3 bendings at 1460 and 1375, respectively, and absorption at 890 cm^{-1}. The nmr spectrum (Figure 56) showed absorptions at δ0.95, δ1.27, δ1.70, and δ4.70.

The ir spectrum of hydrocarbon from P. homomalla (Figure 58) showed C-H and C-H vinyl stretchings to the right and to the left of 3000 cm^{-1}, respectively, absorptions at 1730 cm^{-1}, 1460 cm^{-1} and 1380 cm^{-1}. The nmr spectrum (Figure

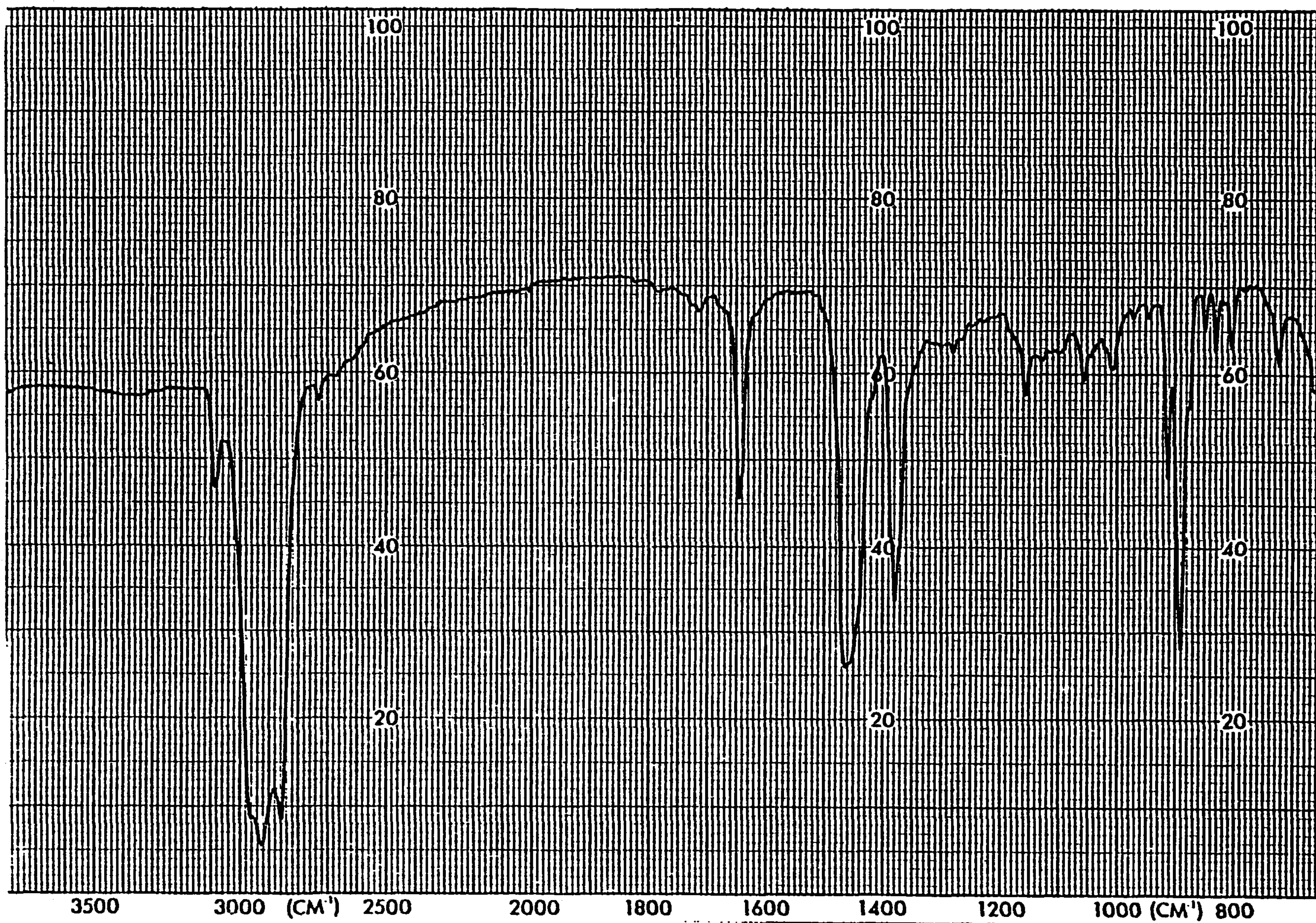
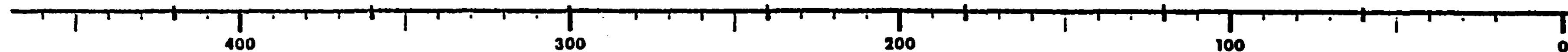


Figure 52. The ir spectrum of hydrocarbon isolated from *Plexaura* REM, San Cristobal.



60 MHz, T-60 NMR Spectrometer

7.0 6.0 5.0 PPM (δ) 4.0 3.0 2.0 1.0 0

Figure 53. The nmr spectrum of hydrocarbon isolated from *Plexaura* REM, San Cristobal

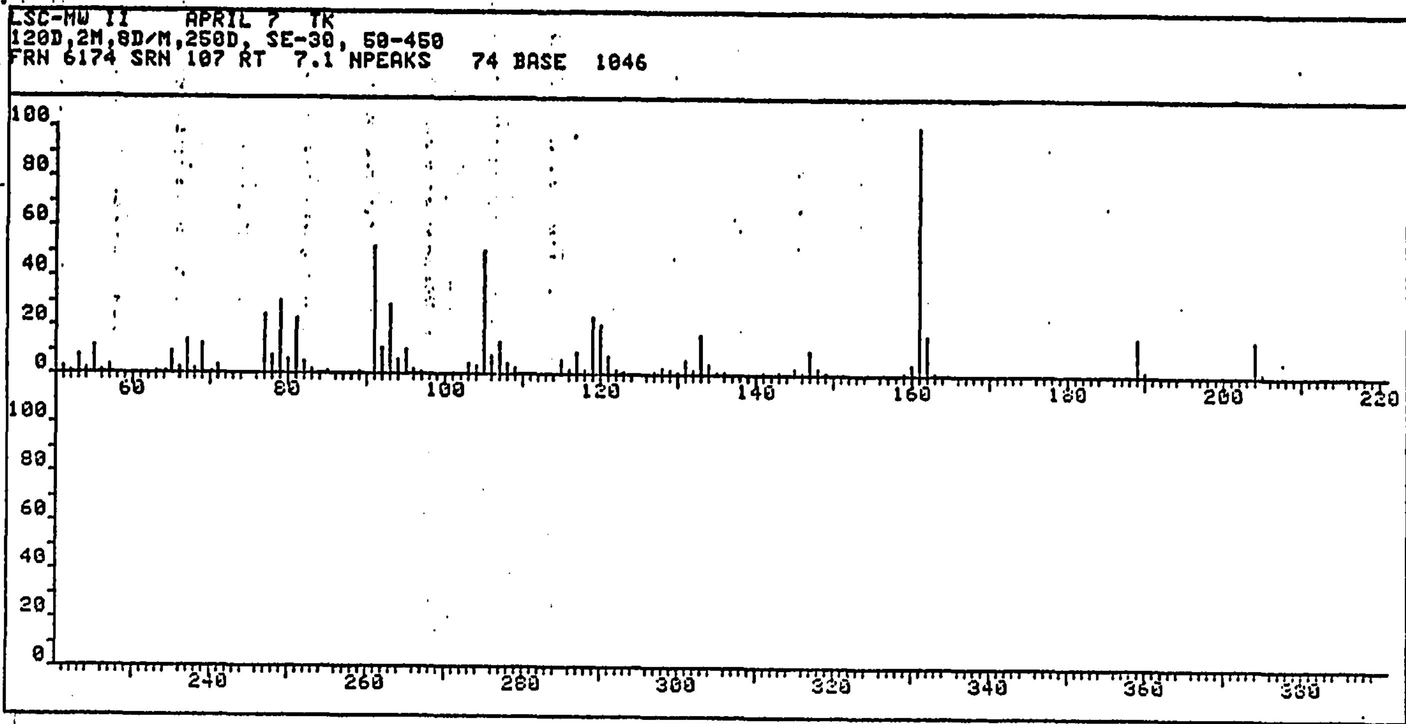


Figure 54. The mass spectrum of hydrocarbon isolated from Plexaura REM, San cristobal.

FRN 6174, SPECTRUM 107, 74PEAKS, RT 7.12 MIN, BASE PEAK 161.1, 1046. COUNTS

MOST INTENSE 74

MASS	%	MASS	%	MASS	%
51.0	2.5	92.1	11.1	131.1	5.7
52.1	1.2	93.1	28.8	132.1	1.8
53.1	7.7	94.2	5.7	133.1	16.5
54.1	2.1	95.1	9.8	134.2	4.7
55.1	12.0	96.2	1.7	135.1	1.4
56.1	1.2	97.1	1.3	136.0	1.1
57.1	3.6	103.1	4.0	141.1	1.0
63.0	1.0	104.2	3.4	143.1	1.0
64.3	1.1	105.1	50.0	145.1	2.8
65.1	9.2	106.1	7.9	146.1	1.0
66.1	2.5	107.1	13.5	147.1	10.1
67.1	13.8	108.1	4.4	148.1	3.1
68.2	2.2	109.1	3.1	149.1	1.5
69.2	12.0	115.1	5.8	159.1	1.1
70.3	1.3	116.1	1.8	160.2	4.2
71.1	3.9	117.1	9.6	161.1	100.0
77.1	24.7	118.0	1.9	162.1	16.7
78.1	7.8	119.1	23.6	163.1	1.3
79.1	29.7	120.1	20.6	189.2	16.0
80.0	5.6	121.1	7.3	190.2	2.1
81.1	23.1	122.0	1.6	204.2	14.5
82.1	5.1	123.1	1.4	205.2	1.8
83.1	1.9	127.0	1.0		
85.2	1.0	128.1	3.1		
89.1	1.1	129.2	2.4		
91.1	51.6	130.1	1.2		

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115.1	5.8	116.1	1.8	117.1	9.6	118.0	1.9	119.1	23.6	120.1	20.6	121.1	7.3
122.0	1.6	123.1	1.4	127.0	1.0	128.1	3.1	129.2	2.4	130.1	1.2	131.1	5.7
132.1	1.8	133.1	16.5	134.2	4.7	135.1	1.4	136.0	1.1	141.1	1.0	143.1	1.0
145.1	2.8	146.1	1.0	147.1	10.1	148.1	3.1	149.1	1.5	159.1	1.1	160.2	4.2
161.1	100.0	162.1	16.7	163.1	1.3	189.2	16.0	190.2	2.1	204.2	14.5	205.2	1.8

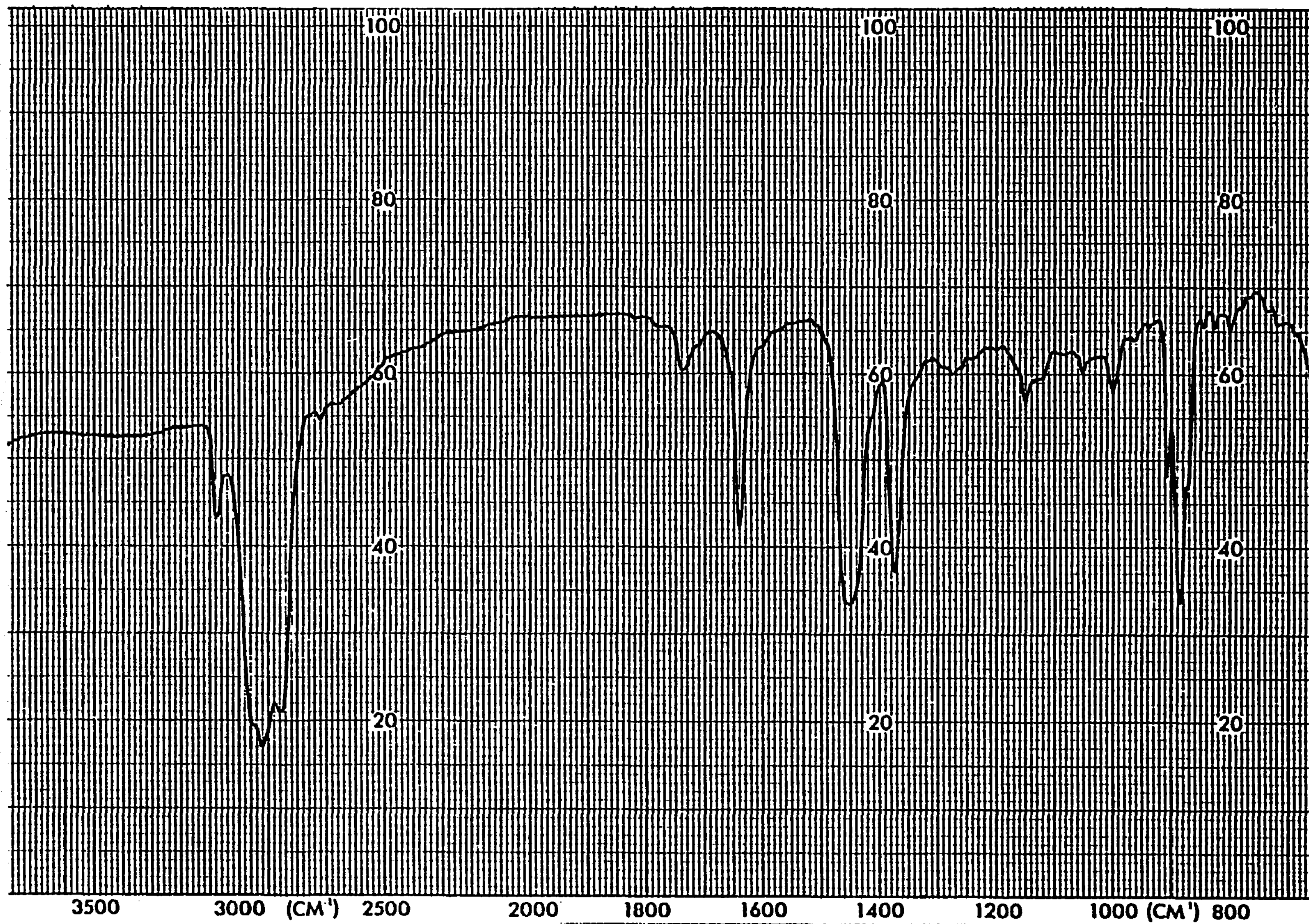

Figure 55. The ir spectrum of hydrocarbon isolated from Plexaura kukentali.

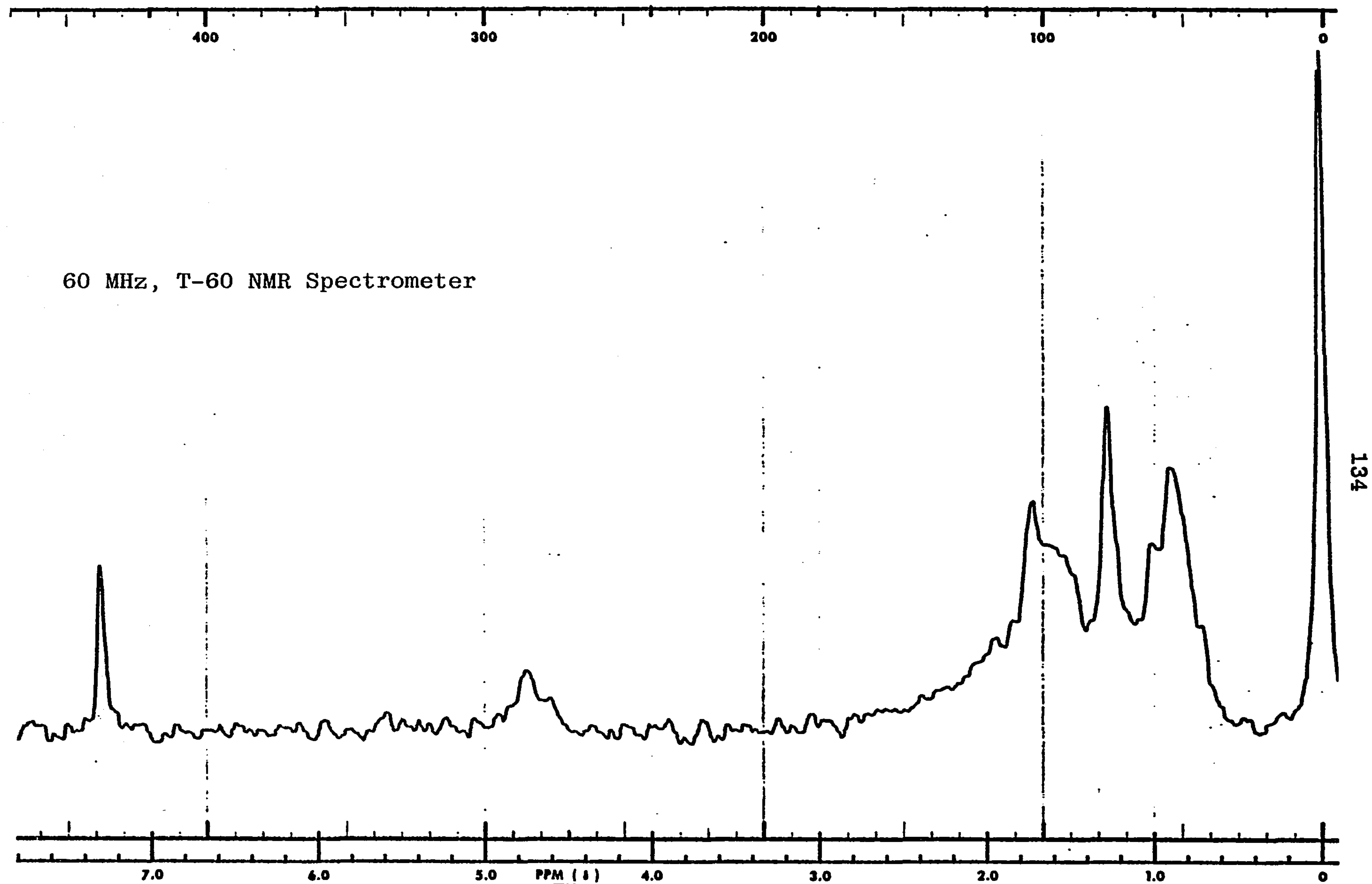

Figure 56. The nmr spectrum of hydrocarbon isolated from Plexaura kukentali.

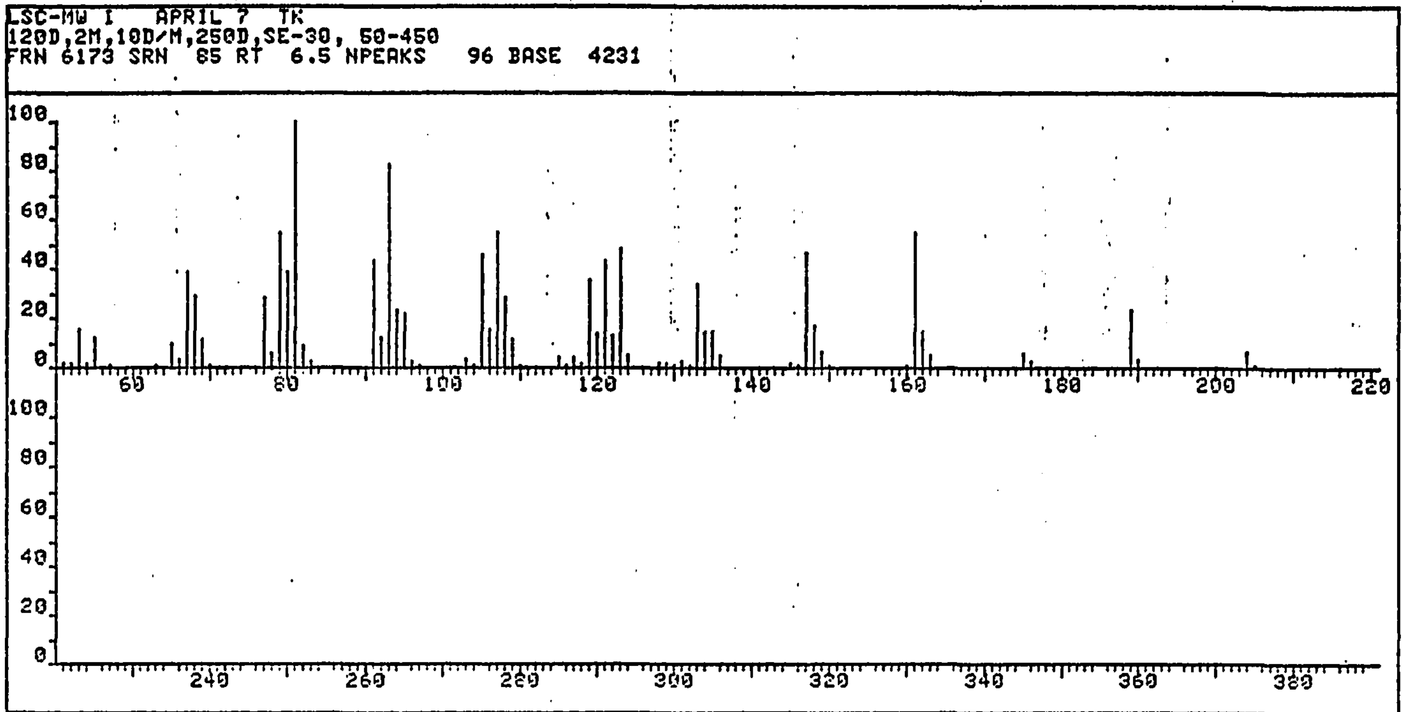

Figure 57. The mass spectrum of hydrocarbon isolated from Plexaura kukenthali.

FRN 6173, SPECTRUM 85, 96PEAKS, RT 6.47 MIN, BASE PEAK 81.1, 4231. COUNTS
MOST INTENSE 96

MASS	%	MASS	%	MASS	%	MASS	%
50.0	.5	81.1	100.0	117.1	4.7	147.1	46.8
51.1	2.2	82.1	9.2	118.1	1.6	148.1	17.5
52.1	1.6	83.2	2.4	119.1	35.7	149.1	6.9
53.1	15.3	84.2	.3	120.2	14.2	150.1	1.0
54.1	1.7	85.2	.4	121.2	43.4	159.1	.4
55.1	12.8	89.1	.7	122.2	13.2	160.2	1.4
56.1	.7	91.1	43.4	123.2	48.4	161.1	54.5
57.1	1.4	92.1	12.4	124.2	5.4	162.1	14.5
62.0	.3	93.1	83.2	125.2	.8	163.1	5.3
63.1	1.3	94.1	23.7	127.1	.6	164.2	.7
64.0	.5	95.1	22.2	128.1	1.7	175.2	6.3
65.1	10.0	96.2	3.1	129.1	1.7	176.2	2.8
66.1	3.9	97.2	1.4	130.1	.8	177.2	.4
67.1	38.5	98.2	.4	131.1	3.0	189.2	23.7
68.2	29.1	102.2	.6	132.1	1.3	190.2	3.8
69.2	11.2	103.1	3.5	133.1	34.2	191.2	.3
70.1	.9	104.2	1.3	134.1	15.0	204.2	7.0
71.2	.8	105.1	45.9	135.1	15.2	205.2	1.2
73.1	.2	106.1	15.5	136.2	5.0		
74.1	.3	107.1	55.1	137.2	.6		
75.1	.3	108.1	28.9	141.2	.6		
76.1	.4	109.1	11.2	142.1	.3		
77.1	28.8	110.1	1.4	143.1	.3		
78.1	6.4	111.1	.5	144.1	.3		
79.1	54.5	115.1	4.3	145.1	2.1		
80.1	38.5	116.1	1.4	146.1	1.0		

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127.1	.6	128.1	1.7	129.1	1.7	130.1	.8	131.1	3.0	132.1	1.3	133.1	34.2
134.1	15.0	135.1	15.2	136.2	5.0	137.2	.6	141.2	.6	142.1	.3	143.1	.3
144.1	.3	145.1	2.1	146.1	1.0	147.1	46.8	148.1	17.5	149.1	6.9	150.1	1.0
159.1	.4	160.2	1.4	161.1	54.5	162.1	14.5	163.1	5.3	164.2	.7	175.2	6.3
176.2	2.8	177.2	.4	189.2	23.7	190.2	3.8	191.2	.3	204.2	7.0	205.2	1.2

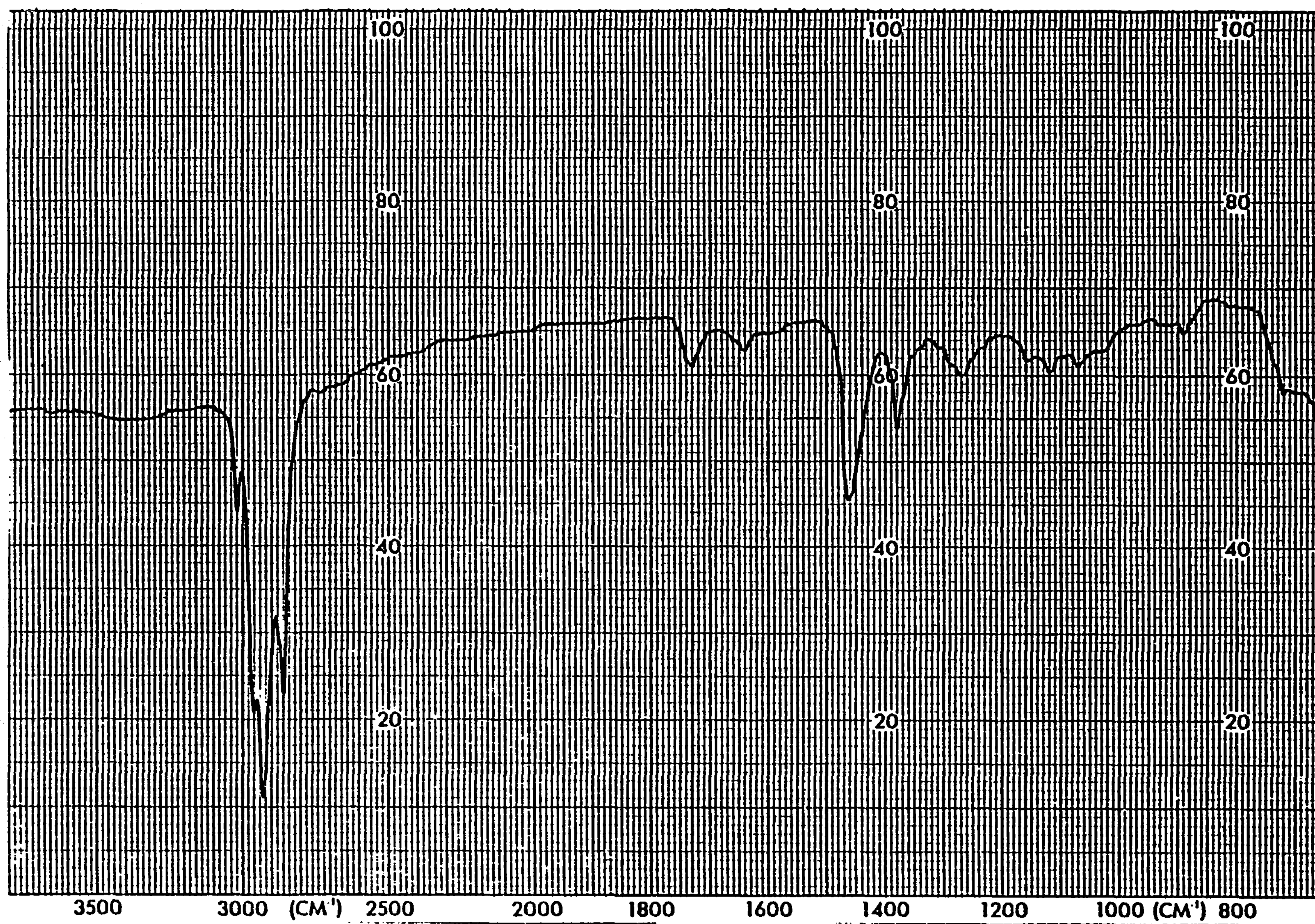

Figure 58. The ir spectrum of hydrocarbon isolated from *Plexaura homomalla*.

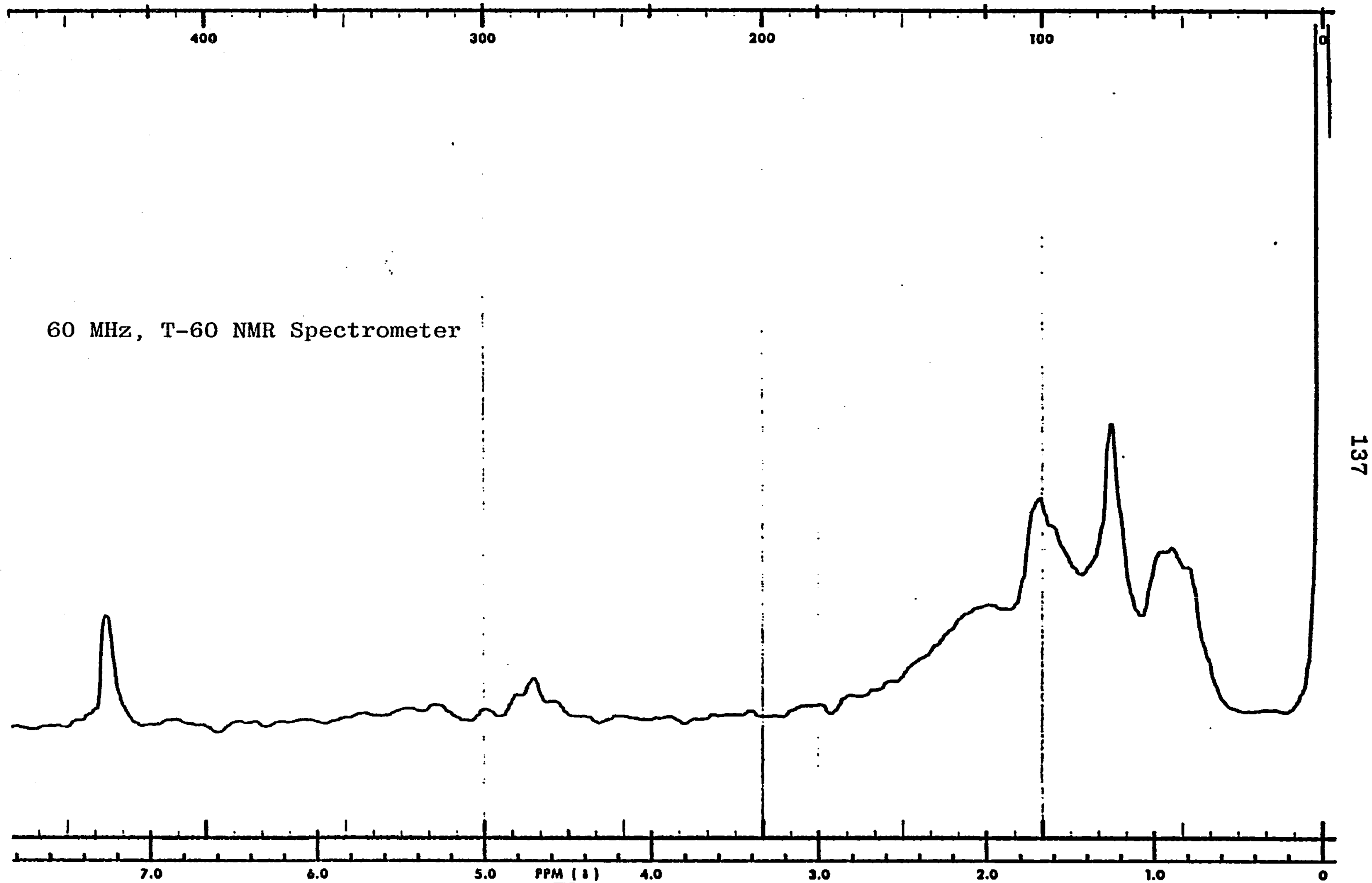

Figure 59. The nmr spectrum of hydrocarbon isolated from *Plexaura homomalla*.

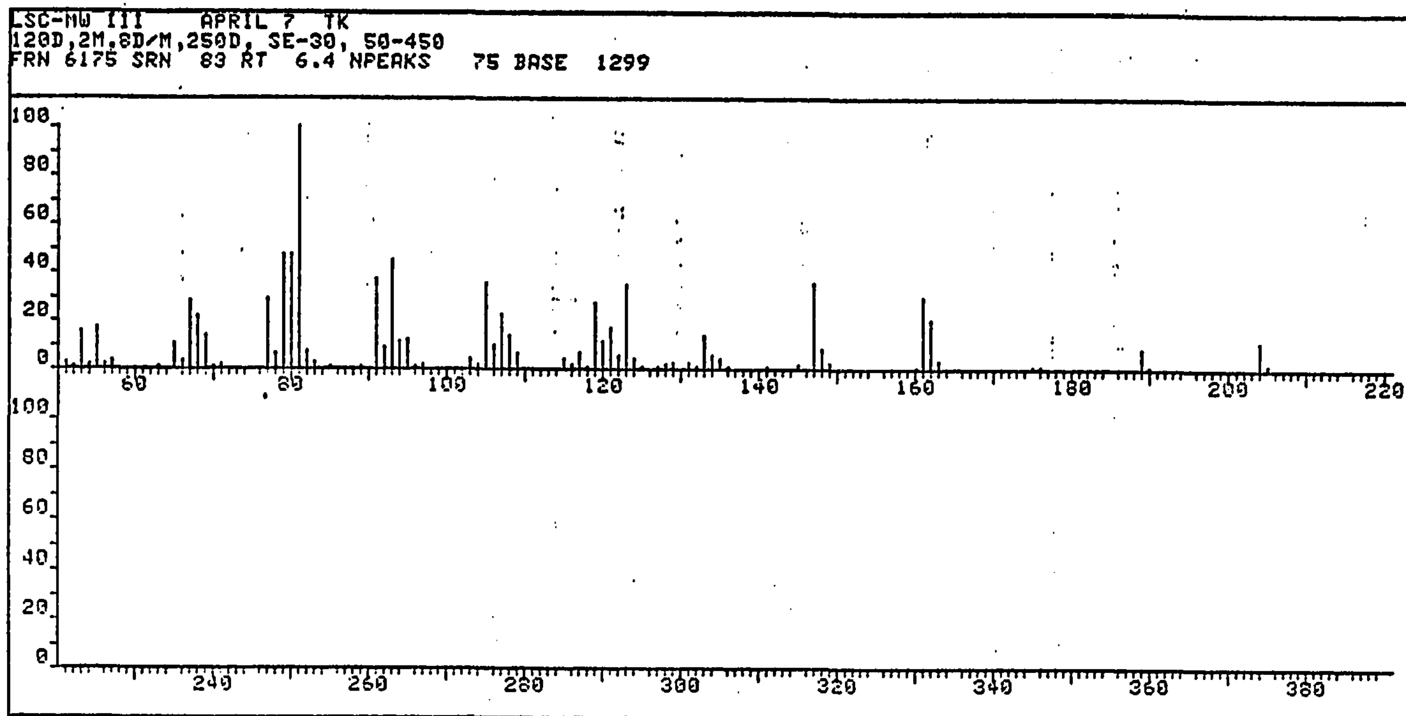

Figure 60. Mass spectrum of hydrocarbon isolated from Plexaura homomalla.

FRN 6175, SPECTRUM 83, 75PEAKS, RT 6.42 MIN, BASE PEAK 81.1, 1299. COUNTS
MOST INTENSE 75

MASS	%	MASS	%		
50.1	.8	92.1	9.0	129.1	2.5
51.1	2.8	93.1	44.9	130.1	.8
52.0	1.5	94.1	11.5	131.1	2.8
53.1	15.9	95.1	12.1	132.1	.9
54.1	2.4	96.1	1.2	133.1	13.6
55.1	17.2	97.1	2.2	134.1	6.3
56.1	1.6	103.2	4.1	135.1	4.2
57.1	3.5	104.1	1.6	136.1	1.5
63.1	1.3	105.2	35.3	141.0	.9
65.1	11.2	106.1	10.4	145.1	1.8
66.1	3.7	107.1	22.9	147.1	35.7
67.1	28.4	108.1	13.6	148.1	8.4
68.2	22.2	109.1	6.5	149.2	2.9
69.2	14.0	115.1	4.8	160.3	1.3
70.1	1.5	116.1	2.0	161.1	30.3
71.1	2.2	117.0	6.8	162.1	20.1
77.1	29.4	118.1	1.5	163.1	3.4
78.1	7.0	119.1	27.9	175.2	1.5
79.1	47.3	120.1	11.9	176.2	1.1
80.1	47.9	121.2	17.2	189.2	8.1
81.1	100.0	122.1	6.3	190.2	1.4
82.1	7.9	123.2	34.9	204.2	11.9
83.1	2.9	124.1	4.1	205.2	2.1
85.1	1.3	125.1	.9		
89.0	1.0	127.1	1.0		
91.1	37.3	128.1	2.2		

LAST 35

116.1	2.0	117.0	6.8	118.1	1.5	119.1	27.9	120.1	11.9	121.2	17.2	122.1	6.3
123.2	34.9	124.1	4.1	125.1	.9	127.1	1.0	128.1	2.2	129.1	2.5	130.1	.8
131.1	2.8	132.1	.9	133.1	13.6	134.1	6.3	135.1	4.2	136.1	1.5	141.0	.9
145.1	1.8	147.1	35.7	148.1	8.4	149.2	2.9	160.3	1.3	161.1	30.3	162.1	20.1
163.1	3.4	175.2	1.5	176.2	1.1	189.2	8.1	190.2	1.4	204.2	11.9	205.2	2.1

59) showed absorptions at δ0.95, δ1.27, δ1.70 and δ4.70.

A major feature of the hydrocarbon component of all *Plexaura* samples under my investigation is the appearance of a nonlinear hydrocarbon with a molecular weight of 204. This is typical of the sesquiterpenes, the major hydrocarbons found in gorgonians. The amount ranged from 0.003% (g hydrocarbons/100 g dry weight animal) for *P. kukenthali* (Table 17) to 0.042% for *P. REM*, San Cristobal. *P. flexuosa* studied by Gross (18) contained a minute quantity of sesquiterpene hydrocarbon, and so did *P. flexuosa* under my investigation. In general the amount of sesquiterpene hydrocarbons in *Plexaura* is relatively insignificant compared to that in the eggs of *Pseudopterogorgia americana* and *Pseudoplexaura crassa* (6).

EXPERIMENTAL

The six gorgonian samples analyzed were collected by Dr. L. S. Ciereszko: (1) Eggs of *Pseudopterogorgia americana*, collected at Cayo Enrique, Puerto Rico, on February 2, 1978; (2) *Plexaura REM*, collected at St. Croix, on April 5, 1978; (3) *Plexaura REM*, collected at San Cristobal on February 23, 1978; (4) *Plexaura kukenthali*, collected at San Cristobal on February 23, 1978; (5) *Plexaura homomalla*, collected at San Cristobal on January 2, 1980; and (6) *Plexaura flexuosa*, collected at San Cristobal on January 2, 1980.

Extraction of Lipids

The dry whole gorgonian samples were crushed in a Waring blendor and weighed. The amounts of sample used for extraction are shown in Table 13. The samples were extracted with hexane (200 ml hexane for each 100 g sample) using a continuous Perculator-Extractor (37), first for 4 hr, and then for 8 hr (Four-hour extraction was shown to be long enough to extract the lipid completely from the samples).

Isolation, Identification and Determination of Diterpenoids

The extracts were allowed to stand at room temperature for 24 hr. The crystalline precipitates were collected on a sintered glass funnel and washed with hexane. The washes were combined with the filtrate and the volume

was reduced to half by using a Büchi Rotavap evaporator. The solution was allowed to stand for 24 hr at room temperature and the crystals which formed were washed as above, and combined with the first crystals. The filtrate was allowed to stand in the refrigerator for another 24 hr and the crystals formed were collected. The crystals were combined, dried and weighed (Table 14). The crystals were tested by tlc (0.25 mm thick Silica Gel H on 5x20 cm plate) using chloroform-methanol (98:2, v/v) as eluting solvent. The diterpenoid was chromatographed on Florisil (20 g, 100/200 Mesh, Floridin Co.) using benzene-ethyl acetate (4:1, v/v) as eluting solvent. Ten ml fractions were collected and each was tested by tlc. Fractions showing the same spot on tlc were combined, evaporated to dryness on a Büchi Rotavap evaporator, dried in a desiccator, *in vacuo*, for 24 hr, weighed, and labelled as fraction I (diterpenoid I) and fraction II (diterpenoid II), respectively. The ir spectrum was determined on a Perkin-Elmer 298 IR Spectrophotometer (0.5 abscissa expansion, 4 min scan time, and solid KBr as sample holder). The nmr spectrum was determined on a T-60 Nuclear Magnetic Resonance Spectrometer (variable spectrum amplitude, 50 rps spinning rate, 250 sec sweep time, 500 Hz sweep width, variable filter, 0.025 RF power level, and chloroform-d_1 containing 1% TMS as solvent). The mass spectrum was determined by a Hewlett-Packard 5985 GC-MS (70 eV, with source temperature 200°C) system with

hexane as solvent. GC conditions: column, 2 mm 6', OV-17; temperature, 180°C; temperature rate, 10°/min; time, 5 min; flowrate, 10 ml/min; attenuation, 8.

Isolation, Identification and Determination of Cetyl Palmitate

The non-branched wax esters were isolated from the lipid solution (after the removal of the diterpenoid crystals) by the urea inclusion method (35,36). A definite amount of lipid was mixed with a saturated solution of urea in chloroform-methanol (3:1, v/v) in the proportion of 1 g lipid per 100 ml solution. The mixture was allowed to stand at room temperature for 24 hr. The white crystals of urea adduct were filtered using a sintered glass funnel, and the filtrate was saved for the investigation of hydrocarbon component. The urea adduct was mixed with distilled water until it dissolved. The lipid component was extracted with diethyl ether in a liquid-liquid extractor for 24 hr. The ether extract was evaporated to dryness on a Büchi Rotavap evaporator. The residue was weighed as a mixture of non-branched wax esters, and was tested by tlc (0.25 mm thick, Silica Gel H, 5x20 cm plate) using hexane-ether (9:1, v/v) as eluting solvent and authentic cetyl palmitate for comparison. For purification of cetyl palmitate, the residue was chromatographed on Florisil (20 g, 100/200 Mesh, Floridin Co., column diameter 2.5 cm) using hexane and then hexane-

diethyl ether (9:1, v/v) as eluting solvents. Ten ml fractions of the eluate were collected and each fraction was tested by tlc as above. All fractions, showing the same spot as that of the authentic cetyl palmitate, were combined, evaporated to dryness using a Büchi Rotavap, and weighed. The ir, nmr and mass spectra were determined as previously described.

Isolation, Identification and Determination of Hydrocarbons

The solution separated from the urea adduct was evaporated to a half volume using a Büchi Rotavap, mixed with water until two phases were formed, and then extracted with diethyl ether by liquid-liquid extraction for 24 hr. The ether extract was evaporated to dryness on a Büchi Rotavap evaporator, and the residue was weighed. The residue was chromatographed on Florisil (40 g for 1 g lipid, 100/200 Mesh, Floridin Co., column diameter 2.5 cm) using hexane as eluting solvent. Ten ml fractions of the eluate were collected and each fraction was tested by tlc (0.25 mm thick, Silica Gel H) using hexane as eluting solvent. Those fractions showing the same spots on tlc were combined, evaporated to dryness on a Büchi Rotavap evaporator. The amount of 25 mg hydrocarbon from P. REM St. Croix (tlc test showed 2 hydrocarbon spots) was applied to tlc (0.5 mm thick, Silica Gel H) and eluted with hexane. The chromatoplate was dried at room temperature for 30 min. The hydro-

carbon spots were scraped from the plate and eluted with 10 ml hexane. The solvent was evaporated by a Büchi Rotavap evaporator. Evaporation was continued under nitrogen and then in a vacuum desiccator for 24 hr. The hydrocarbon was weighed (Table 17) and the ir, nmr and mass spectra were determined as previously described. Sodium chloride disks were used as sample holders in the ir determination.

SUMMARY

Diterpenoid compounds, wax esters and hydrocarbons were examined from eggs of *Pseudopterogorgia americana* and five samples of *Plexaura*: *P*. "*REM*", St. Croix, *P*. "*REM*", San Cristobal, *P*. *kukenthali*, *P*. *homomalla*, and *P*. *flexuosa*. The lipid contents of *Plexaura* samples varied from 1.6% (g lipids/100 g dry weight animal) for *P*. *flexuosa* to 8.8% for *P*. *homomalla*. Crystalline diterpenoids were obtained only from *P*. "*REM*" St. Croix (0.61%) and from *P*. "*REM*" San Cristobal (0.09%), diterpenoid I mw 304 (a hydroxy-diketone) and diterpenoid II mw 306 (a dihydroxyketone).

Wax ester was isolated from the sample by urea inclusion followed by chromatography, and identified as cetyl palmitate: 45% cetyl palmitate in the lipids of eggs of *Ps*. *americana* (g cetyl palmitate/100 g dry weight animal); 1.03% for *P*. "*REM*" St. Croix; 2.13% for *P*. "*REM*" San Cristobal, 2.13% for *P*. *kukenthali*; and <0.1% for *P*. *homomalla* and *P*. *flexuosa*

Two hydrocarbon compounds, mw 204 (48%) and 220 (32%) were isolated from *P*. "*REM*" St. Croix. One hydrocarbon, mw 204 was isolated from the other four *Plexaura* samples: 7.68% hydrocarbon from lipids of *P*. "*REM*" San Cristobal; 0.04% from *P*. *kukenthali*; 0.25% from *P*. *homomalla*; and trace amounts from *P*. *flexuosa*.

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