STUDIES RELATED TO PALYTOXIN

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ABSTRACT

Malo[1] in his book on Hawaiian culture described the use of a potent toxin from a seaweed Limu-make-o-Hana, which was smeared on the spear tips of Hawaiian warriors. A collection of the material from Hana, Maui, Hawaii revealed that the toxic organism was a coelenterate and not an alga. It was tentatively identified as Palythoa vesititus Verril. Palytoxin, the toxic constituent of Palythoa was isolated as an amorphous white powder, $[\alpha]_D$ + 27° (water) and its homogeneity established by chromatography. Palytoxin has an empirical formula of ($C_{30}H_{50}NO_{13}$). On the basis of four nitrogen atoms the molecular formula is $C_{120}H_{200}N_4O_{52}$ (M Wt 2528). It could be converted to an acetate (24 or 25 acetate units), a benzoate (20 benzoate units), a bromobenzoate (20 bromobenzoate units) and other hydroxyl derivatives. Modified Kuhn Roth oxidation of the toxin furnished 1,5-pentanedicarboxylic acid, 1-decanoic acid, octane-1,8-dicarboxylic acid, and dodecanoic acid. Periodate or lead tetraacetate oxidation of palytoxin furnished seven compounds, for four of which structures consistent with spectral and chemical evidence have been written. Palytoxin consumed 33-34 moles or periodate and generated 20 equivalents of acid. A crystalline bromobenzoate, mp 84.5°, of a periodate product of palytoxin was prepared.

From aqueous effluents of toxic Palythoa six compounds were isolated by a combination of column, thin layer and paper chromatography.

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[1]Malo, David, 1951. Hawaiian Antiquities (Moolelo Hawaii). Translated from the Hawaiian by Dr. Nathaniel Emerson 1898, B. P. Bishop Museum Spec. Publ. 2, 2nd Ed., 1951, 278 pp.

Four of these were shown to be m-tyramine, homarine, anemonine and histamine. A similar study on Palythoa mammilosa furnished only p-tyramine and indicated the presence of p-tyrosine.

Thymine was found in the echinoids Ophiacoma insularia, Ophiacoma erinaceous, Echinothrix diadema, and Holothuria atra, while thymidine was found only in Ophiacoma insularia and Ophiacoma erinaceous. Cytosine was found only in Holothuria atra.

Fatty acid analysis of toxic Palythoa indicated the presence of C_6, C_8, C_{10}, C_{14}, C_{15}, C_{16}, C_{17}, C_{18}, C_{20}, and C_{21} saturated and C_{16}, C_{18}, and C_{20} unsaturated carboxylic acids. Orange pigments of toxic Palythoa were found to be essentially α- and β- carotenes.

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INTRODUCTION

Marine biotoxicology is concerned with poisons produced by marine animals and plants. A poisonous marine animal is one whose tissues are toxic either in part or in their entirety (e.g., puffers, ciguateric fishes), while a venomous animal is one which is capable of producing a poison in a highly developed secretary organ or group of cells (e.g., stingrays, Portuguese-Man-of-War).

A. Literature Survey

All major groups of marine animals include representatives which produce toxic secretions (1,2). These are largely complex mixtures of pharmacologically active amines, choline esters, peptides, proteins, and in some instances potent toxic compounds, of unusual structure. The toxic secretions generally result from cellular processes of the animal and are normally employed either for defense or food capture. Sometimes, however, toxins are derived by ingestion, as in case of lamellibranch mollusks and other filter feeders which thrive on the dinoflagellate Gonyaulax catenella, thereby resulting in the well known paralytic shellfish poison saxitoxin. Randall (3) has speculated that ciguatoxin is first synthesized by benthic blue-green algae and is subsequently concentrated in the viscera and flesh of herbivorous acanthurids or surgeon fishes. In turn, they are eaten by larger carnivorous fishes such as the snappers, groupers and barracudas where further concentration occurs in viscera and flesh. Some

toxins may result from bacteria inhabiting host animals but this needs to be substantiated.

Poisoning of victims is caused either by stinging, as in case of coelenterates which bear nematocysts, or by injection, as in case of gastropod mollusks by their highly modified radular apparatus with the teeth altered for injection of venoms (4), or by engulfing the prey with proboscus, thus allowing accumulation of glandular products in the immediate vicinity (5).

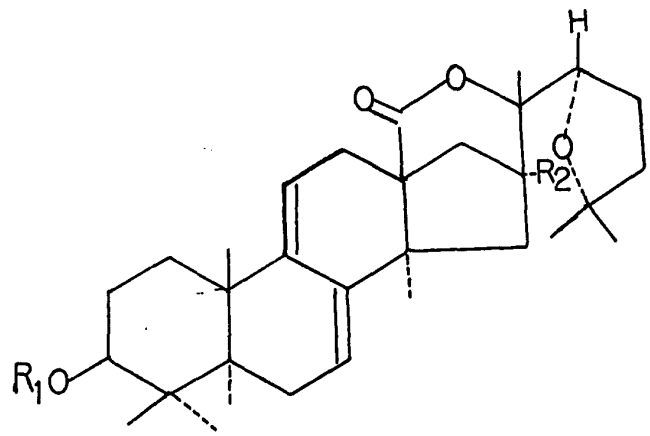
It has been mentioned that most toxic secretions contain pharmacologically active amines. A number of these amines, such as tetramine (I) (6,7), anemonine (II) (8), histamine (III) (9,10), tyramine (IX) (1), m-tyramine (V) (1), epinephrine (VI) (5), norepinephrine (VII) (5), dopamine (VIII) (11), homarine (IX) (6,9), trigonelline (X) (6), N-methylpyridinium ion (XI) (9,12), serotonin (XII) (9,11), γ-butyrobetain (XIII) (6,9), herzynine (XIV) (13) and ergothionin (XV) (13), have been identified over the years. While most of these amines along with "kinins," peptides and choline esters act as pain producers (defensive) and as facilitators of absorption and distribution of toxic components, the purpose of the presence of homarine (IX), trigonelline (X), γ-butyrobetaine (XIII) and some phosphogens such as glycocyamine (XVI) (14), phosphocreatine (XVII) (14), phosphotaurocyamine (XVIII) (14), phosphoarginine (XIX) (14), and phospholombricine (XX) (14) are not completely understood.

Several choline esters, in particular, acetyl choline (XXI) (15), urocanyl choline (XXII) (16,17), senecioyl choline (XXIII) (15), acryl choline (XXIV) (15,16) and pahutoxin (XXV) (18), have been found

in venoms of hydra, gastropod mollusks and the Hawaiian boxfish, Ostracion lentiginosus.

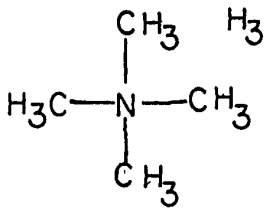
Distribution of these compounds in a few representatives of each phylum are summarized in Table I.

In addition to amines and choline esters marine animals elaborate some highly complex, labile and potent toxins which they normally use to paralyze their prey. Often these toxins are high molecular weight proteins (22,23,24,25,26). In recent years, however, some non-protein toxins (Tables II and III) such as aesterosaponins A and B (27-a,b), from Asteriasamurensis, holothurin B (27a) from Holothuria vagabunda and H. lubrica, tetrodotoxin (25,29), from Tetraodon stellatus, nereistoxin (30), from Lumbriconereis heteropoda and saxitoxin (31), from Saxidomus giganteus have been isolated and studied in some detail. Recently accomplished structure determinations of holothurin A (XXVI), tetrodotoxin (XXVII) and nereistoxin (XXVIII) are the highlights of the marine chemical literature.

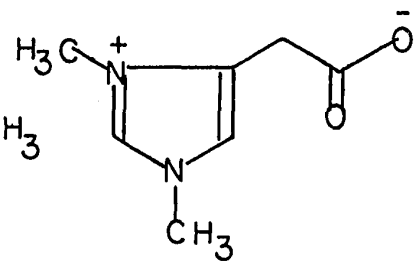


R_1 = H R_2 = OH 22, 25 Oxidohalothurinogenin
R_1 = guinovose -3-O-methyl glucose-glucose-xylose-sulfuric acid
R_2 = OH Holothin

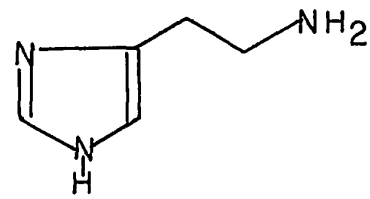
XXVI



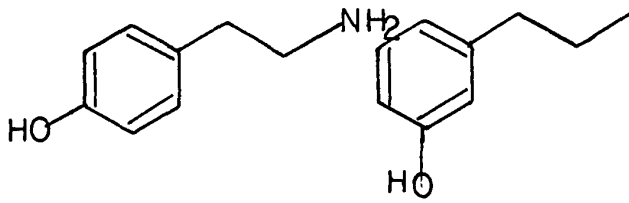
Tetramine
I



Anemonine
II

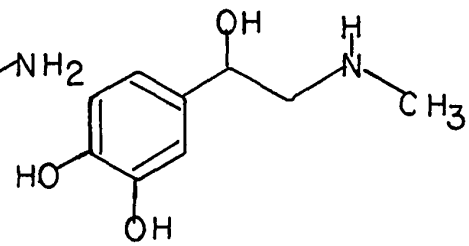


Histamine
III

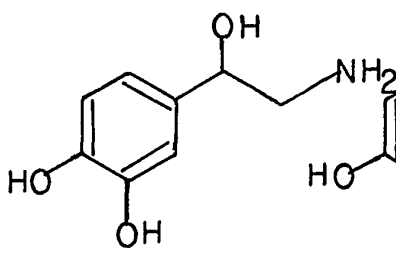


Tyramine
IV

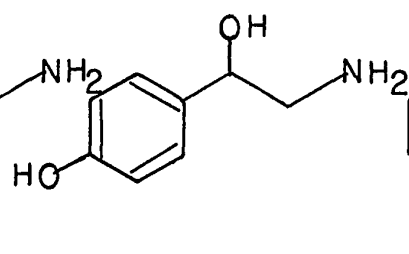
m-Tyramine
V



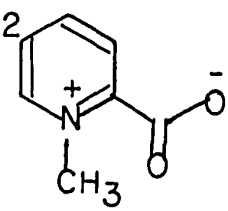
Epinephrine
VI



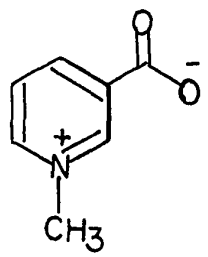
Norepinephrine
VII



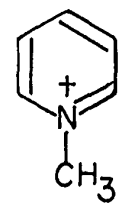
Dopamine
VIII



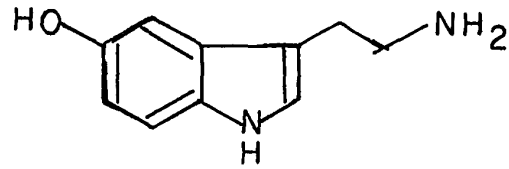
Homarine
IX



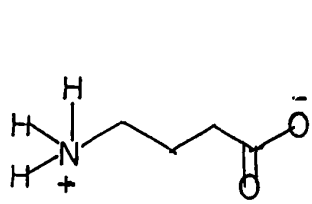
Trigonelline
X



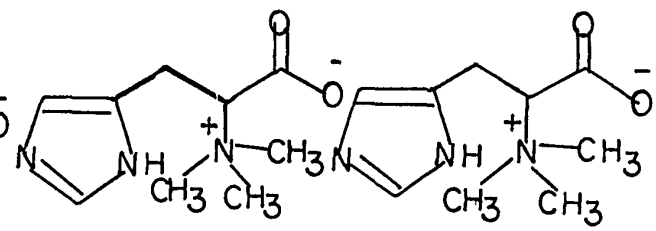
N-Methyl pyridinium
XI



Serotonine
XII

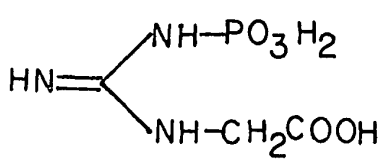


γ-Butyrobetaine
XIII

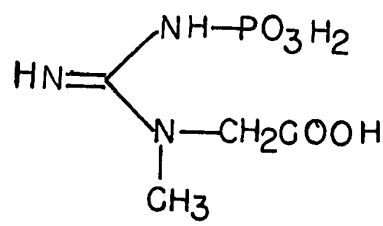


Herzynin
XIV

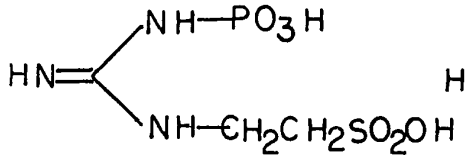
Ergothionein
XV



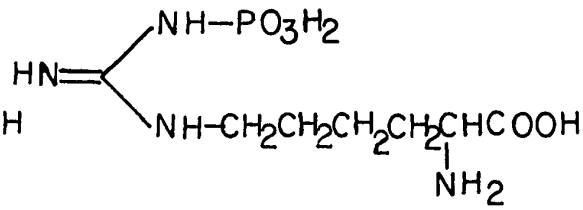
Phosphoglycocyamine
XVI



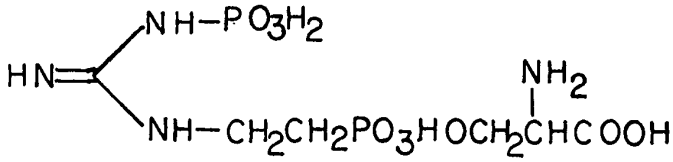
Phosphocreatine
XVII



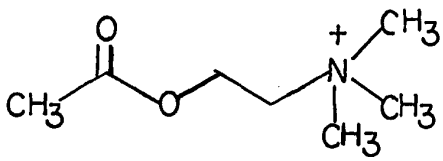
Phosphotaurocyamine
XVIII



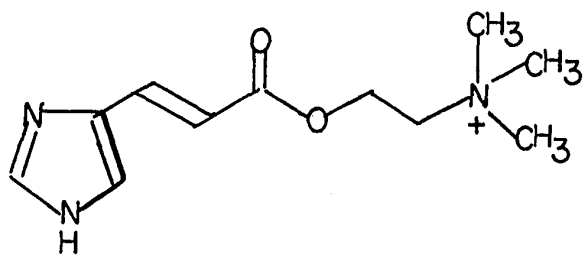
Phosphoarginine
XIX



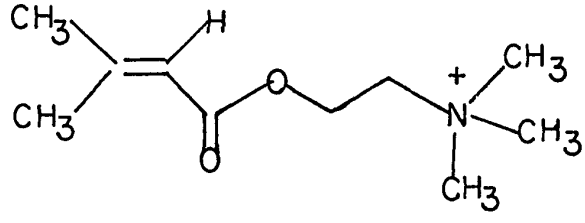
Phospholombricine
XX



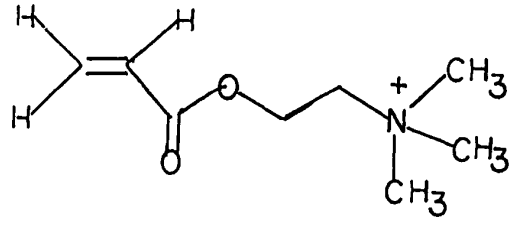
Acetylcholine
XXI



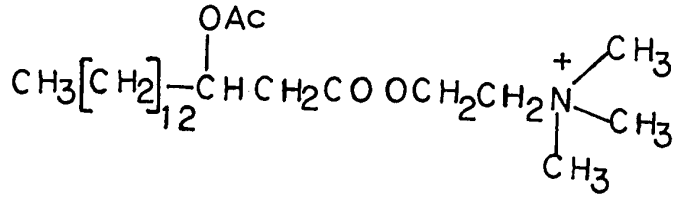
Urocanylcholine
XXII



Senecioylcholine
XXIII



Acrylylcholine
XXIV



Pahutoxin
XXV

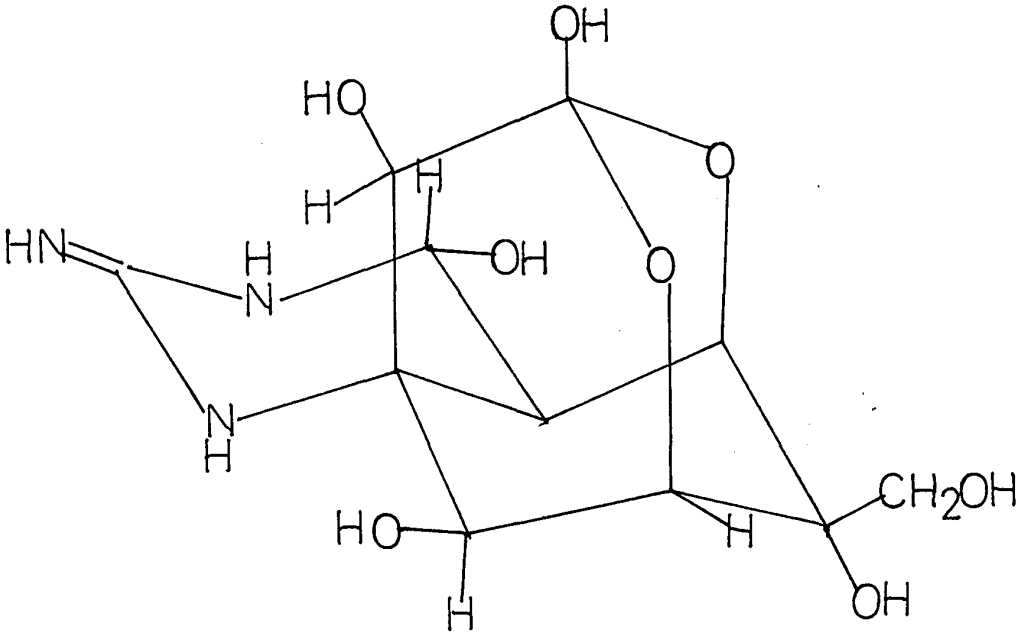
TABLE I

DISTRIBUTION OF PHARMACOLOGICALLY ACTIVE AMINES AND CHOLINE ESTERS IN SOME MARINE ANIMALS

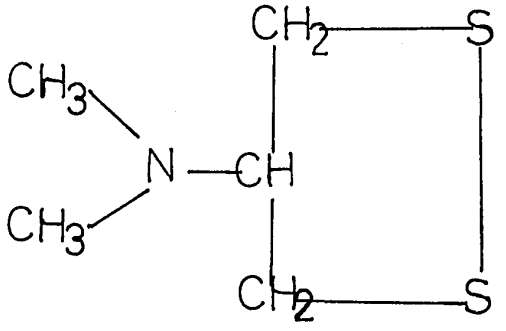
Classification	Observed Compounds
1. COELENTERATES	
Class: Hydrozoa	
Order: Siphonophores	
Family: Physaliidae	
Physalia physalis (6)	Homarine, Trimethyl -ammonium
Class: Anthozoa	
Order: Actinaria	
Family: Actiniidae	
Actinia equina (9)	N-Methyl pyridinium hydroxide, γ-Butyrobetaine, Homarine, 5-HT, and Histamine
Anemonia sulcata (8,9)	Zooanemonin, Homarine, and Histamine
Chondylactis gigantea (6)	γ-Butyrobetaine, Trigonelline, Homarine, and Urocanyl Choline
Subclass: Alcyonaria	
Order: Gorgonaceae	
Plexura flexuosa (6)	γ-Butyrobetaine, Trigonelline, Homarine, and Tetramethyl ammonium
2. MOLLUSKS	
Class: Gastropoda	
Order: Archaeogastropoda	
Family: Buccinidae	
Neptunea antigua (7)	Tetramine
Neptunea arthritica (19)	Tetramine
Order: Stenoglossa	
Family: Muricidae	
Murex trunculus (5,16)	Murexine and Serotonine

TABLE I - Continued

Classification		Observed Compounds
2. MOLLUSKS - Continued		
Class:	Pelecypoda	
Order:	Anisomyaria	
Family:	Mytildae	
	Mytilus edulis (9,12)	Adenine, Arginine, Neosine, Methyl-pyridinium hydroxide and Crangonine
Class:	Cephalopoda	
Order:	Octopoda suborder Cirromorpha	
Family:	Octopodidae	
	Eledone moschata (10)	Histamine
	Octopus bimoculatus (11)	Octopamine, Serotonine, Histamine and Dopamine
	O. vulgaris (5)	Serotonin, Acetyl choline and Tyramine
3. ECHINODERMS		
Class:	Asteroidea	
Order:	Forcipulata	
Family:	Asteriidae	
	Asterias forbesis (20)	Arginine phosphate
Class:	Echinodea	
Order:	Aluodonta	
Family:	Diadematidae	
	Echinothrix diadema (21)	Norepinephrine
	Echinothrix calamaris (21)	Norepinephrine
Order:	Camarodonta	
Family:	Strongylocentrotidae	
	Strongylocentrotus dröbachiensis (20)	Glucose-1-phosphate, Arginine phosphate



XXVII



XXVIII

Among protein toxins, only the structure of eledosine, a peptide from Cephalopod Eledone moschata and E. aldrovandi has been determined and proved by synthesis (XXIX) (26, 32).

H-Pyr-Pro-Ser-Lys-Asp (OH)-Ala-Phe-L-leu-Gly-Leu-Met (NH_2)

Eledosine

XXIX

TABLE II

DISTRIBUTION OF TOXINS IN SOME MARINE ANIMALS

Classification		Observed Compounds
1. PROTOZOA		
Class:	Mastigophora	
Order:	Dinoflagellata	
Family:	Gymnodiniidae	
	Gymnodinium breve (Davis) (33)	$C_{90}H_{162}O_{17}P$ $[\alpha]_{589}^{25°}$ +68°
2. COELENTERATES		
Class:	Hydrozoa	
Order:	Siphonophora	
Family:	Physaliidae	
	Physalia physalis (22)	Protein LD_{50} 200 µg/kg
Class:	Scyphozoa	
Order:	Cubomedusa	
Family:	Chiropidae	
	Chironex fleckri (23)	Not isolated, causes cardiac arrest
Class:	Anthozoa	
Order:	Actinaria	
Family:	Actiniidae	
	Actinia equina (9)	Histamine
	Anemonia sulcata (8,9)	Histamine and Anemonine
3. ANNELIDS		
Class:	Polychaeta	
Order:	Errantia	
Family:	Lumbrineridae	
	Lumbrioconereis heteropoda (30)	Nereistoxin (disulfide)

TABLE II - Continued

Classification	Observed Compounds
4. MOLLUSKS	
Class: Gastropoda/subclass Prosobranchia	
Order: Archaeogastropoda	
Family: Conidae	
Conus geographus (2)	Protein, varies in color from white to black
Family: Buccinidae	
Neptunea antiqua (7)	Tetramine
Neptunea arthritica (19)	Tetramine
Order: Stenoglossa	
Family: Muricidae	
Murex trunculus (16)	β-[imidazolyl-4-(5)] acryl choline
Class: Cephalopoda	
Order: Octopoda/suborder Cirromorpha	
Family: Octopodidae	
Eledone moschata (14.32)	"Eledosine" peptide
E. aldrovandi (14.32)	Eledosine
Octopus bimaculatus (70)	
O. vulgaris (34)	Glycoprotein nature uncertain
Class: Pelecypoda	
Order: Palaeoconcha	
Family: Venerdae	
Saxidomus gigantea (31)	Saxitoxin
5. ECHINODERMS	
Class: Asteroidea	
Order: Forcipulata	
Family: Asteriidae	
Asterias amurensis (27a,b)	Asterosaponin A Asterosaponin B
Class: Echinodea	
Order: Aluodonta	
Family: Diadematidae	
Echinothrix diadema E. calamaris	Injury caused by epidermal investment of calcarious spines

TABLE II - Continued

Classification	Observed Compounds
5. ECHINODERMS - Continued	
Order: Camarodonta	
Family: Toxopneustidae	
Tripneustes gratilla (35) from globiferrous pedicellaria	Protein LD_{50} 10-15 µg/kg
Class: Holothuriodea	
Order: Aspidochirota	
Family: Holothuriidae	
Actinopyga agassizi (28)	Holothurin A
Holothuria vagabunda (27a)	Holothurin B
Holothuria lubrica (27a)	Holothurin B
6. CHORDATES	
Class: Osteichthyes	
Order: Anguilliformes	
Family: Muraenidae	
Gymnothorax javanicus (36)	Ciguatoxin Lipid LD_{50} 100-500 µg/kg
Order: Tetraodontiformes (Plectognathi)	
Family: Tetraodontidae	
Tetraodon stellatus (25)	Tetrodotoxin
Sphaeroides rubripes (25,29)	Complex, polycyclic
Family: Ostraciontidae	
Ostracion lentiginosus (18)	Pahutoxin Choline ester

TABLE III

RELATIVE TOXICITIES OF A SELECTED GROUP OF TOXIC COMPOUNDS

	Toxin	MD µg/Kg	Source	Form of Composition	M.Wt.	Ref.
1	Botulinus Toxin A	0.00003	Bacterium Clostridium Botulinum	Crystalline	900,000	37
2	Tetanus Toxin	0.0001	Bacterium Clostridium Tetani	"	100,000	37
3	Ricin	0.02	Plant Castor Bean	--	72,000	38
4	Cobra Neurotoxin	0.30	Snake, Naja Naja	--	--	38
5	Crotalus Toxin	0.20	Snake, Rattle Snake Non-Protein	--	--	38
6	Palytoxin	0.75	Coelenterate Palythoa vestitus Verril	Non-crystalline	2000-3000	
7	Kokoi Venom	2.7	Frog Phyllobates bicolor	--	400	39, 40
8	Tarichatoxin	8.0	Newt Taricha torosa	$C_{11}H_{17}N_3O_8$	319	41
9	Tetrodotoxin	8.0-20	Fish Sphaeroides	$C_{11}H_{17}N_3O_8$	319	25, 29
10	Saxitoxin	9.0	Shellfish - from Dinoflagellates G. Catenella	$C_{10}H_{17}N_7O_4$	372	
11	Pedicellaria	10-15	Tripneustes gratilla	--	--	35

TABLE III - Continued

	Toxin	MD µg/Kg	Source	Form of Composition	M.Wt.	Ref
12	Physalia	200	Physalia physalis	--	--	22
13	Ciguatoxin	100-500	Gymnothorax javanicus	--	≃+500	36
14	Bufotoxin	390	Toad: Bufo vulgaris	$C_{40}\ H_{60}\ N_4\ O_{10}$	757	38
15	Curare	500	Plant: Chondodendron tomentosum	$C_{38}\ H_{44}\ N_2\ O_6\ Cl_2$	696	38
16	Strychnine	500	Plant: Strychnos nux vomica	$C_{21}\ H_{22}\ N_2\ O_2$	334	38
17	Muscarine	1100	Mushroom Amanita	$C_9\ H_{20}\ O_2\ N\ Cl$	210	--
18	Samandarin	1500	Salamander, Salamandra	$C_{19}\ H_{23}\ N\ O_2$	397	42
19	Diisopropyl Fluoro-phosphate	3000	Gas Synthetic	$(C_3\ H_7)_2\ PO_3F$	184	--
20	Sodium Cyanide	--	Synthetic	NaCN	49	38
21	Holothurin A	8000-10,000	Actinopyga agassizi		1150	28

In conclusion, it must be emphasized that a study of the chemical nature and the mechanism of action of the toxic factors would lead us to effective treatment of human poisoning and to a better understanding of biological processes at the molecular level. Furthermore, powerful toxic substances have frequently become valuable tools in the study of mechanisms of drug action.

B. Research Objective

David Malo in "Hawaiian Antiquities" (43), described the use of a potent toxin which was smeared on spearpoints of Hawaiian warriors. This early account led marine biologists at the Hawaii Institute of Marine Biology and Professor Paul J. Scheuer at the Department of Chemistry to investigate. After a lengthy search they located an extremely toxic coelenterate, confined to a single tidepool in Hana, Maui. It was tentatively identified as Palythoa vestitus Verril. Preliminary study of the toxic principle indicated that the compound was not a protein. The unusual nature and relatively large size of the toxin attracted the author, thus resulting in the present venture of structure determination of palytoxin.

Our observation that some UV active material accompanies palytoxin in the isolation scheme, prompted us to examine them in a hope to find a possible relationship with palytoxin.

In the early stages of our work, extracts of some echinoderms became available in connection with a study of their sterol content in our laboratory. We decided to examine them for UV active water soluble constituents.

C. Acknowledgments

I wish to thank the East West Center for supporting this research by granting an I.S.I. scholarship to the author.

I extend my special thanks to Professor Paul J. Scheuer, my research advisor for his kind advice, patience and understanding during many phases of this work.

Grateful acknowledgment is also made to the following people.

Drs. Kishan C. Gupta for providing us Echinoderm aqueous extracts; Takeshi Yasumoto and Richard E. Moore for many fruitful suggestions; Dr. A. M. Duffield and Professor Carl Djerassi for high resolution measurements on some of our samples, and Mr. K. N. S. Menon for his valuable assistance and suggestions with 100 MHz nuclear magnetic resonance spectra; Dr. Rodney A. Badger for molecular weight measurements and some delightful discussions about photography; and Drs. Otto D. Hensens, Stephen C. Havlicek, and Mr. Nobuhiro Fusetani for providing background music in our laboratory.

CHAPTER II

EXPERIMENTAL SECTION

A. General Information

Melting points were determined on a Fisher-Johns melting point apparatus and are uncorrected.

Optical rotations were measured using an ETL-NPL automatic polarimeter Type 143A.

Ultraviolet (UV) absorption spectra were recorded on an Applied Physics Corporation, Cary 14, Recording Spectrophotometer. Less often, UV spectra were recorded on a Beckmann, D.B. spectrophotometer.

Infrared spectra were run in potassium bromide, in chloroform or as thin films on a Beckmann IR-5 automatic recording spectrophotometer. All infrared intensities are designated as strong (S), medium (M), weak (W), broad (b) and shoulder (Sh).

Nuclear magnetic resonance spectra were recorded mostly on a Varian A-60 (60 MHz) analytical proton spectrometer, and less frequently on a Varian HA-100 (100 MHz) instrument. A 220 MHz spectrum of palytoxin was recorded by Roy L. Johnson at Varian Associates, Palo Alto, California.

The mass spectra, unless otherwise stated, were recorded by Sr. Mary Roger (Brennan) on a Hitachi-Perkin-Elmer RMU- 6D mass spectrometer at the University of Hawaii using a direct inlet system or less frequently by Mr. R. G. Ross on an AEI-MS9 mass spectrometer at Stanford University. All high resolution measurements on various

samples were carried out by Mr. R. G. Ross on an AEI-MS9 mass spectrometer, using $C_{12}F_{27}N$ as an external standard at Stanford University.

All elemental analyses were carried out at Berkeley Analytical Laboratory, California. Analysis for C-CH_3 in palytoxin, acetate-analysis for palytoxin acetate and the Rast molecular weight determination for palytoxin acetate were carried out at Alfred Bernhardt, Mikroanalytisches Laboratorium in Max-Planck Institut at Mülheim in West Germany.

Potentiometeric titrations were carried out by Dr. M. Gorman, at the Lilly Research Laboratories in Indianapolis, Indiana.

All analytical gas chromatography was carried out on a Beckmann GC5 gas chromatograph using a flame ionization detector. The preparative gas chromatography was carried out on an Aerograph, Model 200 gas chromatograph, equipped with a 1 to 10 splitter and a hydrogen flame detector. Less frequently a Varian Aerograph Model 705 gas chromatograph, equipped with a flame ionization detector was used for preparative gas chromatography.

Column chromatography was conducted on Sephadex gels, Dowex cation and anion exchangers, Amberlite MB3, silicic acid, silica gel, alumina, kieselguhr, celite and Microcel C. In all cases, unless otherwise mentioned, the directions provided by the manufacturers were used for preparation of columns. The fractions were usually assayed by dichromate oxidation, quantitative ultraviolet spectra and analytical thin layer and paper chromatography. All thin layer chromatograms were run on 20 x 5 and 20 x 20 cm glass plates, spread with alumina H or silica gel (H and HF) or kieselguhr G at a thickness of 250μ. For

preparative thin layer chromatography, plates spread at a thickness of ca. 300μ were used. The plates were allowed to stand in cyclohexane-ethyl acetate (70:30) overnight, the edges scraped off and the plates activated at 110°C for fifteen minutes prior to use for preparative work. The plates were prepared according to the method of Stahl (71) using a Desaga/Brinkmann variable applicator (Brinkmann Instrument, Inc.). The chromatograms were visualized by spraying the plates either with 50% sulfuric acid in 95% ethanol chlorosulfonic acid or 10% phosphomolybdic acid in 95% ethanol. Analytical and preparative paper chromatography was carried out on Whatman No. 1 paper by the ascending technique. Paper chromatograms, unless otherwise mentioned, were visualized using a silver nitrate dip reagent.

Most of the solvents were purified by conventional means and dried whenever required over a suitable drying agent. All reagent grade solvents were twice fractionated through a 1' fractionating column, and stored in the dark. Petroleum ether (30 - 60°) and cyclohexane were washed with concentrated sulfuric acid, water, 20% sodium hydroxide, dried over sodium hydroxide pellets and finally distilled.

Molecular weights were determined by vapor pressure osmometery using a Hitachi-Perkin-Elmer Model 15 molecular weight apparatus.

B. Procurement of Animals

Toxic zoanthid material was collected from various locations (at different stages of this work). During the early stages of the work and for the standardization of the isolation procedure, the collections were

made from a tidepool, near Hana, Maui, by Dr. Richard E. Moore. During the latter stages of the work, toxic zoanthid material was collected from Atiamono pass, Tahiti from a depth of 40 feet. Initially, the toxin was isolated in this laboratory by the author, Miss Cirvalina Ramos and Dr. James E. Burcsu, but relatively larger quantities of palytoxin became available at later stages of this work through United States Army Laboratories at Edgewood. Unless otherwise mentioned, the toxin used for the structural work was derived from the Tahitian source. All batches were checked for purity by quantitative ultraviolet spectroscopy, analytical paper chromatography, toxicity and in some cases, column chromatography. Palythoa mammilosa was collected from Black Point, Diamond Head Beach Park, Oahu, from a depth of 1 to 3 feet. A few species representing the main classes of Echinodermata were also procured (See Table IV) with the help of Dr. Kishan C. Gupta for structural studies of their water soluble components.

C. Isolation of Palytoxin

1. From Tahitian Zoanthid Material

The polyps (net weight 4.278 kg collected in June 1966 from Atiamona pass, Tahiti) were poured into two large chromatographic columns and soaked in enough ethanol (95%) to cover the animals. After 2 days the columns were drained off and the polyps soaked in 70% ethanol for another two days. The process was repeated twice with 50% ethanol until the extract was almost green. The animals were then ground in a Waring Blendor with 50% ethanol and the extract removed by suction filtration using Celite as a filter aid. The combined

extracts were evaporated to a small volume (500 ml) under vacuum. The concentrate was extracted three times with benzene (200 ml each). The benzene extract (A) was backwashed with water (100 ml). The water wash and the concentrate were combined and extracted twice with 1-butanol (50 ml).

TABLE IV

SOME ECHINODERMS, COLLECTION DATA

Code No.	Genus and Species	Class	Location
Er	Ophiacoma erinaceous	Ophiuroidea	Makaha, Oahu
Ins	Ophiacoma insularia	Ophiuroidea	Makaha, Oahu
Ech	Echinothrix diadema	Echinoidea	Kaneohe Bay, Oahu
Ha	Holothuria atra	Holothuroidea	Kailua Bay, Oahu

a. Chromatography of Water Extract (C) on Polyethylene

The aqueous extract was freed of dissolved butanol in vacuo at ca. 50° and concentrated to a volume 500 ml. It was then passed over two 4x30 cm columns of 200 mesh polyethylene (suction required).

The polyethylene column was prepared by washing Dow Chemical Company's experimental resin Qx 2187 with 5% chloroform in methanol, 95% ethanol, 75% ethanol, 50% ethanol and finally doubly distilled water. The columns were packed with a slurry of washed polyethylene resin in 50% ethanol, followed by a 1/2" thick layer of prewashed sand

at the top. The columns were finally washed with doubly distilled water and used.

The columns were thoroughly washed with doubly distilled water (1 liter each). The eluents and water washings were combined and passed over another 200 mesh polyethylene column (4.0x30 cm). The aqueous effluents and water washes were saved for the study of UV active water soluble constituents.

Finally, the adsorbed toxin was removed from the three columns by elution with 50% ethanol in water (50 ml each). The mixture separated into a brown band consisting of the toxin, inorganic salts and very polar organic material followed by two orange bands and a green band. The toxin fractions were combined and evaporated to a greenish brown glassy mass.

b. Further Purification of Palytoxin on Sephadex Anion Exchange Gel

A column of the anion exchanger DEAE-Sephadex A-25, medium, was prepared before use by successive washings with 0.5N sodium hydroxide, water, 0.1M phosphoric acid, 0.2M sodium phosphate pH 7.0 buffer and finally 0.02M sodium phosphate pH 7.0, until the effluent had pH 7.0.

The crude toxin, a green glassy mass, was dissolved in 1 ml of 0.02M sodium phosphate pH 7.0 buffer and the solution was introduced onto a 2x33 cm column. Elution was continued with 0.02M sodium phosphate pH 7.0 buffer and fractions (5 ml) were collected and assayed for toxicity towards mice, and by dichromate oxidation. Fractions 8 to 10 representing the most toxic fractions were combined and passed over

a 200 mesh polyethylene column (4x30 cm). The column was thoroughly washed with doubly distilled water and the desalted toxin eluted with 50% ethanol-water.

Fractions 11 to 15 were combined, desalted as described above and the material recycled over a DEAE-Sephadex A-25 column (2.0x33 cm).

The total yield of the toxin from the desalted fractions 8 to 10 was 900 mg having a purity of ca. 50%. The recovery of toxicity was greater than 90%.

c. Final Purification of Palytoxin on Sephadex Cation Exchanger Gel

The column of cation exchange gel CM-Sephadex C-25, medium, was prepared before use by successive washing with 0.1N hydrochloric acid, water, 0.5M sodium dihydrogen phosphate and finally 0.02M sodium dihydrogen phosphate.

The 50% pure palytoxin (900 mg) was dissolved in 0.02M sodium dihydrogen phosphate (5 ml) and the solution was introduced onto a CM-Sephadex C-25 column (2.0x35 cm). Elution was continued with 0.02M sodium dihydrogen phosphate. Fractions (5 ml) were collected and assayed for toxicity towards mice, dichromate oxidation and ultraviolet absorption spectrum. Fractions 35 to 56 were combined and desalted over a 300 mesh polyethylene column (4.0x30 cm). The recovery of the toxicity was only 15%, but the toxin could be rechromatographed on the cation exchanger with essentially no loss of toxicity. The yield of palytoxin was 123 mg. (0.002875%), in the form of a white glass. The white glass on freeze-drying furnished a hygroscopic white amorphous powder.

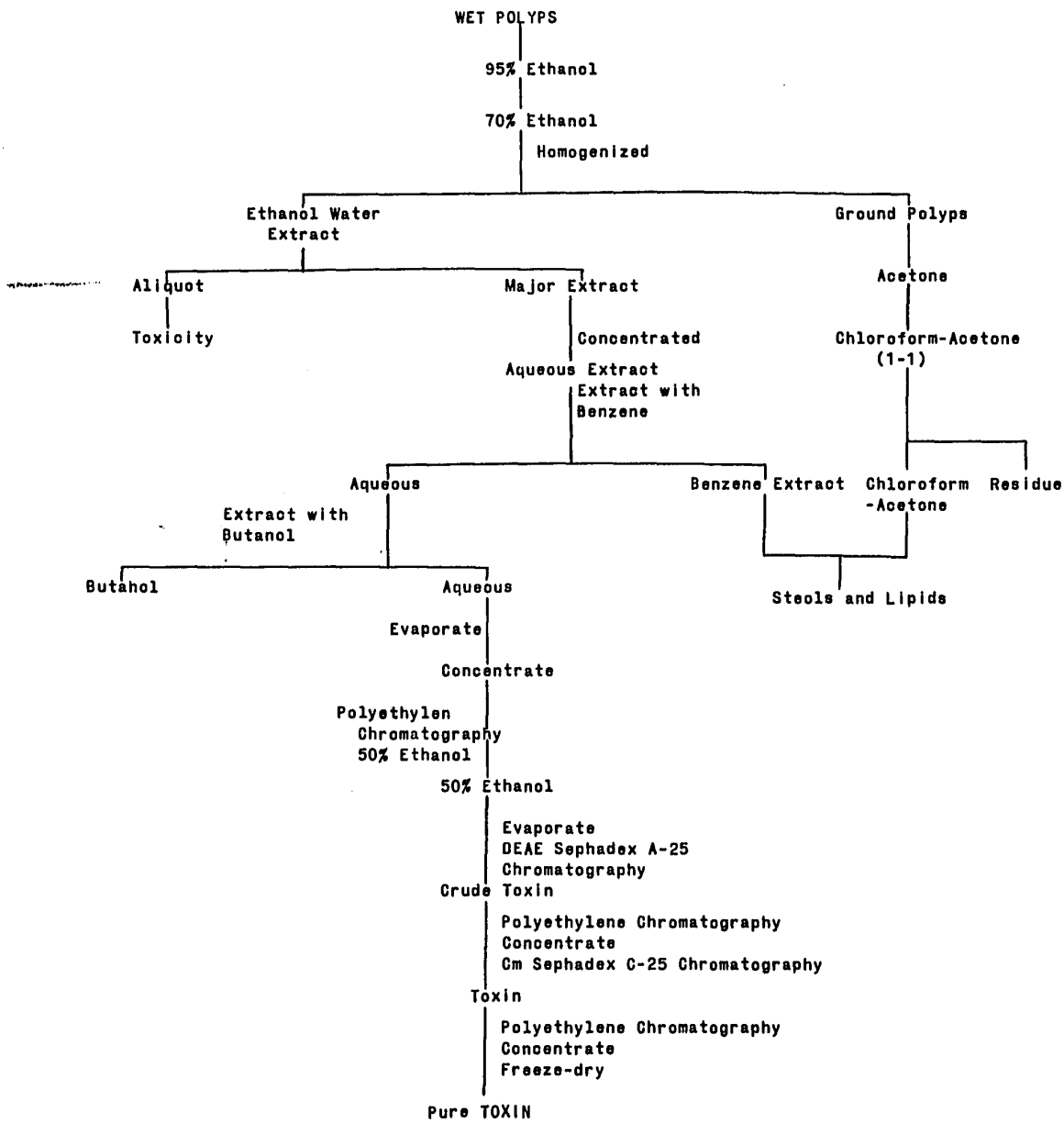


Fig. 1. Isolation Scheme of Palytoxin

2. Purification of Jamaican Palytoxin by Gel Filtration

A sample of palytoxin (76.0 mg) isolated from Jamaican toxic zoanthids was purified by repeated chromatography over a Sephadex G-25 (Fine) column (2.0x84 cm). Elution was continued with doubly distilled water. Fractions (11.5 ml) were collected and assayed by ultraviolet spectroscopy. Initial chromatography gave the following distribution by weight of toxin.

Fractions 13 - 20	(D-A)	7.0 mg.
Fractions 21 - 30	(D-B)	5.0 mg.
Fractions 31 - 40	(D-C)	15.0 mg.
Fractions 41 -	(D-D)	27.0 mg.

D-A showed maxima at 263nm and 233nm in its ultraviolet spectrum with a ratio different from that of palytoxin. D-B was essentially transparent. D-C corresponded to impure palytoxin, while D-D appeared by ultraviolet spectrum to be pure toxin.

D-D was rechromatographed over Sephadex G-25 (Fine) column (2.0x90 cm). Fractions (11.5 ml) were collected and assayed by ultraviolet spectroscopy. Fractions 12 to 15 were combined and rechromatographed over the same column to furnish the pure toxin (ca. 20 mg).

D-A (7.0 mg) was rechromatographed over a Sephadex G-25 (Fine) column (2.0x88 cm). Elution was continued with doubly distilled water. Fractions (9.8 ml) were collected and assayed by ultraviolet spectroscopy and combined as follows:

Fractions 12 to 16	5.0 mg.
Fractions 19 to 21	2.0 mg. (palytoxin)

Fractions 12 to 16 were designated palytoxin II.

D. Homogeneity of Palytoxin

1. Paper Chromatography of Palytoxin

Palytoxin could be chromatographed on Whatman No. 1 paper (silica gel H and kieselguhr G) with a variety of solvent systems. In each case only one spot could be detected. The results are summarized in Table V.

2. Thin-Layer Chromatography of Palytoxin

The results are shown in Table VI.

3. Column Chromatography of Palytoxin

Palytoxin, purified according to the above isolation scheme was chromatographed over Sephadex G-25 (Fine) (1.5x75 cm) and CM-Sephadex C-25 (medium) columns (2.0 x 33.5 cm). Elution was continued with doubly distilled water and 0.02M sodium dihydrogen phosphate respectively. Fractions (7.5 ml and 10 ml) were collected and assayed by dichromate oxidation and ultraviolet absorption spectra. Column chromatograms indicated the presence of only one symmetrical peak in each case. In case of Sephadex G-25 (Fine) column chromatography palytoxin eluted between 75 to 115 ml, whereas in case of CM-Sephadex C-25 column chromatography, the toxin eluted between 180 to 260 ml.

4. Countercurrent Distribution of Palytoxin

Palytoxin (25 mg) was subjected to countercurrent distribution in a 100-tube Craig apparatus using a 1-butanol-water system. The volume of the upper phase was ca. 1.4 times greater than that of the lower. The partition coefficient for the distribution of palytoxin between 1-butanol and water was calculated to be 0.21 (based on UV).

TABLE V

PAPER CHROMATOGRAPHY OF PALYTOXIN

Solvent Systems	R_F-Values
Ethanol-Water (30:70)	0.90
1-Propanol-Butyl acetate-Water (7:1:3)	0.37
1-Propanol-Amyl acetate-Water (7:1:3)	0.30
Isobutyl alcohol-Ethyl acetate-Water (7:1:3)	0.58
1-Butanol-Acetic acid-Water (4:1:2)	0.44
Ethyl acetate-Pyridine-Water (2:1:2)	0.31
2-Propanol-Pyridine-Acetic acid-Water (8:8:1:4)	0.58
1-Butanol-Acetic acid-Water (4:1:5)	0.391
1-Butanol-Pyridine-Water (45:25:40)	0.479

TABLE VI

THIN LAYER CHROMATOGRAPHY OF PALYTOXIN

Solvent System	R_F-Values	
	Kieselguhr G	Silica Gel H
n-Amyl alcohol-Pyridine-Water (7:7:6)	0.850	0.150
1-Butanol-Pyridine-Water (9:5:8)	0.970	0.230
Ethyl acetate-Pyridine-Water (2:1:2)	0.910	0.860

After 300 transfers the experiment was stopped and the ultraviolet absorption spectrum of the aqueous layer of each tube was examined. The countercurrent distribution chromatogram depicted essentially a symmetrical peak corresponding to tubes 40 to 100.

E. Spectral Characteristics of Palytoxin

1. Ultraviolet Absorption Spectra of Palytoxin

Quantitative ultraviolet spectra of palytoxin were run in distilled water, 0.5N sodium hydroxide and 0.5N hydrochloric acid (Fig. 2).

In distilled water:

- λ_{max} 263nm (ε 8.4xM)
- λ_{min} 255nm (ε 8.0xM)
- λ_{max} 233nm (ε 14.1xM).
- λ_{min} 210nm (ε 7.1xM).

In 0.5N hydrocholoric acid:

- λ_{max} 263nm (ε 2.5xM),
- λ_{min} 255nm (ε 2.5xM),
- λ_{max} 233nm (ε 12.8xM).
- λ_{min} 210nm (ε 6.9xM).

In 0.5N sodium hydroxide:

- λ_{max} 222nm (ε 13.6xM)
- λ_{min} 220nm (ε 13.0xM)

2. Infrared Spectrum of Palytoxin

The spectrum was determined in KBr (Fig. 3) and had the following bands:

3300 (s), 2900 (s), 2850 (sh), 2700 (sh), 1650 (m),
1575 (sh), 1490 (sh), 1430 (b), 1370 (b), 1320 (broad),
1200 (w), 1060 (s), 892 (w), 855 (b) cm^{-1}.

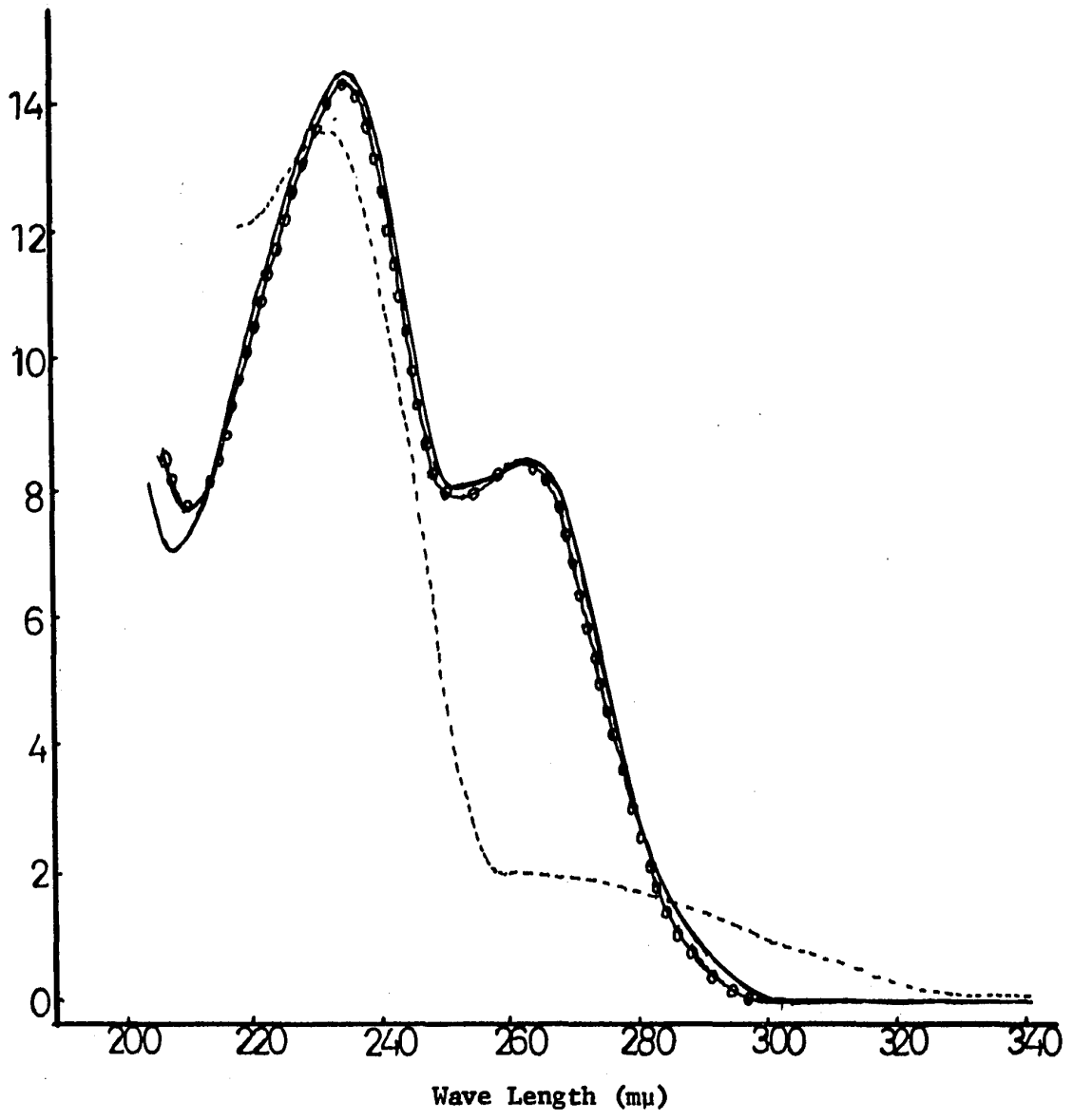


Fig. 2. Quantitative U.V. Spectrum at p.H. 13(----), p.H. 1(_____), and p.H. 6.96 (_____o_____o_____).

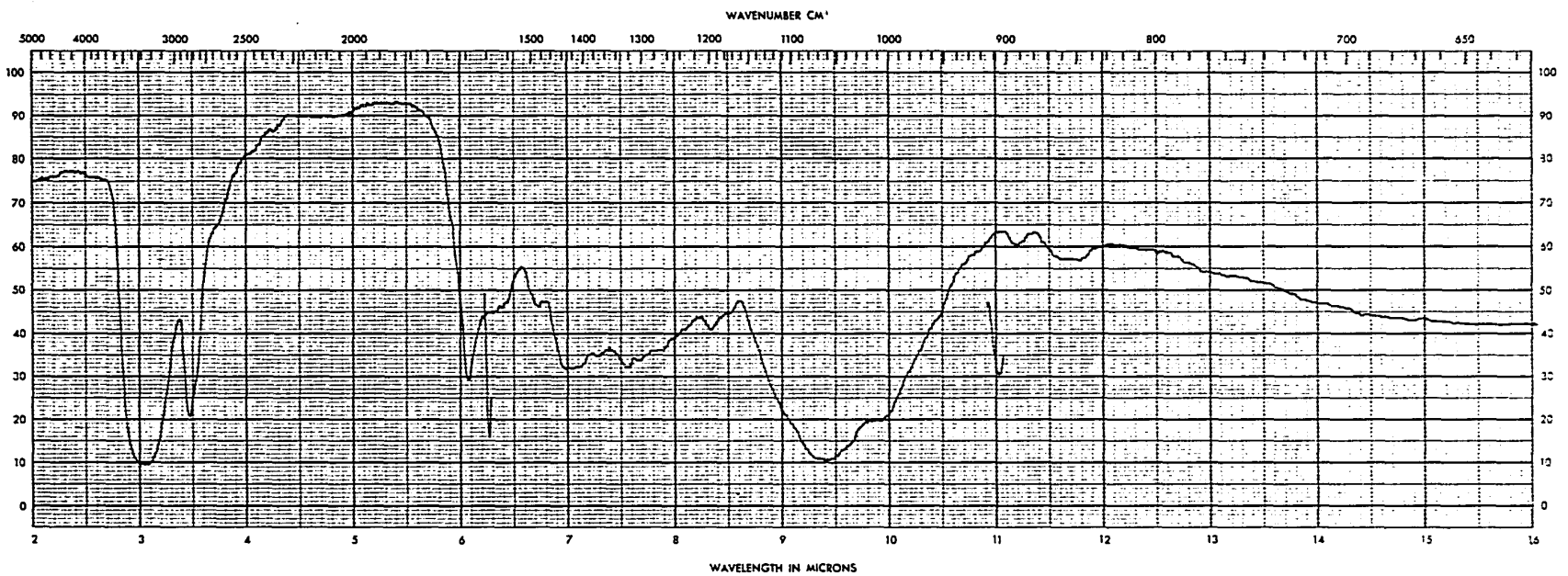


Fig. 3. Infrared Spectrum of Palytoxin in Potassium Bromide

3. Specific Optical Rotation

A palytoxin sample had $[\alpha]_D$ + 27° (c 1.00, water).

4. Nuclear Magnetic Resonance Spectra of Palytoxin

The nuclear magnetic resonance spectra of palytoxin were run in pyridine-d_5 (HA100, Fig. 5, Table VIII; 220 MHz, Fig. 4, Table VII).

F. Elemental Analyses of Palytoxin

A sample of Tahitian palytoxin was purified by preparative paper chromatography over Whatmann No. 1 paper followed by column chromatography over polyethylene Q x 2187 resin. Finally the sample was dried under vacuum.

Calcd. for $C_{30}H_{60}NO_{15}$:

C, 53.46; H, 8.97; N, 2.08; O, 35.61.

Found: C, 54.06, 53.94; H, 8.85, 8.97; N, 2.06, 2.12

A 6.5 mg sample of purified palytoxin (D-D) after drying at 80° under vacuum for 6 hr was analyzed.

Calcd. for $C_{33}H_{52}NO_{16}$:

C, 51.85; H, 7.38; N, 1.97; O, 35.56

Found: C, 52.19, 52.34; H, 7.44, 7.60; N, 1.66, 1.83; S, 0.05

G. Chemical Properties of Palytoxin

1. Sensitivity of Palytoxin towards Heat

Palytoxin (2.0 mg, λ_{max} 263 16.8xM) was heated in water (10 ml) to 100° for 3 hr. The quantitative ultraviolet spectrum of the solution indicated that no palytoxin had decomposed.

TABLE VII

220 MHz NMR SPECTRUM OF PALYTOXIN IN PYRIDINE-d_5

Nature of Signal	Chemical Shift	Relative Number of Protons
Singlet	0.863 δ	3
Singlet	1.000 δ	3
Singlet	1.159 δ	17
Singlet	1.227 δ	3
Complex (broad)	1.369 δ	10
Complex (broad)	1.639 δ	17
Complex (broad)	2.386 δ	3-4
Complex (broad)	2.772 δ	6
Complex (broad)	3.773 δ	9
Complex (broad)	4.318 δ	27-28
Complex (broad)	5.040 δ	7
Singlet (broad)	5.9318 δ	52

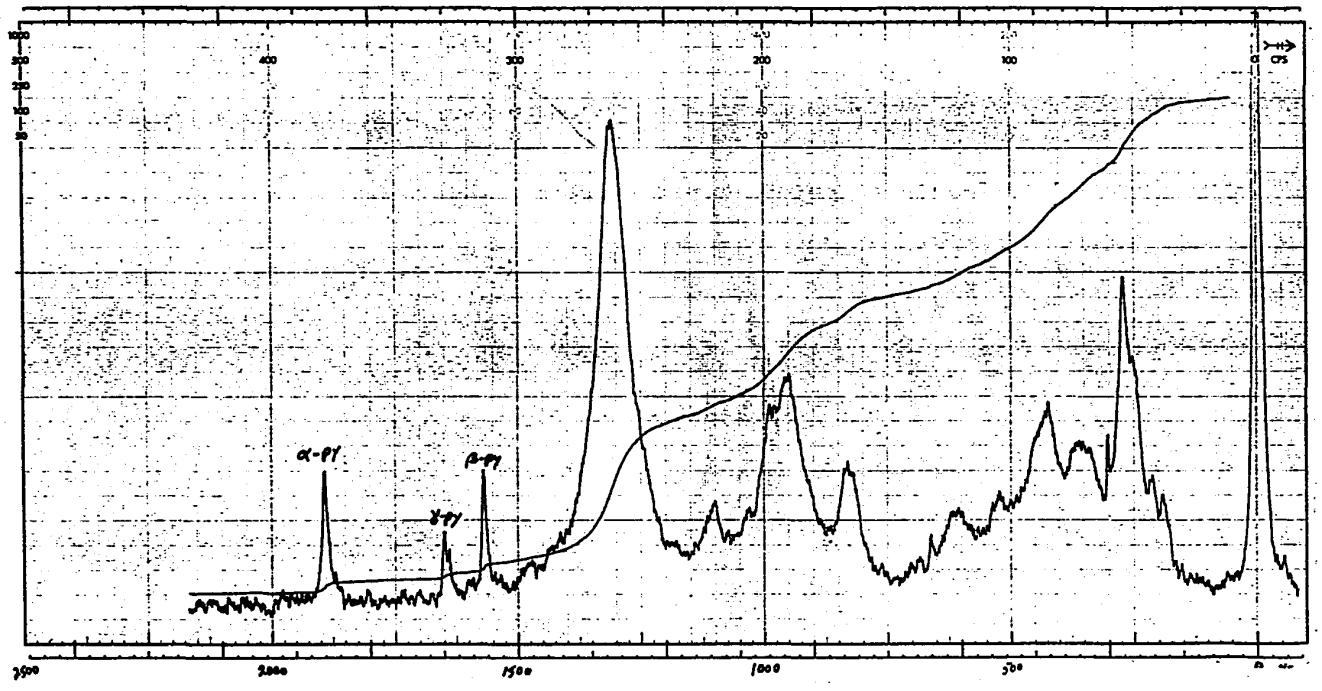


Fig. 4. 220 MHz Nuclear Magnetic Resonance Spectrum of Palytoxin.

TABLE VIII

100 MHz NMR SPECTRA OF PALYTOXIN,

δ VALUES IN DIFFERENT SOLVENTS

Pyridine-d_5	Dimethyl Sulfoxide-d_6
	0.60 (d, J=6.0 Hz)
0.98 (c)	1.00 (s,b)
1.45 (c)	1.36 (c)
1.78 (c)	1.90 (c,b)
2.50 (c,b)	2.70 (c,b)
3.60 (t, J=5 Hz)	3.50 (s,b)
4.12 (c,b)	5.20 (b)
5.40 (s,b)	5.50 (b)

b = broad, c = complex, d = doublet,
s = singlet, t = triplet.

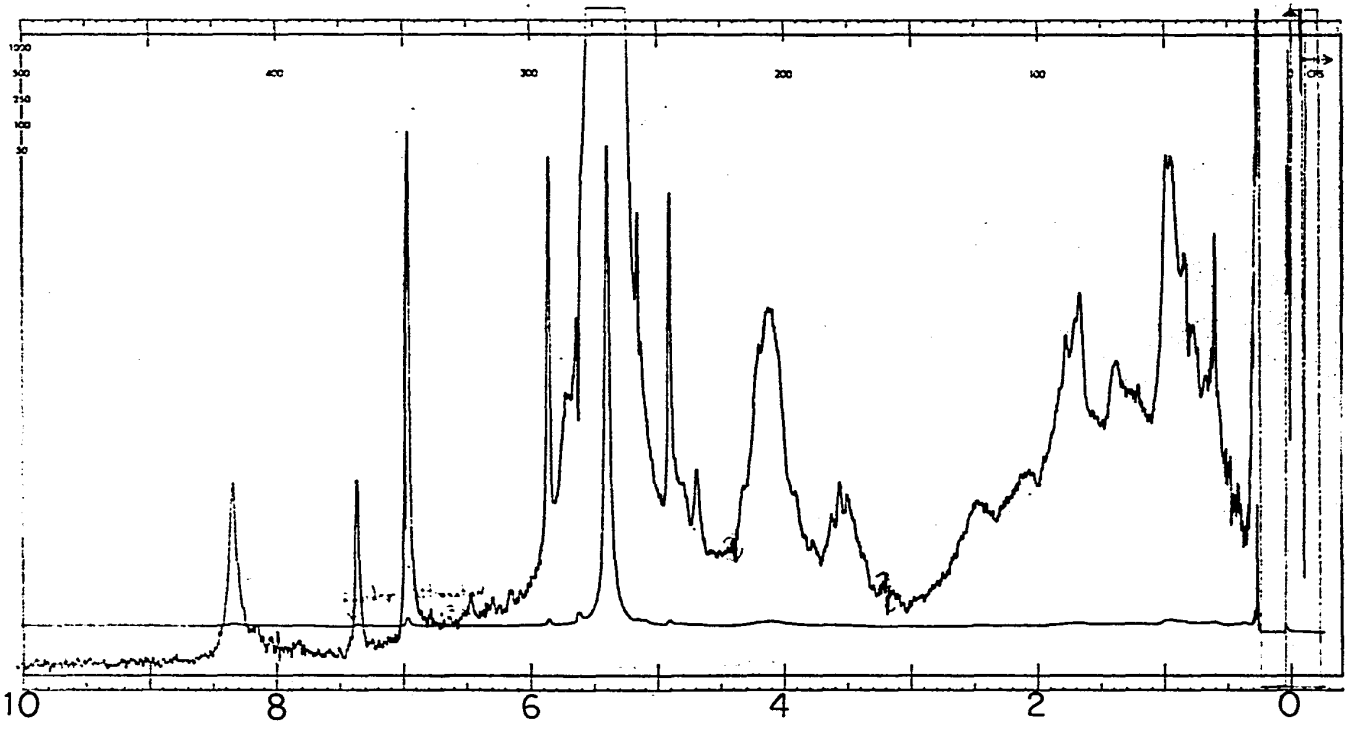


Fig. 5a. 100 MHz Nuclear Magnetic Resonance Spectrum in Pyridine-d_5

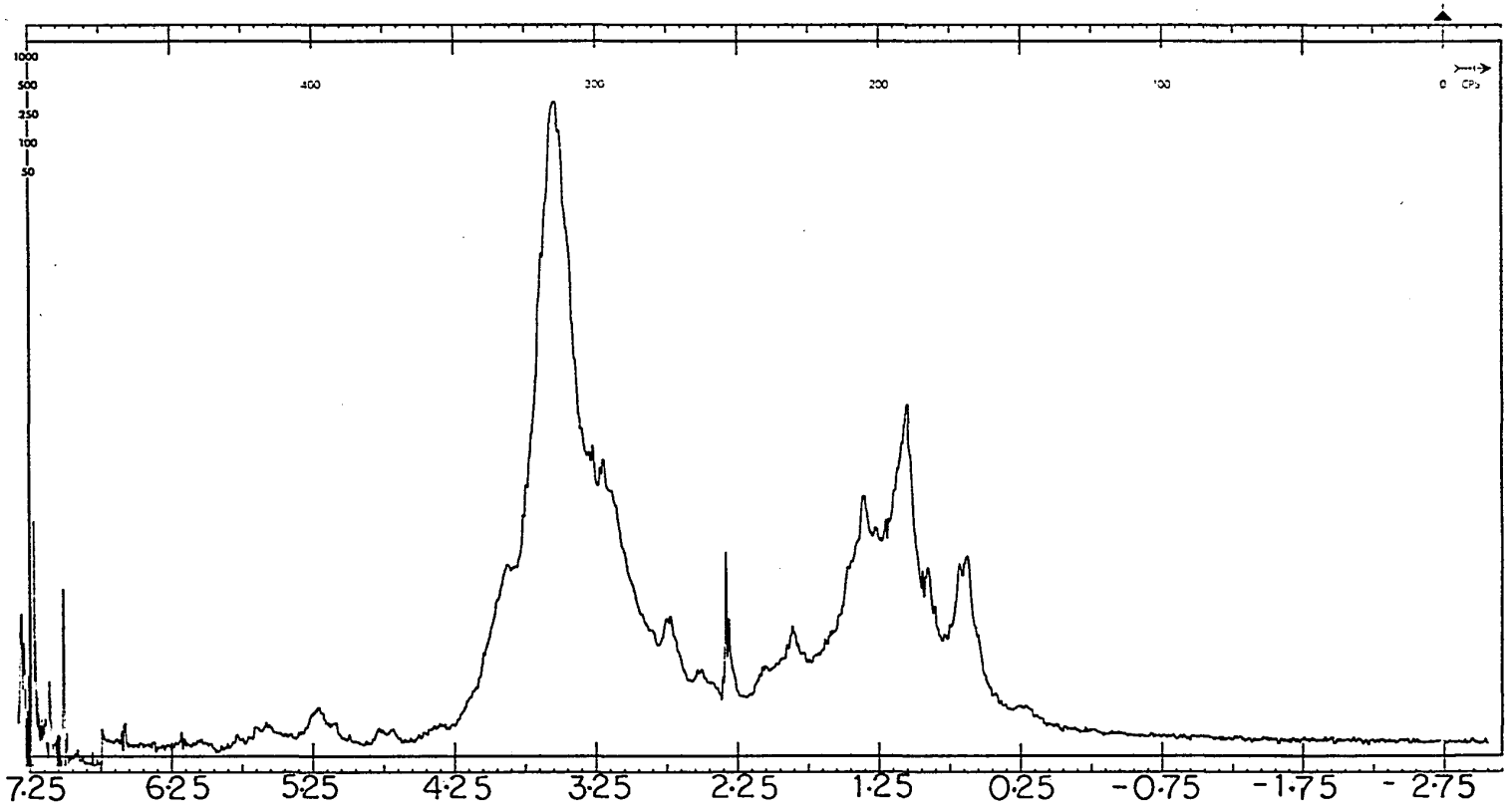


Fig. 5b. 100 MHz Nuclear Magnetic Resonance Spectrum in Dimethylsulfoxide-d_6

2. Sensitivity of Palytoxin towards Acid and Base

Palytoxin was found to be sensitive to both acid and base. Changes of absorbance at 263nm with time in 0.04N sodium hydroxide and 0.20N hydrochloric acid are represented in Fig. 6.

The quantitative ultraviolet spectrum of palytoxin in 0.5N sodium hydroxide depicts the instantaneous irreversible disappearance of absorption at 263nm and the hypsochromic shift of the 233nm (ε 14.1xM) maximum to 222nm (ε 13.6xM) in the reaction mixture.

3. Spot Tests

Spot tests were performed to determine the nature of various functional groups in palytoxin.

a. Test for Oxo Function (72)

2,4-Dinitrophenyl hydrazine reagent was prepared by dissolving 2,4-dinitrophenyl hydrazine (400 mg) in 2N-hydrochloric acid (100 ml).

The palytoxin paper chromatogram on spraying with 2,4-dinitrophenyl-hydrazine reagent did not show any spot. However the sprayed chromatogram on heating depicted the area corresponding to palytoxin as a yellow spot.

b. Test for 1,2-Diketones (72, 73)

(i) 1,2 and 1,4-diketones give yellow and pink spots respectively with o-phenylenediamine reagent. A palytoxin paper chromatogram was sprayed with o-phenylenediamine spray prepared by dissolving o-phenylenediamine (50mg) in 10% aqueous trichloroacetic acid (100 ml). The chromatogram on heating to 100° for 2 min depicted a yellow spot.

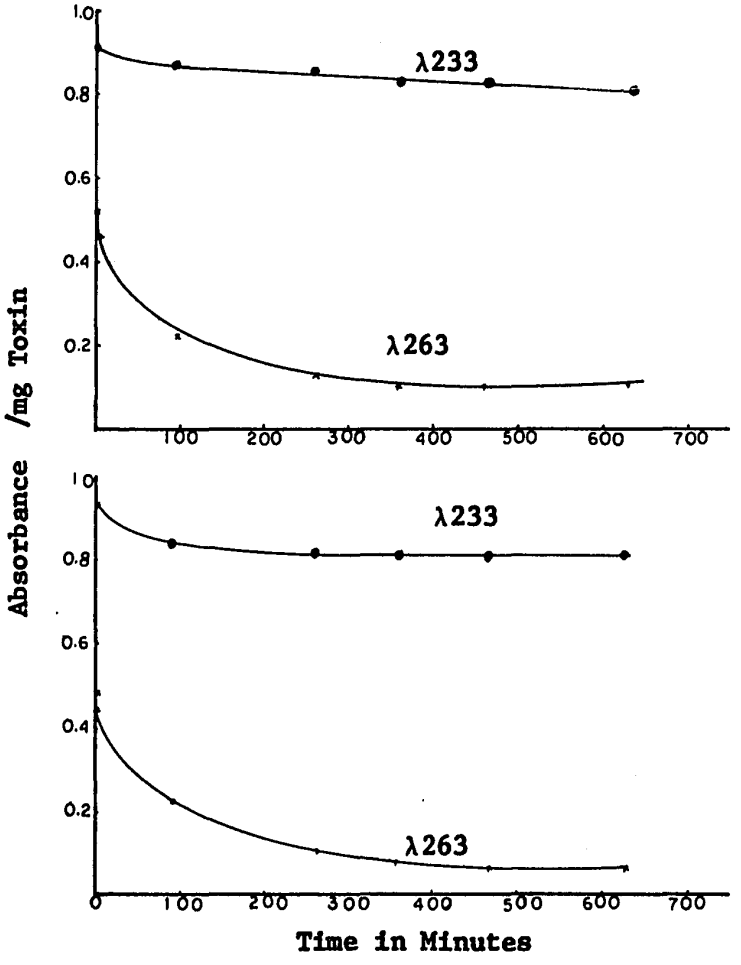


Fig. 6. Rates of Hydrolysis of Palytoxin by:

a. 0.20N Hydrochloric Acid
b. 0.04N Sodium hydroxide

(ii) A drop of palytoxin solution (10 mg/ml water) was treated in a 1 ml centrifuge tube with a drop of hydroxylamine hydrochloride solution (1g NH_2OH HCl, 1g NaOAc and 2 ml H_2O). After warming on a water bath the clear solution was spotted on Whatmann No. 1 paper, air dried and spotted with 5% nickel acetate solution. A pink spot appeared. A blank using only hydroxylamine also gave a pink spot.

c. Test for Carbohydrates

(i) Silver Nitrate (72)

A palytoxin paper chromatogram was drawn through a solution prepared by mixing saturated aqueous silver nitrate solution (0.1 ml) acetone (20 ml) and a drop of water. After drying, the paper chromatogram was sprayed with sodium hydroxide solution (2 g) in methanol (100 ml). Palytoxin appeared as a brownish black spot either after standing for a few minutes or on exposure of the chromatogram to a tungsten lamp.

(ii) Permagnate-Periodate (72)

The spray reagent was prepared by mixing 2% aqueous sodium metaperiodate and 1 part of 1% potassium permagnate in 2% sodium carbonate solution. A palytoxin paper chromatogram depicted a greenish white spot on pink background. The spot turned brown on white background on washing the chromatogram with water.

(iii) Benzidine-Periodate (72)

Palytoxin chromatogram was sprayed with aqueous sodium metaperiodate solution (2%). After 10 min standing in air, the chromatogram was sprayed with a mixture of 0.1M benzidine in methanol-

acetone-0.2N hydrochloric acid (10-2-1). Palytoxin appeared as a white spot on a blue background.

(iv) Triphenyltetrazolium Chloride (75)

The spray reagent was prepared by mixing equal volumes of a 2% aqueous solution of triphenyltetrazolium chloride and 1N sodium hydroxide. The dried palytoxin chromatogram was sprayed with the reagent and kept in a moist atmosphere at 40° for 20 min. The excess reagent was washed out with water and the paper was dried at 25°. Palytoxin showed only a very faint pink spot.

(v) Benzidine (75)

The spray reagent was prepared by dissolving benzidine (500 mg) in glacial acetic acid (200 ml) and absolute ethanol (80 ml). A palytoxin paper chromatogram was sprayed with the reagent and heated at 100 - 105° for 15 min. Only a faint yellow spot was observed.

(vi) p-Anisidine hydrochloride (75)

The spray reagent was prepared by dissolving p-anisidine (0.5g) in phosphoric acid (2 ml) followed by diluting the solution with ethanol to 50 ml. The precipitated anisidine phosphate was removed by filtration. The filtrate (A) was saved. The precipitate was dissolved in a minimum quantity of water and diluted with an equal volume of alcohol. Phosphoric acid was added to a final concentration of 2% (B). A and B were combined and the palytoxin chromatogram was sprayed with the reagent. The chromatogram did not show any spot.

(vii) Anthrone Reagent (75)

Anthrone (300 mg) was dissolved in boiling acetic acid (10 ml). Ethanol (20 ml), phosphoric acid (3 ml) and water (1 ml)

were added to the solution. The palytoxin paper chromatogram was sprayed with the above solution and heated to 110° for 6 min. Palytoxin appeared as a light yellow spot. A yellow color is characteristic of ketoses and oligosaccharides.

(viii) Aniline Hydrogen Phthalate (75)

The reagent was prepared by mixing 2N aniline in 1-butanol: 2N phosphoric acid in 1:2 v/v ratio. The palytoxin chromatogram was sprayed with the reagent and heated at 115° for 10 min. No spot could be observed.

(ix) The Molisch Test (75)

Molisch reagent (1%α-napthol in concentrated sulfuric acid--let stand for 2 hr before use : 2 drops) was treated with 1 drop of palytoxin (5 mg/ml water). The solution became brown and later turned black.

A similar reaction with sucrose, fructose, and glucose resulted in a violet color.

(x) p-Toluidine Blue (75)

The palytoxin paper chromatogram was dipped into a 0.125% w/v solution of toluidine blue in methanol. No spot could be observed.

d. Test for Amines and Other Nitrogenous Groups

(i) p-Dimethylaminobenzaldehyde (75)

The paper chromatogram of palytoxin was sprayed with a 1% p-dimethylaminobenzaldehyde solution in 1N-hydrochloric acid. Palytoxin appeared as a yellow spot.

(ii) p-Dimethylaminobenzaldehyde (75)

A drop of palytoxin solution (5 mg/ml) was treated

with two drops of p-dimethylaminobenzaldehyde solution (5% in concentrated hydrochloric acid). Palytoxin gave a violet color; however the color faded to yellow on standing.

(iii) Ninhydrin (75)

A palytoxin chromatogram was sprayed with 0.2% ninhydrin solution in water-saturated butanol. The chromatogram on heating to 110° for 3 to 5 min showed a purple spot. The test is positive only with relatively large amounts of palytoxin.

(iv) Dragendorff (75)

The Dragendorff reagent was prepared by boiling bismuth carbonate (2.6 g) and sodium iodide (7.0 g) with glacial acetic acid (25 ml) for a few minutes. After 12 hr the precipitated sodium acetate crystals were filtered. The clear red-brown filtrate (20 ml) was mixed with 8 ml ethyl acetate and stored. Stock solution (10 ml) was mixed with acetic acid (25 ml) and ethyl acetate (60 ml) to give the spray reagent.

The palytoxin paper chromatogram on spraying with Dragendorff spray did not show any spot.

(v) Konig Reagent (75)

The palytoxin paper chromatogram was exposed to cyanogen bromide vapors and then sprayed with a solution prepared by dissolving p-aminobenzoic acid (2 g) in 0.75N hydrochloric acid (75 ml) and diluting the resulting solution to 100 ml with 95% ethanol. Palytoxin failed to show up as a fluorescent spot.

(vi) Pauly's Test (74)

The spray reagent was prepared by dissolving diazotized

sulfanilic acid (100 mg) in 2% sodium carbonate (50 ml). The palytoxin paper chromatogram on spraying with Pauly's reagent did not show any spot.

(vii) Jaffé Reagent (72)

A palytoxin paper chromatogram was sprayed with Jaffé's reagent prepared by mixing a 3% ethanolic solution of picric acid with a 10% sodium hydroxide solution (5:1). The paper chromatogram on heating to 100° for 1 hr did not show any spot.

(viii) Sakaguchi Test (72)

Solution I was prepared by mixing a 0.02% solution of 1-naphol in 20% ethanol with water and 10% sodium hydroxide (1:1:2). Solution II was prepared by dissolving bromine (2 g) in ice cold 5% sodium hydroxide solution (100 ml). The palytoxin paper chromatogram was sprayed successively with solution I and II. No spot could be observed.

(ix) Griess Reagent (73)

A drop of palytoxin solution (5 mg/ml water) was treated with one drop of Griess solution prepared by mixing equal volumes of α-napthylamine in 30% acetic acid and one drop of 5N hydrochloric acid. Nitroso compounds develop a red violet color. The test was negative for palytoxin.

(x) Test for Imines and Nitriles (73)

A drop of palytoxin solution (5 mg/ml water) was mixed with sulfur (30 mg) in a test tube. The test tube was covered with a filter paper soaked in ferric nitrate. The test tube was heated from the upper end and gradually proceeding to the bottom. Compounds containing C=N and C≡N evolve thiocyanic acid and stain ferric nitrate

impregnated paper purple. Palytoxin gave no color; cyanoacetic acid gave a purple spot.

H. Derivatization

1. Acetylation of Palytoxin

Palytoxin (12.0 mg) in pyridine (2.5 ml) was reacted with acetic anhydride (1.5 ml) at room temperature for 24 hr. The mixture was evaporated to a gummy residue. The gummy residue on repeated evaporation with absolute ethanol (3x1 ml) furnished a white solid. The white solid was dissolved in chloroform (5 ml). The chloroform layer was backwashed with two portions of saturated sodium bicarbonate (3 ml), potassium bisulfate (3 ml) and saturated sodium chloride (3 ml). The chloroform layer after drying over potassium carbonate was filtered and evaporated to an oily mass. The oily mass on repeated evaporation with ethanol furnished an amorphous white solid. The white solid was purified by column chromatography over a Sephadex LH 20 column (2.0x45 cm, flow rate 9.5 ml/hr, 10% methanol in chloroform). The fractions were assayed by dichromate oxidation and by absorbance at 263nm in its ultraviolet spectrum. The fractions corresponding to volumes 42 to 52 ml were combined and evaporated to a glass. The glass was precipitated as an amorphous white solid from petroleum ether; m p 100-105°.

An analytical sample was prepared by preparative thin layer chromatography of the above amorphous white solid over silica gel H, chloroform-menthanol-acetic acid (90-5-3). A purified sample of palytoxin acetate (4.0 mg) was dried under vacuum for 6 hr and analyzed.

Calcd. for $C_{42}H_{62}N_1O_{19}$:

C, 57.07; H, 6.96; N, 1.58; O, 34.39.

Found: C, 57.15, 56.69; H, 7.04, 6.86; N, 1.48, 1.64.

The results of a computer program indicating the various possibilities for the composition of palytoxin acetate corresponding to the above found analysis are presented in Table IX.

a. Ultraviolet Spectrum

A quantitative ultraviolet absorption spectrum was run in methanol (Fig. 7).

λ_{max} 263 (6.5xM), λ_{max} 233 nm (8.84xM).

b. Infrared Spectra

The ir spectrum in potassium bromide (Fig. 8) had the following bands:

3350(w), 2925(w), 2850(sh), 1735(s), 1660(w), 1515(b,w), 1415(w,b), 1370(s), 1235(s,b), and 1030(m,b) cm^{-1}.

The ir spectrum in chloroform had the following bands: 3500(w), 3400(w), 2930(m), 2850(sh), 1735(s), 1660(w,b), 1430(sh,b), 1370(s), 1250-1180(b,s), 1030(b,s) cm^{-1}.

c. Nuclear Magnetic Spectra

Nuclear magnetic resonance spectra of palytoxin acetate were run in deuterochloroform (A-60 and HA 100). (Fig. 9, Table X)

d. Mass Spectrum of Palytoxin Acetate

A mass spectrum of palytoxin acetate was run on an AEI MS9 Mass Spectrometer using a direct inlet system at 70 EV. Peaks as high as m/e 1000 can be seen.

TABLE IX

POSSIBLE COMPOSITION OF PALYTOXIN ACETATE

Case No.	Molecular Formula				M. Wt.	Unsaturation
	C	H	N	O		
1	57.06	6.95	1.56			
	0.10	0.10	0.10			
2	42	61	1	19		
	57.07	6.96	1.58	34.39	883.938	13
	0.01	0.01	0.02			
3	44	63	1	20		
	57.07	6.86	1.51	34.56	925.975	14
	0.01	- 0.09	- 0.05			
4	82	118	2	37		
	57.13	6.90	1.63	34.34	1723.823	25
	0.07	- 0.06	0.07			
5	82	120	2	37		
	57.07	7.01	1.62	34.30	1725.839	
	0.01	0.06	0.06			
6	84	122	2	38		
	57.07	6.96	1.58	34.39	1767.876	24
	0.01	0.01	0.02			
7	86	124	2	39		
	57.07	6.91	1.55	34.47	1809.91	26
	0.01	- 0.04	- 0.01			
8	86	126	2	39		
	57.01	7.01	1.55	34.44	1811.929	25
	- 0.05	0.06	- 0.01			
9	88	126	2	40		
	57.07	6.86	1.51	34.56	1851.95	27
	0.01	- 0.09	- 0.05			
10	88	128	2	40		
	57.01	6.96	1.51	34.52	1853.966	26
	- 0.05	0.01	- 0.05			
11	90	130	2	41		
	57.01	6.91	1.48	34.60	1896.00	27
	- 0.05	- 0.04	- 0.08			

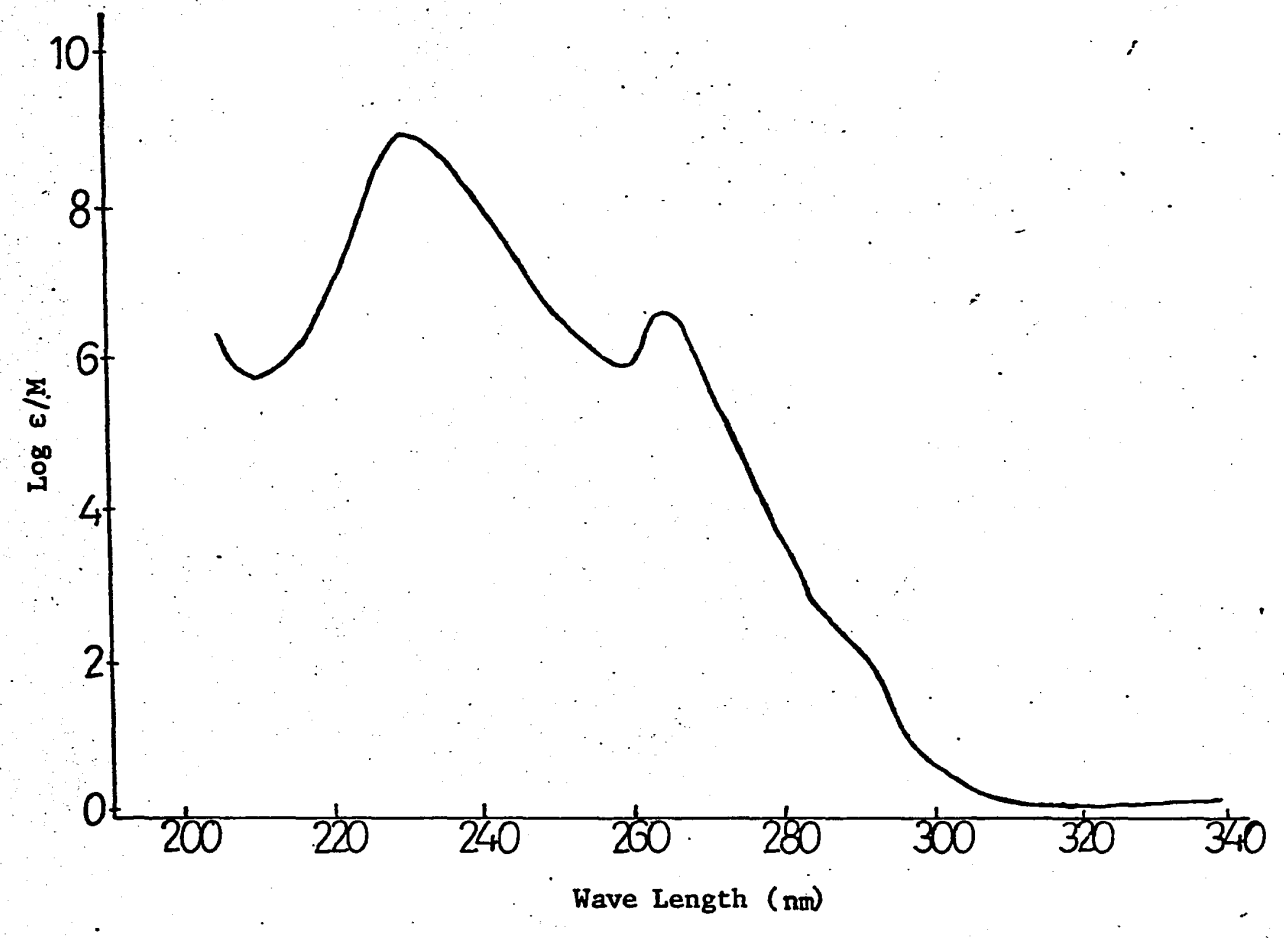


Fig. 7. Ultraviolet Spectrum of Palytoxin Acetate in Methanol.

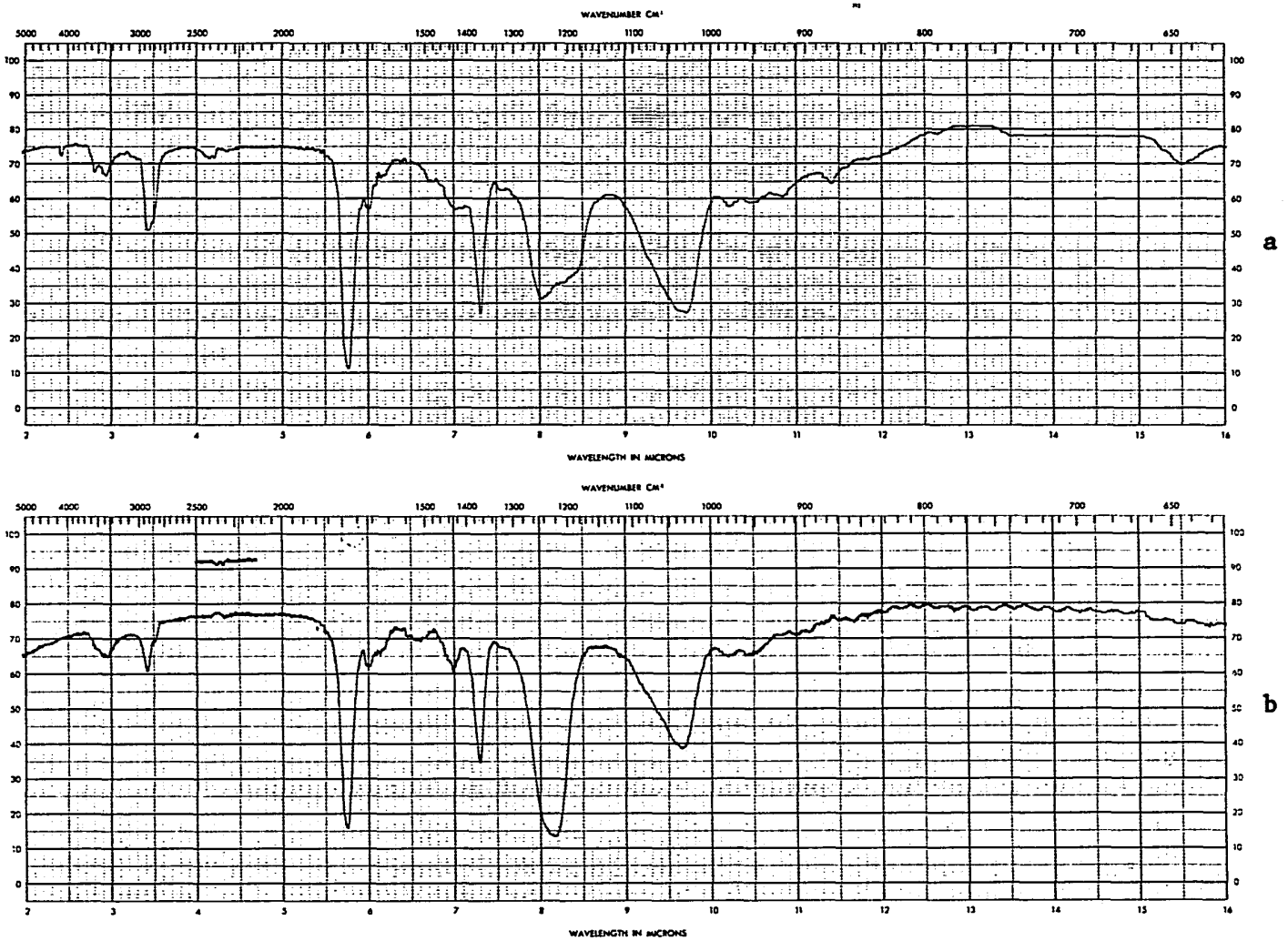


Fig. 8. Infrared Spectra of Palytoxin Acetate in:

a. Chloroform
b. Potassium Bromide

TABLE X

NMR SPECTRA OF PALYTOXIN ACETATE IN DEUTEROCHLOROFORM

60 MHz Spectrum		100 MHz Spectrum	
Chemical Shift	Relative Proton Ratios	Chemical Shift	Relative Proton Ratios
0.91 (s,b)	12	0.68 (s)	
1.28 (s,b)	21	1.00 (c,b)	27
1.60 (c,b)		1.10 (s)	
1.78 (c,b)	8	1.27 (c,b)	34
2.01 & 2.05 (s)	97	1.51 (s)	6
2.50 (c)		1.78 (c,b)	
3.35 (b)		2.025 (s,b)	107
4.17 (c,b)	6	3.300 (b)	
5.20 (c,b)		4.200 (c)	14
5.60 (c,b)	32	5.10 (c,b)	18

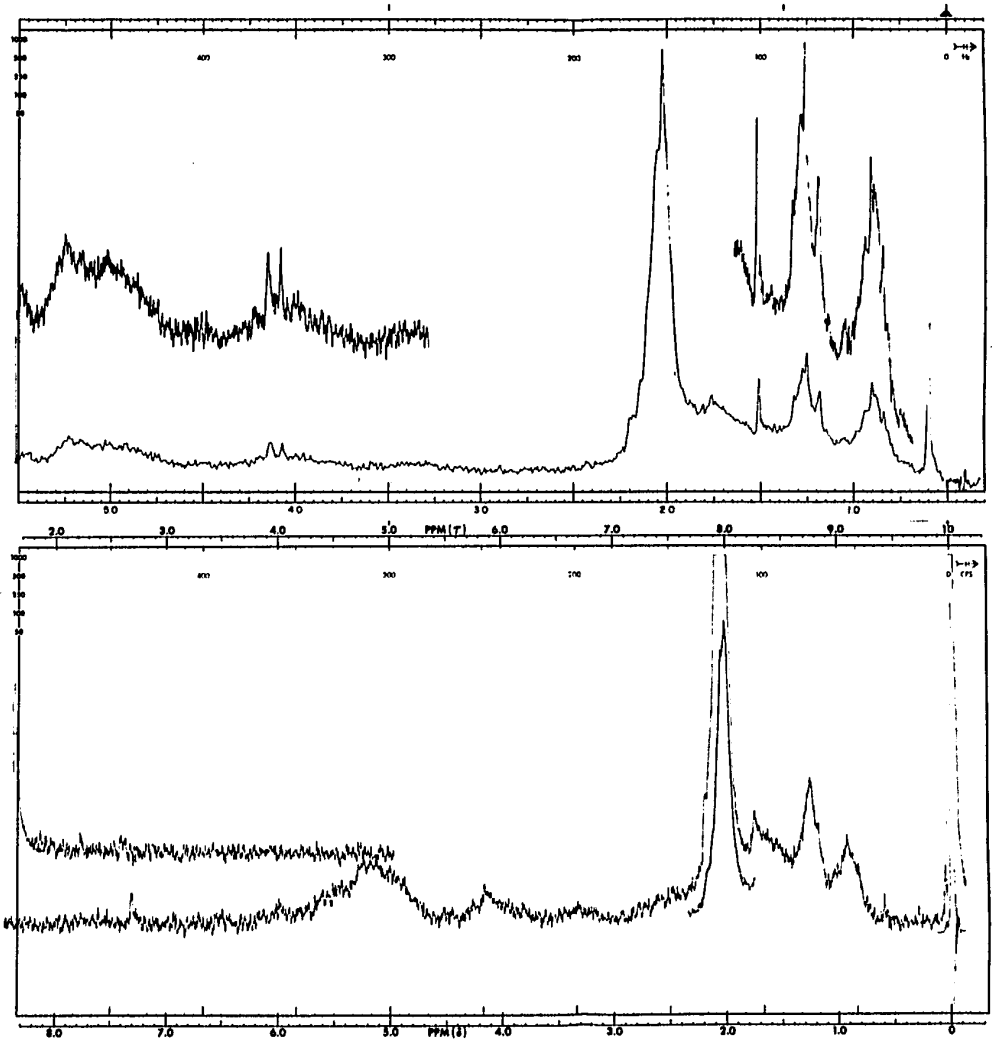


Fig. 9. NMR Spectra of Palytoxin Acetate in Deuterochloroform

a. 60 MHz
b. 100 MHz

m/e	%	m/e	%	m/e	%	m/e	%	m/e	%
492	1	313	2.5	233	3.0	179	4.0	151	7.0
464	1.2	300	2.0	230	3.0	175	3.0	149	7.0
436	1.5	288	3.0	219	2.5	167	3.0	147	3.0
408	2.0	275	2.0	211	5.5	165	4.0	145	3.0
380	4.0	268	2.5	205	2.5	163	3.5	139	6.5
352	2.5	258	4.0	201	3.0	161	4.0	137	8.5
324	3.5	256	2.0	191	4.0	155	6.0	135	7.5
315	2.0	240	4.0	183	23.0	153	3.5	133	7.0
115	14.5	109	21.0	83	39.5	69	53.0	55	61.0
114	14.5	97	38.0	82	30.0	67	21.0	43	98.0
113	19.0	95	32.5	81	33.0	60	57.0	41	41.0
112	7.5	85	39.5	70	18.0	57	100.0		

e. Molecular Weight of Palytoxin

(i) On The Basis of the Quantitative Ultraviolet Spectra of Palytoxin and Palytoxin Acetate

Palytoxin and palytoxin acetate both depict maxima at 263 nm (9.1xM and 6.5xM) and 233 nm (15xM and 8.8xM). If it is assumed that the gain in weight on acetylation parallels the loss of absorbance of the 263 nm maximum on acetylation, the weight gained on acetylation of 1 mg toxin is 0.3846 mg. Molecular weight per acetate unit is 105.0 and for 25 acetate units the molecular weight of palytoxin is 2625.

(ii) Gel Filtration

Palytoxin acetate on column chromatography over a Sephadex LH 20 column (2.0x45 cm; 10% methanol in chloroform) eluted

between 42 to 52 ml, whereas lactose octaacetate and tripalmitin eluted between 52-62 ml and 60-70 ml respectively.

(iii) By Isopiestic Distillation

A Hitachi-Perkin-Elmer Model 115 Molecular Weight apparatus was used in an attempt to determine the molecular weight of palytoxin acetate. A calibration curve was prepared with various concentrations of benzil in dry benzene. A solution of palytoxin acetate (2.166 mg) in dry benzene (0.226109 g) was used for the determination of ΔR.

R_2		R_1	$(\Delta R) = 0.387$
1.750	1.764	1.360	
1.757	1.767	1.370	
1.760	1.762	1.375	

m_o (from graph) $= 0.21 \times 10^{-2}$

$$m = \frac{W_x}{W_x + W_s} \times \frac{1}{0.21 \times 10^{-2}} = 4520.52.$$

$W_x = 2.1666$ $W_s = 0.226109$

(iv) By Depression of Freezing Point

A Rast molecular weight determination of palytoxin acetate provided a molecular weight of 498.

f. Chemical Properties of Palytoxin Acetate

(i) Rearrangement of Palytoxin Acetate over Silica Gel H

Palytoxin acetate when chromatographed on silica gel H plates lost the 233 nm maximum in its ultraviolet absorption spectrum.

Palytoxin acetate (1 mg) on stirring with degassed freshly prepared catalytic platinum (20 mg) in methanol (10 ml) rearranged to a compound with only a shoulder at 263 nm (8.9xM) and no peak at 233 nm in its ultraviolet absorption spectrum. The compound also differed in its Rf values from palytoxin acetate.

Solvents	Rf - values	
	Rearranged Palytoxin acetate	Palytoxin acetate
Chloroform-Methanol (8-2)	0.855	0.827
Chloroform-Methanol-Acetic Acid (90-5-3)	0.583	0.616

Infrared spectrum in potassium bromide had the following bands: 3400(w), 2930(m), 2850(sh), 1740(s), 1650(w), 1420(w), 1370(m), 1215(s), and 1040(s) cm^{-1}.

(ii) Hydrogenation of Palytoxin Acetate over Catalytic Platinum

Palytoxin acetate (11.17 mg) was stirred with platinum oxide (60 mg) in methanol (15 ml) under hydrogen at 1 atm for 5.5 hr. The mixture was filtered, the residue was washed with methanol (5 ml), the filtrates were combined and evaporated to a glassy mass (11 mg). Palytoxin acetate and hydrogenated palytoxin acetate had the same Rf values as follows: Chloroform-Methanol-Acetic Acid (90-5-3), 0.663; Chloroform-Methanol (8-2), 0.930 on silica gel H and 0.600 using Chloroform-Methanol-Acetic Acid (90-5-3) on silver nitrate impregnated silica gel H. Hydrogenated palytoxin acetate was positive to the hydroxamate ester test but negative to Dragendorff and ninhydrin sprays.

Hydrogenated palytoxin acetate was found to be transparent in the ultraviolet.

Infrared Spectrum in Chloroform. The ir spectrum in chloroform had the following bands:
3400(w), 2930(s), 2850(sh), 1740(s), 1660(w), 1510(s), 1420(b), 1370(m), 1265-1200(s,b), 1020(b), and 950(w) cm^{-1}.

Nuclear Magnetic Resonance Spectrum in deuterochloroform is presented in Table XI.

TABLE XI

60 MHz NMR SPECTRUM OF HYDROGENATED PALYTOXIN ACETATE IN DEUTEROCHLOROFORM

Chemical Shift	Relative Proton Ratios	Chemical Shift	Relative Proton Ratios
0.87δ (d)	19	1.98δ (s,b)	75
1.20δ (s,b)	36	3.28δ (c,b)	--
1.55δ (c,b)	--	4.10δ (c,b)	--
1.65δ (c,b)	--	5.00δ (c,b)	27

Mass Spectrum (MS 9) of Hydrogenated Palytoxin Acetate: The mass spectrum of hydrogenated palytoxin acetate indicates peaks even beyond m/e 1000. (Table XII)

TABLE XII

70 EV MASS SPECTRUM OF HYDROGENATED PALYTOXIN ACETATE

m/e	%	m/e	%	m/e	%	m/e	%	m/e	%
757	0.25	580	1.27	452	1.00	354	1.4	257	3.5
719	0.200	579	2.50	451	1.00	353	2.3	253	2.5
718	0.200	578	1.90	439	1.70	352	1.0	247	3.8
704	0.20	577	1.00	424	1.00	342	1.0	244	1.9
691	0.15	576	0.60	411	1.00	341	3.6	241	3.6
690	0.20	565	0.90	410	1.00	340	1.6	239	5.75
689	0.15	564	1.90	408	1.00	339	1.9	238	3.20
677	0.30	563	1.10	407	2.20	338	1.2	230	3.1
676	0.18	552	1.00	406	2.00	327	1.8	225	2.0
675	0.20	551	3.00	399	1.20	325	1.0	221	2.0
662	0.40	550	1.00	398	1.40	314	1.9	219	3.5
661	0.70	538	0.70	397	1.20	313	3.3	217	2.0
660	0.75	537	2.30	396	1.60	311	2.4	211	3.0
659	0.65	536	1.20	395	2.4	303	3.94	206	2.7
647	0.18	524	0.40	383	2.0	299	1.9	205	3.4
646	0.25	523	2.20	382	1.7	296	1.3	191	13.6
645	0.15	522	1.00	381	1.3	285	2.9	183	5.4
644	0.30	521	0.80	380	1.4	283	1.7	175	5.5
631	0.40	509	0.80	370	1.4	282	1.3	171	3.6
608	1.00	495	1.30	369	2.4	274	2.0	159	12.7
607	2.12	467	2.00	368	3.3	267	4.0	151	5.0
606	1.27	466	1.00	367	2.7	264	1.45	149	9.0
605	0.60	453	1.00	355	1.6	260	1.5	147	5.5
137	7.27	112	10.9	95	25.5	81	27.0	56	16.0
135	10.0	111	16.4	93	9.0	71	51.0	55	42.0
133	18.0	109	16.0	91	14.5	69	69.0	55	42.0
125	14.5	107	13.0	89	25.0	68	9.0	45	69.0
124	15.5	103	11.0	87	44.0	60	65.5	43	100.0
123	12.7	98	27.0	85	27.0	59	8.0	42	38.0
121	11.0	97	31.0	83	33.0	57	65.0	28	76.0
119	9.0								

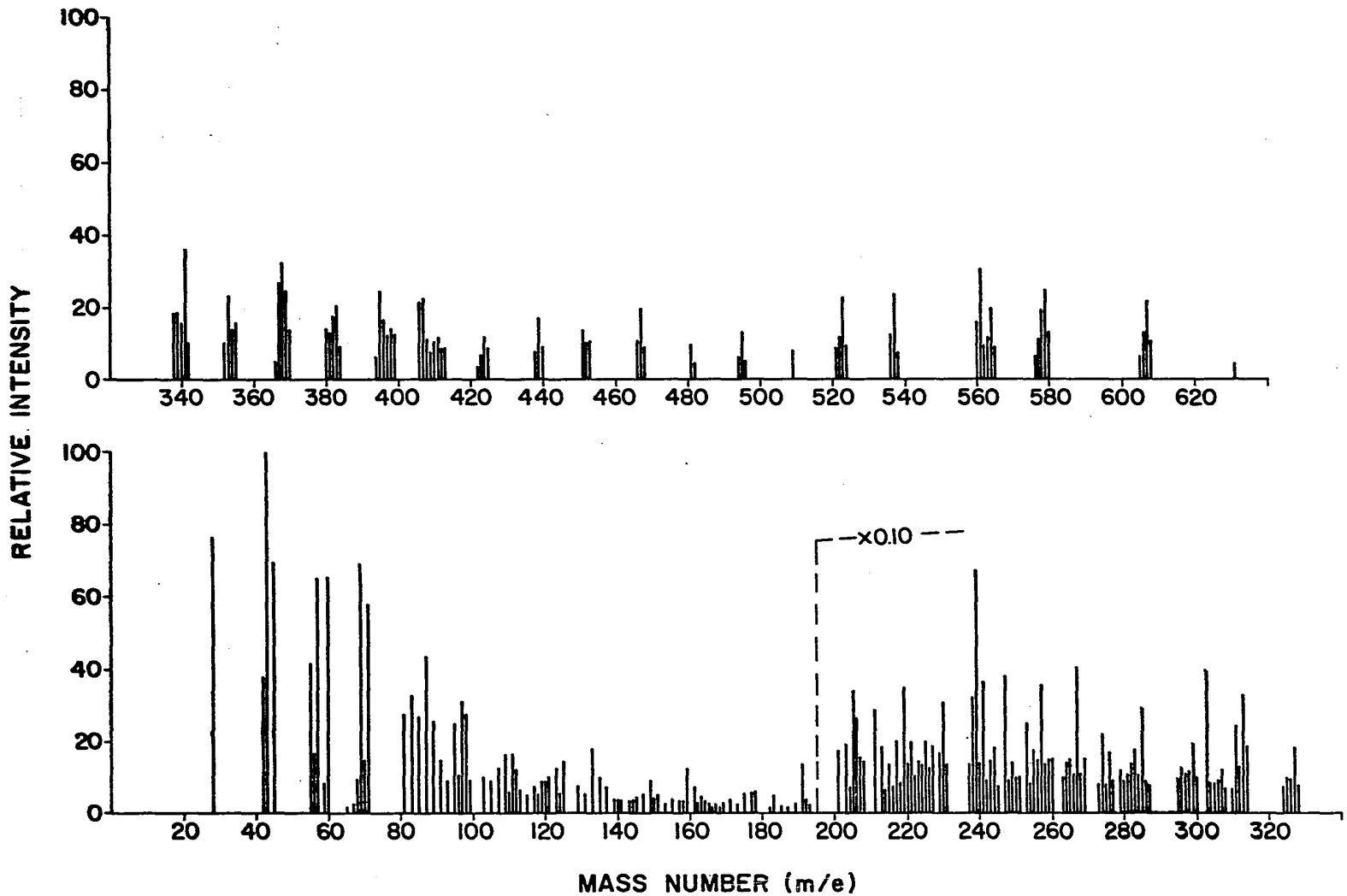


Fig. 10. 70 EV Mass Spectrum of Palytoxin Acetate Hydrogenated

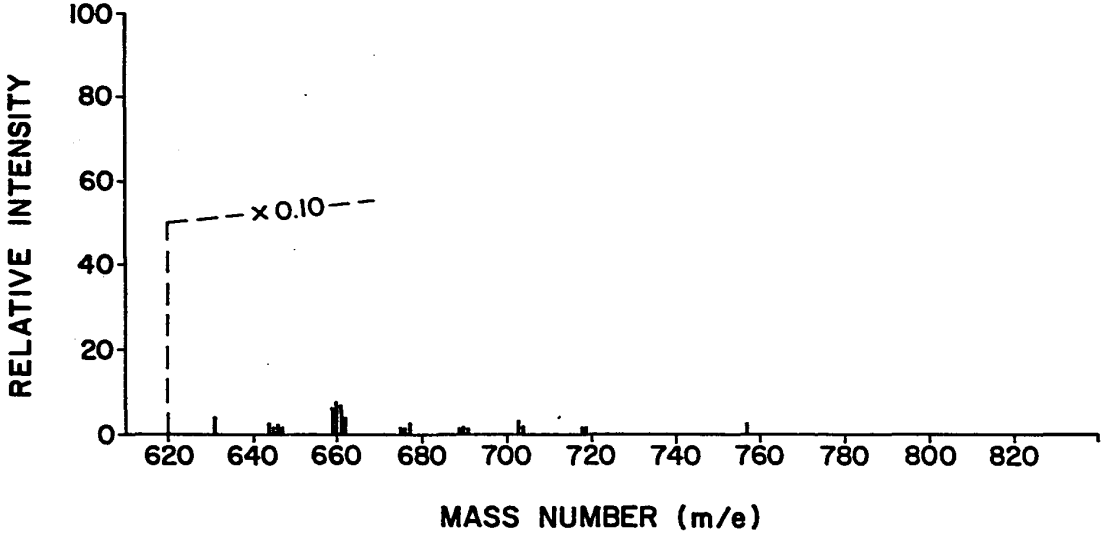


Fig. 10. (continued)

(iii) Attempted Hydrolysis of Palytoxin Acetate by Triethyloxonium Fluoborate

Palytoxin acetate (6.65 mg) was stirred with triethyloxonium fluoborate (60 mg) in dichloromethane (3 ml) for 12 hr. The mixture was evaporated to dryness. The residue was mixed with water (5 ml) and extracted with three portions (5 ml) of ether. The ether extract was dried over anhydrous potassium carbonate and evaporated to a pale solid (5 mg). The solid was negative to ninhydrin, Dragendorff, and DNP spray reagents.

Ultraviolet absorption spectrum in methanol; The compound was transparent in the ultraviolet.

Infrared spectrum; The following bands were observed:

3400(w), 2930(m), 2850(sh), 1735(s), 1660(w), 1428(w), 1370(m), 1220(s), 1120(m), 1070-1030(s,b) cm^{-1}

Nuclear magnetic resonance spectrum in deuterochloroform depicted the following signals.

0.90δ(c,b), 1.02δ(c,b), 1.22δ(c,b), 2.00δ(c,b), 4.10δ(c,b), 5.00δ(c,b)

(iv) Attempted Introduction of Bromine in Palytoxin Acetate

Hydrobromic acid gas after purification by passage over anhydrous calcium chloride and red phosphorus, was passed through a solution of palytoxin acetate (5.0 mg) in acetic anhydride (1.0 ml) for 30 min. After 12 hr standing at room temperature, the mixture was freed from hydrobromic acid gas by bubbling nitrogen through the solution. It was then poured into water (5 ml). The mixture after neutralization with sodium carbonate was extracted twice with chloroform

(5 ml each). The chloroform layer after drying over potassium carbonate was evaporated to a pale glass, (3.0 mg).

Ultraviolet spectrum in methanol. The glass was transparent in the ultraviolet.

Infrared spectrum in chloroform. The following bands were noted: 3400(w), 2920(m), 2850(sh), 1740(s), 1675(w), 1600(b), 1440(b), 1370(w), 1255(s), 1060(b) cm^{-1}.

Nuclear magnetic resonance spectrum in deuterochloroform depicted the following signals.

0.90δ(s,b), 1.25δ(s,b), 1.65δ(c,b) 2.05δ(s,b), 3.80δ(c,b), 5.00δ(c,b)

(v) Attempted Transfer Bromoacetylation of Palytoxin Acetate

Bromoacetic anhydride (0.5 ml) and glacial acetic acid (2 drops) were added to a cool stirred solution of palytoxin acetate (13 mg) in acetic anhydride (0.3 ml). The mixture was agitated for 2 hr at 2°, and for 12 hr at room temperature. The mixture was poured over ice (5 g), scrubbed and extracted with chloroform. The chloroform layer was backwashed with saturated potassium carbonate (5 ml) and saturated sodium chloride (5 ml), dried over anhydrous magnesium sulfate, filtered and evaporated to a brown gummy mass. The gummy mass was thrice evaporated with absolute ethanol (1 ml each) to remove the last traces of acetic anhydride.

The above gummy mass was chromatographed over a Sephadex LH 20 column (2x45 cm, 10% methanol in chloroform as eluent). Fractions corresponding to 25 to 40 ml were combined and evaporated to a pale gummy mass. Analytical thin layer chromatography over silica gel H using chloroform-methanol:acetic acid (90:5:3) indicated the

presence of only one compound (R_f 0.508). Paper chromatography over DMSO impregnated paper employing diisopropyl ether and petroleum ether-benzene (95:5) proved useless.

Ultraviolet spectrum in methanol.

λ_{max} 265 nm

λ_{sh} 230 nm λ_{min} 250 nm

Infrared spectrum in potassium bromide showed these bands: 3400(w), 2930(m), 2850(sh), 1742(s), 1380(m), 1235(s), 1030(s), and 715 cm^{-1}.

(vi) Attempted Methanolysis of Palytoxin Acetate

Palytoxin acetate (6.7 mg) was treated with methanolic hydrochloric acid gas (3 ml, 5.3N in hydrochloric acid) for 18 hr. The mixture changed color from white to light yellow to orange to brownish yellow. The mixture was evaporated to a brownish black gummy mass (7.0 mg). The gummy mass was insoluble in chloroform but soluble in water and methanol. It consisted of at least three compounds as shown by thin layer chromatography over silica gel H plates, R_f's 0.000, 0.245 and 0.645 using butanol-acetic acid-water (4:1:5) as developer. No attempt was made to purify the mixture.

(vii) Reaction of Palytoxin Acetate with Methyl Iodide

Methyl iodide (0.5 ml) was reacted with palytoxin acetate (1 mg) in methanol (1 ml). The solution was evaporated to dryness and the residue tested against Dragendorff's reagent.

	Palytoxin	Palytoxin Acetate	Palytoxin Acetate + CH_3I
Dragendorff Reagent	no color	pale	orange
Ninhydrin Reagent	purple	no spot	no spot

Mass Spectrum. A 70 EV. spectrum indicates peaks even beyond m/e 500.

m/e	%	m/e	%	m/e	%	m/e	%	m/e	%
369	4	152	26	113	23	85	66	56	22
280	22	151	23	112	14	72	44	55	43
203	11	141	19	103	23	71	33	43	100
201	13	134	27	101	37	70	29	41	79
167	56	131	23	87	21	57	91	28	46
159	14								

2. Benzoylation of Palytoxin in Sodium Hydroxide

Freshly distilled benzoyl chloride (0.1 ml) was added in one portion to an ice cold solution stirred in the cold for 15 min prior to addition of palytoxin (15 mg) in 5% aqueous sodium hydroxide solution (0.75 ml). After stirring the mixture for 15 min 5% sodium hydroxide (1.2 ml) was added. The reaction mixture was stirred at 2° for 1 hr and at room temperature for 45 min. A gummy precipitate separated during the process. The mixture was poured into water (5 ml), scrubbed and extracted with chloroform (two 10 ml portions). The chloroform layer was washed with 10% sodium hydroxide (10 ml) and saturated sodium chloride (10 ml). It was then dried over anhydrous magnesium sulfate, filtered and evaporated to a white glass (18 mg).

The white solid was chromatographed over a Sephadex LH 20 column (2.0x45 cm; flow rate 18 ml/hr). Fractions corresponding to volumes 18 to 33 ml were combined and evaporated to a solid (13.6 mg). Preparative thin layer chromatography over kieselguhr G plates, chloroform methanol (95:5), followed by precipitation from petroleum ether furnished a white solid m.p. 123-128°.

Ultraviolet spectrum in methanol.

λ_{max} 281.8, 273.0, 227.3 nm

λ_{min} 258, 215 nm

Infrared spectrum in chloroform.

3400(m), 2910(m), 2850(sh), 1710(s), 1600(w), 1440(w), 1260-1190(s,b), 1110(s,b), 1100(s,b), 1060(s), 1020(s) and 975(b) cm^{-1}.

Nuclear magnetic resonance spectrum in deuterochloroform depicted the following signals.

1.20δ(c,b), 1.40δ(c,b), 3.00δ(c,b), 4.00δ(c,b), 5.50δ(c,b), 7.0δ(s), 7.10δ(c,b), 7.60δ(c,b)

Analysis

A 5.0 mg sample after drying under vacuum was analyzed.

for $C_{66}H_{72}NO_{22}$:

Found: C, 64.14, 64.03; H, 5.78, 5.86; N, 1.13, 1.13%.

3. Benzoylation of Palytoxin in Pyridine

To a solution of palytoxin (15 mg) in dry pyridine (1.5 ml) was added benzoyl chloride (0.5 ml). The mixture was warmed to 70° for 5 min. After 72 hr standing at room temperature the mixture was poured over ice-water (7 g). After scrubbing for 20 min, the mixture was

neutralized with saturated potassium carbonate and extracted with chloroform (2x5 ml). The chloroform layer was backwashed with saturated potassium carbonate (2x5 ml), dried over sodium sulfate and evaporated to a yellow mass. On preparative thin layer chromatography over silica gel HF (cyclohexane-ethyl acetate 1:1), the yellow mass furnished one band (Rf 0.568). This band on rechromatography over silica gel HF, using the same solvent system, followed by precipitation from petroleum ether gave an amorphous white solid, mp 122-126°.

Ultraviolet spectrum in dichloromethane.-

λ_{max} 282.0 (8.5xM) and 273 (10.0xM) nm.

Infrared spectrum in potassium bromide.-had the following bands. 3400(w), 3050(w), 2925(sh), 2850(w), 1720(s), 1595(w), 1449(w), 1316(w), 1266(s), 1176(w), 1110(s,b), 1096(s), 1070(s), 1029(m), 708(s), and 686(w) cm^{-1}.

Mass spectrum.-70 EV mass spectrum had the following peaks.

m/e	%	m/e	%	m/e	%	m/e	%	m/e	%
456	0.22	341	0.10	283	0.35	198	0.60	57	12
407	0.10	334	0.20	267	0.20	122	40.0	51	17
371	0.10	325	0.10	239	0.20	105	100.0	28	12
351	0.21	295	0.10	226	0.25	78	33		

Elemental Analysis.-A 3 mg sample after drying under vacuum for 6 hr was analyzed.

Analysis

for $C_{60}H_{51}NO_{15}$:

Found: C, 70.17; H, 5.05; N, 1.37%.

4. 4-Bromobenzoylation of Palytoxin in Sodium Hydroxide

Freshly prepared, recrystallized 4-bromobenzoyl chloride (300 mg) was added in one portion to a cooled stirred solution of palytoxin (34 mg) in aqueous 5% sodium hydroxide (1.5 ml). The mixture was stirred for 15 min and 5% sodium hydroxide (1.5 ml) was added. The mixture was stirred for another hour at ice bath temperature. The mixture was poured into water (9.0 ml) and extracted with three portions of chloroform (7.0 ml each). The chloroform extract was back-washed with two portions of 5% sodium hydroxide (7.0 ml), once with 15% sodium hydroxide (10 ml), and once with saturated sodium chloride (5 ml). The chloroform layer after drying over sodium sulfate was evaporated to a pale solid (35 mg). The pale solid was chromatographed over a Sephadex LH 20 column (2.0x45 cm). Fractions (6.8 ml) were collected and assayed by dichromate oxidation. Fractions 5 to 7 were combined and evaporated to a pale solid (32.0 mg). The pale solid on preparative thin layer chromatography over silica gel H using cycohexane-2-butanone (45:60) furnished three bands; A (10 mg), B (2.9 mg), and C (6.0 mg). A was precipitated as an amorphous white solid (7.5 mg) from peteroleum ether, mp 131-135°, and examined for its spectral data.

Ultraviolet spectrum in dichloromethane.-Ultraviolet spectrum indicated maxima at 245.6 (56.14xM), and 235 (48.81xM) nm.

Infrared spectrum.-Infrared spectrum showed the following bands. 3400(w), 2920(w), 2850(sh), 1720(s), 1580(m), 1480(s), 1400(s), 1240(s), 1170(m), 1112(sh), 1100(s), 1070(sh), 845(m), 758(s), and 680(s) cm^{-1}.

for $C_{68}H_{66}Br_5N_1O_{22}$:

Found: C, 49.0; H, 3.92; N, 0.84; Br, 25.18%.

5. 4-Bromobenzoylation of Palytoxin in Pyridine

To a cooled solution of palytoxin (12 mg) in dry pyridine (1.5 ml) was added freshly prepared 4-bromobenzoyl chloride (90 mg) in one portion. The mixture was warmed to 70° for a few min and left at room temperature for 24 hr. It was poured into ice cold water (5 ml) whereby a white solid separated. The solution was twice extracted with chloroform (10 ml each). The chloroform extract was washed with two portions of saturated potassium chloride (5 ml each), dried over sodium sulfate, filtered and evaporated to a pale solid. The solid on crystallization from chloroform deposited white needles, mp 218°.

The supernatant liquid on repeated preparative thin layer chromatography over silica gel HF (cyclohexane-ethyl acetate, 65:35) furnished two fluorescent bands which could be stained with 50% sulfuric acid (Rf's 0.75 and 0.58 respectively, band A 11.0 mg, band B 10.0 mg). Both bands A and B were glassy. Band B on rechromatography over silica gel HF plates, followed by precipitation from n-hexane furnished a white amorphous solid (8.0 mg). Attempts to crystallize it from ethyl acetate - petroleum ether failed, mp 137-141°.
Ultraviolet spectrum of band B in chloroform.-Ultraviolet spectrum indicated a maximum at 246 nm (57.8xM).
Infrared spectrum of band B in potassium bromide.-Infrared spectrum had the following bands.

3400(w), 2925(w), 2850(sh), 1710(s), 1580(m), 1480(w), 1390(w), 1260(s), 1170(m), 1110(sh), 1092(s), 1070(sh), 1010(s), 845(m), 752(s), and 680(w) cm^{-1}.

Mass spectrum of band B.-70 EV mass spectrum depicted peaks above m/e 600.

m/e	%	m/e	%	m/e	%	m/e	%	m/e	%
491	0.33	279	0.41	200	80.0	76	29.0	50	33.0
443	0.66	269	0.33	185	100.0	75	30.0	44	7.00
441	1.00	258	2.20	183	100.0	65	10.0	38	10.00
439	0.66	256	2.30	157	38.0	56	12.0	28	30.00
385	0.37	202	80.0	155	38.0				

Elemental Analysis of band B.-A 4.0 mg sample of band B after drying under vacuum was analyzed.

Found: C, 47.28; H, 3.27; N, 0.27; Br, 29.8%

6. Mesylation followed by Acetylation of Palytoxin

Palytoxin (20 mg) in dry pyridine (0.5 ml) was treated with methanesulfonyl chloride (100 mg) in pyridine (2.5 ml) at room temperature. The mixture after 48 hr standing in a refrigerator was poured over crushed ice (5 g). After scrubbing thoroughly, it was extracted with chloroform (20 ml). A yellow solid separated during the process. The solid was washed with water (2x10 ml), dissolved in a chloroform methanol mixture (95:5), filtered and evaporated to dryness. The dry mass was mixed with ethanol (5 ml) and evaporated to dryness. The process was repeated three times, thereby giving an amorphous solid, mp 140-145° (12 mg). The white solid was found to be insoluble in

methanol, chloroform, ethyl acetate and water but readily dissolved in a chloroform methanol mixture (95:5). Attempts to crystallize it from chloroform-methanol (95:5), dichloromethane-methanol and ethyl acetate failed.

Infrared spectrum in potassium bromide indicated the following bands. 3400(m), 3000(sh), 2925(m), 2900(sh), 1710(sh), 1640(w), 1350(s), 1215(sh), 1175(s), 1050(b), 960(b,m), 915(b,w), and 850(b) cm^{-1}.

The white solid was chromatographed over a Sephadex LH 20 column (2.0x70 cm). Fractions (4.8 ml) were collected and assayed by dichromate oxidation. Fractions 10 and 11 were combined and evaporated to a white solid (ca 10.0 mg). The white solid was acetylated by acetic anhydride (0.2 ml) in pyridine (0.5 ml) for 24 hr. The acetate after work-up in the usual fashion and preparative thin layer chromatography over silica gel HF (chloroform-methanol 80:20), was precipitated from peteroleum ether as a white solid, ca 4.0 mg. The compound softens on heating to 125°, solidifies to a brown mass and then melts with decomposition (browning) at 145-150°.

Ultraviolet spectrum in methanol.-The compound is transparent in ultraviolet.

Infrared spectrum in potassium bromide had the following bands. 3400(m), 2925(w), 2850(sh), 1730(s), 1650(b,w), 1415(b), 1370(b,s), 1235(b,s), 1172(s), 1040(b,m), 960(b,w), and 910(b,w) cm^{-1}.

Mass spectrum.-No spectrum could be obtained.

7. Tritylation followed by Acetylation of Palytoxin

A solution of palytoxin (7.34 mg) and trityl chloride (15 mg) in pyridine (0.5 ml) was heated to 65° for 3 min and allowed

to stand at room temperature for 24 hr. Acetic anhydride (0.4 ml) was added, the mixture was shaken and left at room temperature for another 24 hr. The mixture was then poured onto crushed ice (5 g), scrubbed and extracted with chloroform (2x5 ml). The chloroform extract was dried over anh magnesium sulfate, filtered and evaporated to dryness. Thin layer chromatography over silica gel G using benzene as developer indicated the presence of three compounds (Rf's 0.00, 0.120, and 0.55). Preparative thin layer chromatography using the above conditions furnished two bands:
band I (Rf. 0.55) and band II (Rf 0.12).

Band I. Infrared spectrum in chloroform indicated the following bands.

3500(m), 3020(sh), 2995(m), 2910(m), 2840(sh), 1940(w), 1800(w), 1800(w), 1710(w), 1595(m), 1490(s), 1440(s), 1328(s,b), 1145(s,b), 1080(w), 1030(m), 1010(s,b), 910(s,b), 890(s,b) cm^{-1}.

Nuclear magnetic resonance spectrum in deuterochloroform depicted the following bands.-broad 2.25δ(1H), singlet 7.55δ(15H)

Mass spectrum.-70 EV mass spectrum indicated the following peaks.

m/e(%);

m/e	%	m/e	%	m/e	%	m/e	%
260	27	155	17	105	100	58	12
243	11	154	19	91	5.0	51	5.0
183	81	152	12	77	86.0	43	4.0
165	18						

Band II. Infrared spectrum in chloroform depicted the following bands.

3450(w,b), 2920(s), 2830(s), 1720(m,b), 1600(w), 1550(w,b), 1380(w,b), 1285(m,b), 1200(m,b), 1125(w,b), 1075(w,b) cm^{-1}.

Nuclear magnetic resonance spectrum in deuterochloroform depicted the following bands.

0.78δ(b), 1.18δ(s,b), 3.60δ(b), 7.27δ(b).

8. 4-Bromobenzenesulfonation of Palytoxin

Palytoxin (9.5 mg) in dry pyridine (0.4 ml) was added to a frozen mixture of freshly crystallized dry 4-bromobenzenesulfonyl chloride (50 mg) in pyridine (0.4 ml). The mixture was cooled in a propanediol-dry ice bath for 30 min and then placed in a refrigerator for 48 hr. The mixture was then evaporated to dryness. Water (1.0 ml) was added, the mixture was scrubbed and mixed with water (4 ml), and extracted with three portions of chloroform (5 ml each). The chloroform layer was washed twice with sodium carbonate (10 ml each, 10%), once with 1N hydrochloric acid (10 ml) and twice with saturated sodium chloride solution (5 ml each). The chloroform layer was then dried over sodium sulfate and evaporated to a light yellow glass. The glass can be readily visualized by 50% sulfuric acid as a black spot on silica gel HF layers. Both 4-bromobenzoyl chloride and 4-bromobenzenesulfonic acid cannot be visualized by 50% sulfuric acid, thereby indicating that the product indeed is derived from the toxin. Analytical thin layer chromatography over silica gel HF using ethyl acetate-cyclohexane (35:65, Rf 0.0), chloroform-methanol (70:30, Rf 1.0) chloroform-methanol (90:10, Rf 0.00 tailing) and toluene-methyl ethyl ketone (60:40, Rf 0.00) indicated either extensive tailing or the material moved almost with the solvent front. Attempt to crystallize the solid from chloroform, 2-propanol and isobutyl alcohol failed.

9. Attempted Bromoacetylation of Palytoxin

a. With bromoacetyl chloride

Palytoxin (6.5 mg) in pyridine (1.0 ml) was reacted with bromoacetyl chloride (0.5 ml) at room temperature. The mixture turned almost black. After 24 hr standing at room temperature, the mixture was poured onto ice (5 g), scrubbed for 15 min and extracted with chloroform (10 ml). The chloroform layer on evaporation did not furnish any solid.

b. With bromoacetic anhydride

Palytoxin (5.0 mg) in dry pyridine (1.0 ml) was reacted with bromoacetic anhydride (0.3 ml). After 24 hr standing the black mixture was evaporated to a black gum. The gum could not be purified by thin layer chromatography over silica gel H using a variety of solvent systems.

10. Attempted Formation of Isopropylidene Derivative of Palytoxin

Palytoxin (6.0 mg) was stirred with anhydrous copper sulfate (60 mg) in dry alcohol-free acetone (4.5 ml) at room temperature under nitrogen for 168 hr. The mixture was filtered, the residue washed thrice with acetone (5 ml each). The filtrates were combined and evaporated to dryness. No residue was left.

11. Attempted formation of Trimethylsilyl Ether of Palytoxin

Palytoxin (15 mg) in dry pyridine (1 ml) was treated with hexamethyldisilazane and trimethylchlorosilane (0.1 ml). Pyridine (0.5 ml) was added to dissolve the precipitated solid. The mixture after standing at room temperature for 12 hr was freed of suspension

by centrifugation and evaporated to a gum. The gum was triturated with chloroform (10 ml). The chloroform layer was concentrated to a small volume (0.5 ml) and examined by analytical gas chromatography over SE 30 3% on chromosorb W (1/8" x 5', column temperature 280°). No peak could be detected. The attempted chromatography over silica gel H plates failed. The compound failed to move with a variety of solvent systems.

12. Formation of Methyl Ether of Palytoxin

Palytoxin (8.58 mg) in dimethylformamide (0.8 ml) was stirred with ether-washed sodium hydride under nitrogen for 10 min. Methyl iodide (0.8 ml) was added over a period of 10 min. After 5 min the mixture became semi-solid. After 48 hr standing at room temperature, the mixture was decomposed by dropwise addition of methanol; it was evaporated to dryness and the residue partitioned between water (5.0 ml) and chloroform (10 ml). The chloroform layer after back-washing with water (5 ml) was dried over sodium sulfate and evaporated to a brown gummy mass. The gum on thin layer chromatography over silica gel HF using chloroform-methanol-acetic acid (80:10:10) as the developer indicated the presence of three compounds (Rf 0.850, 0.600 and 0.511). Preparative thin layer chromatography employing the same conditions gave three bands, Band I (Rf 0.850), Band II (Rf 0.60), Band III (Rf 0.511). The experiment was repeated with palytoxin (40 mg) to acquire more of bands I, II and III.

Band I (Rf 0.85). Ultraviolet spectrum.-λ_{max} 276 (sh), λ_{max} 230 nm (sh).

Infrared spectrum in chloroform exhibited the following bands.

3400(w), 2940(s), 2800(sh), 1725(m), 1710(sh), 1640(w), 1565(sh), 1450(m), 1380(w), 1100(s,b) cm^{-1}.

Nuclear Magnetic Resonance Spectrum indicated the following signals:

Nature of Signal	Chemical Shift	Relative Proton Ratio
Triplet (J=5.3 CPS)	0.875 δ	9
Singlet (broad)	1.250 δ	43
Complex (broad)	1.700 δ	6
Complex (triplet, J=CPS)	3.600 δ	3
Complex	3.300 δ	

Mass Spectrum. 70 EV mass spectrum depicted peaks as high as m/e 500.

m/e	%	m/3	%	m/e	%	m/e	%
364	0.7	270	2.1	139	8.0	81	31.0
350	0.9	267	2.0	137	10.0	75	10.0
336	1.2	239	3.0	127	8.0	73	30.0
334	0.9	221	6.0	125	15.0	71	57.0
323	1.2	213	4.0	123	14.0	69	63.0
322	1.2	198	4.0	121	7.0	67	24.0
320	0.9	185	4.0	112	10.0	60	22.0
309	1.4	183	6.0	111	27.0	57	100.0
308	1.4	179	5.0	109	20.0	55	88.0
295	1.6	165	6.0	97	44.0	43	98.0
285	2.8	163	7.0	87	12.0	42	69.0
281	1.7	149	38.0	85	37.0		
280	1.7	141	8.0	83	45.0		

Band II. Ultraviolet spectrum in methanol indicated a shoulder at 275 nm and a small peak at 228 nm.

Infrared spectrum in chloroform depicted the following signals. 3400(w), 2900(s), 2800(sh), 1740(b,w), 1710(b,w), 1365(w), 1275(b), 1090(s,b) cm^{-1}.

Nuclear Magnetic Resonance Spectrum in deuterochloroform (HA 100) indicated the following signals.

Chemical Shift	Relative Proton Ratio
1.25δ(c)	1.6
1.70δ(c)	3.7
2.00δ(c)	3.2
3.00δ(c)	12.6

Mass spectrum.-70 EV mass spectrum shows peaks as high as m/e 500.

m/e	%	m/e	%	m/e	%	m/e	%	m/e	%
256	0.9	205	0.8	97	7	71	11	47	31
240	1.0	181	1.2	87	12	69	11	45	20
239	1.0	163	6	85	7	57	23	41	16
223	0.7	149	16	83	100	55	16	35	10
222	0.7	111	4						

Band III. Ultraviolet spectrum in methanol.-λ_{max} 275 nm and 228 nm.

Infrared spectrum in chloroform.-3400(b,w), 2920(s), 2850(m), 1750(w), 1710(s), 1600(m), 1420(b), 1291(b), 1095(m,b) cm^{-1}.

Nuclear magnetic resonance spectrum in deuterochloroform depicted the following signals.

1.25δ(c), 1.55δ(c), 2.375δ(c)

Mass spectrum.-70 EV mass spectrum shows peaks as high as m/e 500.

m/e	%	m/e	%	m/e	%	m/e	%	m/e	%
396	0.8	265	1.4	237	2.9	165	6	147	6
386	0.6	264	1.8	236	4.6	155	5	141	6
368	1.3	257	3	223	1.8	153	6	139	8
278	1.8	255	2.1	208	2.3	151	7	137	9.5
277	1.7	238	2.5	201	3.9	149	9	135	9
125	15	97	40	81	31	57	100	43	18
123	13	95	30	71	50	55	66	41	36
111	25	85	36	69	62	45	79	29	14
109	19	83	48	67	22				

I. Hydrolytic Studies on Palytoxin

1. Kinetics of the Acid and Base Hydrolysis of Palytoxin

Rates of hydrolysis of palytoxin in 0.2N aqueous hydrochloric acid, and 0.04N sodium hydroxide were followed by observing the decay in the optical density of the 263 nm and 233 nm peaks with time. The plots of the rates of reactions are presented in Fig. 6.

2. Hydrolysis of Palytoxin by 5N-Hydrochloric Acid

A mixture of palytoxin (2.8 mg) in 5N-hydrochloric acid (1.0 ml) was heated on a water bath for 2 hr. The mixture turned black and a brownish black solid floated on the solution. The mixture was evaporated to a brown solid. The brown solid was mixed with ethanol (1 ml) and evaporated to dryness. The process was repeated thrice. The residue was finally mixed with water ().5 ml) and the brown insoluble solid was separated by centrifugation. To the supernatant solution was added Amberlite MB-3 (60 mg). After 10 min

standing the mixture was filtered and the residue washed with water (0.5 ml). The water wash and the filtrate were combined and evaporated to dryness. The residue was dissolved in water (0.1 ml) and examined by thin layer (on sodium acetate impregnated kieselguhr G plates) and paper chromatography. The residue depicted a spot with an Rf value similar to that of D-ribose, positive to aniline hydrogen phthalate and benzidine-phosphoric acid sprays on both paper and thin layer chromatography.

TABLE XIII

COMPARISON OF ACID HYDROLYSIS PRODUCT WITH D-RIBOSE

	Paper Chromatography		Kieselguhr Layers	
	Hydrolyzed Palytoxin	D-Ribose	Hydrolyzed Palytoxin	D-Ribose
Butanol-acetic acid-water (4:1:5)	0.238	0.238	0.789	0.789
1-Propanol-Ethyl acetate-water (7:1:3)	0.580	0.580		
Ethanol-water (8 : 2)	0.653	0.653	0.870	0.870
Ethyl acetate-2-propanol: water (2 : 1) 65 : 35			0.760	0.760

The brown residue was found to be insoluble in water, methanol, ethanol, chloroform, benzene, pyridine and dichloromethane.

Similar hydrolytic experiments with perhydro palytoxin and manganese dioxide oxidized palytoxin also indicated the presence of a component matching D-ribose in Rf values.

3. Hydrolysis of Palytoxin with 2N, followed by 4N Hydrochloric Acid

A solution of palytoxin (2.9 mg) in 2N-hydrochloric acid was stirred at room temperature for 2 hr. The mixture was freed of hydrochloric acid by repeated evaporation with water. Finally it was dried to a colorless glass;
Ultraviolet spectrum in water.-λ_{max} 263 nm (sh) and 233 nm (sh).
It had the same Rf value as the toxin. The glass was mixed with 4N-hydrochloric acid (4 ml), stirred at room temperature for 12 hr. The mixture after being freed from hydrochloric acid was evaporated to a glass. On paper chromatography over Whatman No. 1 paper, the glass depicted one spot, with 1-butanol-ethyl acetate-water (7:1:4, Rf 0.46) and 1-butanol-acetic acid water (4:1:5, Rf 0.24) and two spots with 1-butanol-acetic acid-water (6:1:2, Rf 0.08 and 0.12). The glass depicted four spots over cellulose thin layer plates; butanol-ethyl acetate-water (7:1:3, Rf 0.68, 0.51, 0.45 and 0.34). No attempt was made to separate these components on a preparative scale.

4. Hydrolysis of Hydrogenated Palytoxin by 3N-Hydrochloric Acid

A solution of hydrogenated palytoxin (6.2 mg) in 3N hydrochloric acid (2 ml) was heated on a hot water bath at ca. 90° for 2 hr. The mixture was evaporated under vacuum and freed of hydrochloric acid by repeated evaporation with distilled ethanol. Finally the mixture was freeze dried to a straw yellow amorphous solid:
Infrared spectrum in potassium bromide depicted the following bands. 3322(s,b), 2899(m), 2841(sh), 1709(w), 1637(w) and 1071 cm^{-1}.

The product mixture was negative to aniline phthalate, 2:4 dinitrophenylhydrazine, but questionably positive to ninhydrin. The mixture depicted two spots positive to iodine and potassium permagnate indicators with 1-butanol-acetic acid-water (4:1:5) or 1-butanol-pyridine-water (45:25:40) as the solvent systems. One of the spots could be visualized with silver nitrate and the other with ninhydrin. The mixture on paper chromatography over DEAE paper with ethanol-water (75:25) and visualization with ninhydrin showed two spots (Rf's 0.34 and 0.72).

Preparative paper chromatography over DEAE paper with ethanol-water (75:25) provided three bands Rf's 0.000, 0.35, and 0.72.

Band I (Rf. 0.000). Infrared spectrum in potassium bromide depicted the following bands.

3390(s,b), 2907(m), 1656(w,b), 1585(w), 1408(-), 1263(s,b), 1072(s,b), and 868(w) cm^{-1}.

Band II (Rf. 0.350). Band II gives an intense purple color with ninhydrin.

Infrared spectrum in potassium bromide depicted the following signals.

3356(s,b), 2933(m), 2865(sh), 1656(w,b), 1618(w), 1576(w,b), 1404(w), 1381(w), 1348(m,b), 1111(s), and 1060(s,b) cm^{-1}.

Band III (Rf. 0.72). Infrared spectrum in potassium bromide exhibited the following signals.

3356, 2933, 2865, 1656, 1618, 1575, 1404, 1381, 1348, 1111, and 1060 cm^{-1}.

The band area (Rf. 0.00) was eluted a second time with ammonium hydroxide. The eluate was acidified (turned yellow) and freeze dried to a yellow solid.

Infrared spectrum in potassium bromide depicted the following signals. 3115(s,b), 1754(sh), 1709(m,b), 1626(b,m), 1399(s), 1125(w,b), 1087(w), and 961(w,b) cm^{-1}.

Analytical paper chromatography of the yellow solid, using a solvent system 1-butanol-acetic acid-water (4:1:5) showed five spots with permanganate spray (Rf 0.18, 0.29, 0.60, 0.75 and 0.86) and one spot with ninhydrin spray (Rf 0.2).

A similar experiment with hydrogenated palytoxin (6.0 mg) was conducted, and an attempt was made to fractionate the reaction mixture by chromatography over a DEAE-Sephadex-A25 Cl-form, column (1x60 cm, flow rate 8 ml/hr, eluent 0.01M phosphate, sodium chloride pH 7.0 gradient). The column chromatography indicated the presence of three compounds eluting between 13 ml to 28 ml, 37 to 42.5 ml and 43.0 to 47.5 ml in a ratio of 20:2:1 by weight, respectively.

5. Methanolysis of Palytoxin

Palytoxin (9.60 mg) was reacted at room temperature with methanolic hydrogen chloride (4 ml, 5N) for 19 hr. The mixture was evaporated to dryness and freed of hydrogen chloride by repeated evaporation with distilled ethanol. It was acetylated, after drying under vacuum for 4 hr by acetic anhydride (0.8 ml) in pyridine (1.2 ml) for 22 hr. The mixture was worked up as usual to give a brown mass. Preparative thin layer chromatography of the brown mass over silica gel G, using chloroform-methanol-acetic acid (90:5.3) provided four bands (Rf's 0.700, 0.616, and 0.000).

Band I (Rf 0.700). Ultraviolet spectrum in methanol.-λ_{max} 290 nm and 245 nm.

Infrared spectrum in potassium bromide.

3400(w), 2930(s), 2850(sh), 1740(s,b), 1670(sh), 1430(b,m), 1370(s), 1260(b) and 1050(b,s) cm^{-1}.

Nuclear magnetic resonance spectrum in deuterochloroform depicted the following signals.

0.855δ(s,b), 1.22δ(s,b), 2.00δ(s,b), 2.22δ(s,b), 2.40δ(c,b), 3.50δ (c,b), 5.15δ(c,b), 5.80δ(c,b), 6.30δ(c,b)

Mass spectrum showed the following ions.

m/e	%	m/e	%	m/e	%	m/e	%	m/e	%
486	1.2	318	2.5	201	9	129	31	83	36
485	1.2	302	2.5	184	38	127	21	71	56
458	3	288	7	171	11	125	14	63	57
457	2	276	5.5	163	9	123	10	60	65
430	5.5	275	7	153	11	98	31	57	96
402	4	258	12	151	15	97	34	45	51
374	5.5	257	12	149	33	96	15	44	24
359	2.2	221	7	142	17	87	33	43	100
346	3	214	10	141	22	85	46	36	23
331	2.5	212	10.5	135	16	84	29	31	69

Band II (Rf 0.616). Ultraviolet spectrum in methanol.-λ_{max} 290 nm.

Infrared spectrum in potassium bromide.

3400(w), 2930(w), 2850(sh), 1740(s), 1685(m), 1630(m), 1420(b), 1370(s), 1250(b), 1050(b,s) cm^{-1}.

Nuclear magnetic resonance spectrum in deuterochloroform exhibited the following signals.

0.85δ(b), 1.30δ(s,b), 2.05δ(s,b), 3.10δ(b)

Mass spectrum.-70 EV mass spectrum indicates peaks even higher than m/e 500.

m/e	%	m/e	%	m/e	%	m/e	%	m/e	%
382	1.6	185	10	135	12	109	19	59	33
368	1.8	181	5	131	8	97	40	57	100
284	3.5	179	7	129	19.5	85	39	55	79
279	4	175	7	125	14	83	50	43	91
264	5	171	7.5	123	13	81	27	41	82
256	14	168	21.5	118	14	71	58	29	29
213	6	155	12	115	14	69	59	28	21
201	5	151	10	113	16.5	67	22	18	95
195	5	141	16	111	24	60	33		

Band III. Ultraviolet spectrum in methanol. Transparent.

Infrared spectrum in potassium bromide exhibited the following bands. 3400(w), 2930(s), 2850(sh), 1740(s,b), 1685(sh), 1630(sh), 1450(b,m), 1370(s), 1250(b,m), and 1050(s) cm^{-1}.

Nuclear magnetic resonance spectrum showed the following signals. 0.80(b), 1.20(b), 2.10(b), 2.40(b), 3.30(b), 3.70(b), 4.60(b), 5.00(b)

Mass spectrum.-70 EV mass spectrum indicated peaks as high as m/e 500. Some of the more important peaks are:

m/e	%	m/e	%	m/e	%	m/e	%	m/e	%
279	8	201	11.5	151	23	131	19	103	22
269	2.5	183	7	149	100	116	10	101	35
267	3	167	26	147	12.5	113	16	97	25
221	4.5	162	9.5	141	11.5	111	13	95	19
203	7.5	153	15	135	25	109	11	87	22.5

m/e	%	m/e	%	m/e	%	m/e	%	m/e	%
85	83	60	13	55	64	43	43	28	34
71	39	57	64	51	37	29	25	18	64
69	31.5								

6. Photocatalyzed Hydrolysis of Palytoxin

Palytoxin (7.0 mg) in 0.2N hydrochloric acid (1 ml) was photolyzed (256 nm) at room temperature. Photolysis was followed by analytical paper chromatography. After 12 hr the precipitated white fibrous mass was centrifuged. It was found to be insoluble in chloroform, dichloromethane, methanol, ethanol, benzene, petroleum ether and pyridine. Infrared spectrum in potassium bromide was almost identical to that of the palytoxin.

The supernatant on paper chromatography using butanol-acetic acid-water (4:1:5) as the developer indicated the presence of four compounds (Rf 0.410, 0.283, 0.094, and 0.000). All of these spots are positive to silver nitrate. The spot, Rf. 0.00, can also be visualized by 2,4-dinitrophenylhydrazine spray. The spots at 0.094 and 0.283 can be visualized by ninhydrin reagent. The spot (Rf 0.41) is visualized by silver nitrate even without spraying the chromatogram with sodium hydroxide. All of the spots are negative to Dragendorff reagent.

7. Hydrolysis of Palytoxin with 50% Acetic Acid

A solution of palytoxin (37.1 mg) in 50% acetic acid (3.0 ml) was heated to 90° for 72 hr. The progress of the reaction was followed by gradual deepening of color (light yellow to yellow to

brown) and periodic withdrawal of samples for paper chromatography. As the reaction proceeded, the paper chromatogram showed a gradual diffused spot positive to ninhydrin and benzidine-periodate sprays. The reaction mixture was freeze dried to give the crude product (31.0 mg), which on preparative paper chromatography using butanol-acetic acid-water (4:1:5) as developer furnished a compound (Rf 0.367).

Ultraviolet spectrum in distilled water. λ_{max} 275 nm and 225 nm.

Infrared spectrum in potassium bromide.

3400(s), 2925(w), 2850(sh), 1725(sh), 1640(b,w), 1380(b,w), 1240(b), 1072(b,c) cm^{-1}.

Nuclear magnetic resonance in pyridine-d_5 exhibited the following signals.

1.25δ(s,b), 1.65δ(s,b), 1.95δ(s), 2.55δ(b), 3.45δ(b), 4.45δ(b)

The above compound (Rf 0.367) was acetylated by acetic anhydride (1.5 ml) in pyridine (2.0 ml) for 24 hr. The mixture was worked up as usual and the acetate of the hydrolyzed palytoxin was purified by thin layer chromatography over silica gel H using chloroform-methanol-acetic acid (90:5:5). A band corresponding to Rf 0.78 was collected.

Infrared spectrum in potassium bromide.

3400(w), 3000(w), 2925(w), 2850(sh), 1740(s), 1375(m), 1240-1210(s), 1120(b,w), and 670(w) cm^{-1}.

Mass spectrum.-70 EV mass spectrum shows peaks even beyond m/e 500.

Some of the more important peaks are:

m/e	%	m/e	%	m/e	%	m/e	%	m/e	%
382	0.25	169	0.70	99	7	83	100	49	25
368	0.50	165	0.75	97	5	73	10	47	52
264	0.60	151	1.5	95	5	69	8	45	12
256	0.55	121	5	86	76	60	5	43	18
204	0.55	119	12	85	91	57	14	40	11
185	0.80	113	10.5	84	91	55	11	39	17
181	0.50	101	3						

Analysis

Calcd. for $C_{45}H_{66}No_{22}$:

C, 55.60; H, 6.82; N, 1.44; O, 36.29 %.

Found: C, 55.74; H, 6.91; N, 1.44 %.

8. Attempted Hydrolysis of Palytoxin by 50% Formic Acid

A solution of palytoxin (8.8 mg) in 50% formic acid (0.5 ml) was heated to 90° in a sealed tube for 2 hr. The mixture was basified to pH 9.0 with ammonium hydroxide and freeze dried to a white solid. The white solid was chromatographed over a Sephadex G 10 column (2.0x60 cm, fraction size 7.8 ml, eluent doubly distilled water). Fractions 11 and 12 were combined and evaporated to a hygroscopic pale solid (4 mg). Analytical paper chromatography using 1-butanol-acetic acid-water (4:1:5) as the solvent system indicated the presence of three compounds corresponding to Rf's 0.0476, 0.170 and 0.3537. No attempt for further purification of the mixture was made.

9. Hydrolysis of Palytoxin by Methoxide anion in Dimethyl sulfoxide

To a solution of sodium methoxide (35 mg) in dimethyl sulfoxide (0.5 ml) under a positive pressure of nitrogen was added a palytoxin (7.0 mg) solution in dimethyl sulfoxide (0.7 ml). Glacial acetic acid (0.3 ml) was added to the mixture after 7 hr stirring at room temperature. The mixture was evaporated to dryness under vacuum at a waterbath temperature of 50 - 60°. The residue was dissolved in water (0.7 ml) and freed of suspension by centrifugation. The solution was freeze dried, dissolved in 50% ethanol and subjected to analytical paper chromatography.

	Palytoxin	D-Ribose	Hydrolysate
Butanol-acetic acid-water (4:1:5)	0.333	0.3111	0.333 & 0.75
Butanol-pyridine-acetic acid water (8:8:1:4)	1.000	----	1.000

The above hydrolysate was swirled with Amberlite MB3 (50 mg) for 10 min, filtered and lyophilized to a white solid (5.0 mg). Ultraviolet spectrum in water.-λ_{max} 310 nm, 267 nm and 228 nm. Infrared spectrum in potassium bromide.
3400(s), 2950(w), 1645 to 1560(m,b), 1400(b), 1075(b) cm^{-1}.

10. Hydrolysis of Palytoxin by Sodium Hydroxide

Palytoxin (50.0 mg) was added to a precooled 5% sodium hydroxide solution (1.5 ml). The mixture after stirring at ice water temperature for 25 min was brought to room temperature and stirred for another 50 min. The mixture after neutralization with freshly distilled

acetic acid, was evaporated to dryness, and chromatographed over a Sephadex G 10 column (2.0x60 cm, distilled water as eluent, fraction size 5.2 ml, flow rate 24 ml/hr). Fractions 12 to 15 (A) and 16 to 20 (B) were combined and showed respectively Rf values of 0.270 and 0.143 on paper using butanol-acetic acid-water (4:1:5) as the developer. Part A was preparatively paper chromatographed under the above conditions to furnish a white hygroscopic solid.

Infrared spectrum in potassium bromide depicted the following bands. 3300(b,s), 2925(m), 1625(sh), 1560(b,m), 1400(b,m), and 1075(b,m) cm^{-1}.

Nuclear magnetic resonance spectrum in pyridine-d_5 (HA 100) exhibited the following signals.

0.90δ(b), 1.35δ(b), 1.65δ(b), 2.45δ(b), 3.50δ(b), 4.20δ(b)

Calcd. for $C_{35}H_{64}NO_{21}$:

C, 50.40; H, 7.73; N, 1.69; 0, 40.24 %.

Found: C, 50.12, 49.93; H, 7.61, 7.73; N, 1.63, 1.71 %.

Both bands A and B after rechromatography over the same Sephadex G 10 column were acetylated by acetic anhydride in pyridine for 24 hr. The acetates were worked up as usual.

Band A. The acetate of band A was purified by column chromatography over a Sephadex LH 20 column (2.0x45 cm, fraction size 5.5 ml). Fraction 9 contained essentially all the acetate. It was further purified by preparative thin layer chromatography over silica gel H using chloroform, methanol, acetic acid (80:17:3) and finally precipitated from petroleum ether, as a white solid, mp.

Ultraviolet spectrum in methanol.-Transparent.

Infrared spectrum in potassium bromide.

3450(s,b), 2940(w), 2850(sh), 1735(s), 1650(sh), 1430(s,b), 1370(m), 1225(s,b), and 1035(m,b) cm^{-1}.

Calcd. for $C_{42}H_{58}NO_{19}$:

C, 57.32; H, 6.64; N, 1.59; O, 34.55 %.

Found: C, 56.88; H, 6.58; N, 1.60 %.

Band B. The acetate of band B was preparatively thin layer chromatographed over silica gel H, using chloroform-methanol-acetic acid (80:17:3) as the developing system. Finally, it was precipitated from petroleum ether, as a white solid mp. 98-101°.

Ultraviolet spectrum in methanol.-Transparent.

Infrared spectrum in potassium bromide.

3450(w,b), 2940(w), 2850(sh), 1735(s), 1650(sh), 1530(w,b), 1430(w,b), 1370(s), 1225(s,b), and 1035(m,b) cm^{-1}.

for $C_{54}H_{63}NO_{27}$:

Found: C, 55.40; H, 6.40; N, 1.20 %.

J. Reductive Experiments on Palytoxin

1. Lithium Aluminum Hydride Reduction of Palytoxin

Palytoxin (2.57 mg) placed in a perforated glass capsule was washed down from a Soxhlet extractor over a slurry of peroxide-free dioxane (25 ml) and lithium aluminum hydride (75 mg). The mixture was refluxed for 6 hr and cooled to room temperature. The excess lithium aluminum hydride was destroyed by ethyl acetate (2 ml). The mixture was stirred for 2 hr, filtered through a sintered funnel and the filter

cake was washed with dioxane (10 ml). The filtrate and the washings were combined and evaporated to dryness. The dry mass was dissolved in water (UV: λ_{max} 271.5 nm, λ_{min} 232 nm), filtered and dialyzed against distilled water through a cellophane membrane. Both retentate and the diffusate were found to be transparent in UV.

2. Sodium Borohydride Reduction of Palytoxin

Sodium borohydride (20 mg) in ice cold water (4 ml) was added to a solution of palytoxin (0.9 mg) in water (3 ml). Simultaneously dilute sulfuric acid was added and the pH of the solution maintained at 7.0. The mixture was stirred for a total of 4.5 hr. It depicted only a shoulder at 273 nm in its ultraviolet spectrum.

K. Oxidative Reactions

1. Manganese Dioxide Oxidation of Palytoxin

To a solution of palytoxin (1.4 mg) in twice distilled water (1 ml) was added freshly prepared active manganese dioxide (64 mg). The mixture was allowed to stand with occasional stirring for 1 hr. It was then filtered and the residue washed with three portions of distilled water (0.5 ml each). The filtrates were combined and evaporated to a dry mass. The residue was examined for its ultraviolet spectrum.

λ_{max} 263 nm (8.3xM) λ_{max} 233 nm (6.8xM).

The white solid could not be further oxidized with manganese dioxide (69 mg) in water (1 ml), over a period of 3 d, as indicated by the retention of the quantitative ultraviolet spectrum. Paper chromatography over Whatman No. 1 paper using 1-propanol-ethanol-water

(7:1:2, Rf 0.239) and ethanol-water (8:2, Rf 0.26) indicated the presence of only one compound. The compound was found to be negative to Brady's reagent and to the hydroxamate ester test, but positive to permagnate-periodate and silver nitrate sprays.

Infrared spectrum in potassium bromide.

3400(s,b), 1650(m,b), 1150(s,b), 1000(sh), cm^{-1}.

2. Oxidation of Palytoxin by Fuming Nitric Acid

Palytoxin (2.59 mg) was heated to 92° in fuming nitric acid (0.6 ml) for 4 hr under a slight positive pressure of nitrogen. Nitrogen peroxide was removed by blowing nitrogen through the solution and then by aspirator vacuum. It was then evaporated to dryness. No residue could be observed.

3. Oxidation of Palytoxin by Molecular Oxygen in the Presence of Catalytic Platinum in Base

Palytoxin (31.0 mg) was dissolved in sodium bicarbonate (0.1N, 4 ml) and water (16 ml). Degassed freshly prepared catalytic platinum (270 mg) was added to the above solution. The mixture was heated to 65° and a stream of pure oxygen was bubbled through the solution for 7 d. The solution was then filtered and the residue washed with water (10 ml). The mixture was passed over a freshly prepared Amberlite IR 120 column (1x12 cm). The eluates were evaporated to a small volume and chromatographed over a Sephadex G 25 (Fine) column (1.5x28 cm, flow rate 19 ml/hr, fraction size 3.1 ml). Fractions 11 to 18 were combined, evaporated to a small volume and freeze dried to a hygroscopic light yellow granular solid (21 mg). The behavior of this solid on paper and tlc is summarized as follows.

Solvent System	Paper		Kieselguhr G	
	Palytoxin	Cat. oxidized Palytoxin	Palytoxin	Cat. oxidized Palytoxin
1-Propanol-Ethyl acetate-water (7:1:4)	0.459	0.510		
1-Butanol-Pyridine-water (45:25:40)	0.393	0.514		
Pyridine-Ethyl acetate-Acetic acid-Water (5:5:1:3)	0.3525	0.246		
1-Butanol-Ethanol-Water (5:1:4)	0.130	0.304	0.625	0.125

The compound was negative to DNP spray but positive to bromocresol green, benzidine-periodate and silver nitrate reagents.

Ultraviolet spectrum in water.-Transparent.

Infrared spectrum in potassium bromide depicted the following bands. 3350(m,b), 2940(w), 2850(sh), 1720(s,b), 1640(sh), 1370(b), 1220(m,b), 1100(s,b), 1010(sh,b) cm^{-1}.

Nuclear magnetic resonance spectrum in deuterium oxide had the following signals: δ values,

0.7(b), 1.1(b), 1.5(b), 2.2(b), 3.0(b), 3.6(b), 4.5(b,s)

Potentiometeric Titration. - Potentiometeric titration did not indicate the presence of any titratable group between pH. 3.5 and 13. On allowing the sample to sit at pH 12 for several hr, a group appeared to be present at pH 6.0, which still represents a small quantity of the sample or indicates a huge molecular weight.

a. Attempted Formation of β-Napthylhydrazone of Calalytically Oxidized Palytoxin

Catalytically oxidized palytoxin (2.6 mg) was refluxed with freshly sublimed β-napthylhydrazine in methanol (10 ml), for 2 hr. The mixture was evaporated to dryness. Analytical thin layer chromatography of the residue indicated the presence of twelve compounds. Since preparative thin layer chromatography could not effectively be performed, no attempt at further purification of the mixture was made.

b. Attempted formation of 2,4-dinitrophenylhydrazone of oxidized palytoxin

Catalytically oxidized palytoxin (1.5 mg) was dissolved in water (0.2 ml). 2,4-Dinitrophenylhydrazine reagent (2 drops) was added and the mixture allowed to stand at room temperature for 48 hr. During this period a fine precipitation occurred. The mixture was extracted with benzene (10 ml) containing a few drops of ethyl acetate. The benzene extract was dried over potassium carbonate and evaporated to dryness. Analytical thin layer chromatography over silica gel G indicated that the residue consisted of more than 6 compounds. Separation of these products was not attempted.

c. Esterification of catalytically oxidized palytoxin

Catalytically oxidized palytoxin (3.5 mg) was esterified by excess diazomethane. The methyl ester thus obtained was acetylated by acetic anhydride (0.1 ml) in pyridine (0.2 ml). The crude acetate on preparative thin layer chromatography over silica gel H furnished the pure acetate (1.5 mg).

Ultraviolet spectrum in methanol. - Transparent.

Infrared spectrum in potassium bromide depicted the following bands. 3400(w,b), 1720(s), 1440(m,b), 1420(sh), 1350(m), 1280(sh), 1200(s), 1120(m), 1070(m,b) cm^{-1}.

4. Catalytic Oxidation at pH 7

A similar oxidation of palytoxin (4.73 mg) was conducted at pH 7.0 at room temperature. The reaction mixture was examined by analytical thin layer chromatography and infrared spectroscopy.

	Paper		Kieselguhr G	
Solvent System	Palytoxin	Oxidized Palytoxin	Palytoxin	Oxidized Palytoxin
1-Propanol-Ammonium hydroxide (1:1)	0.113	0.1066		
1-Butanol saturated with Water	0.860	0.860		
1-Butanol-Pyridine-Water (60:40:30)	0.207	0.207		
1-Butanol-Acetic acid-Water (4:1:5)	0.259	0.222 & 0.311		
Pyridine-Amyl alcohol-Water (7:7:6)			0.606	0.530 & 0.625

Infrared spectrum in potassium bromide exhibited the following bands. 3400(b,s), 2950(m), 2850(sh), 1720(sh), 1650(w,b), 1420(w,b), 1075 (b,s), 1010(w,sh) cm^{-1}.

5. Oxidation of Palytoxin by Nitric Acid

Palytoxin (46.13 mg) was heated to 80° with conc nitric acid (1.5 ml) for 4 hr. The mixture was cooled and freed of nitrogen peroxide by aspirator vacuum. Water (5 ml) was added and the mixture extracted with ether (30 ml). The aqueous layer was evaporated to

dryness, the residue was mixed with ethanol (3 ml) and evaporated to dryness. The process was repeated thrice. The residue was dissolved in water and freeze dried to furnish a yellow solid (27.0 mg). The yellow solid was chromatographed over Sephadex G 10 (2.0x60 cm, fraction size 5.5 ml, flow rate 50 ml/hr). Fractions 10 to 22 were combined, evaporated to a small volume and then freeze dried to a pale solid. The solid was preparatively chromatographed over Whatman No. 1 paper. Two bands A (11 mg), B (10 mg) corresponding to Rf's 0.266 and 0.233 (1-butanol-acetic acid-water 4:1:5) were collected, and found to be positive to silver nitrate and potassium permagnate. The two bands showed Rf values of 0.483 and 0.451 respectively with a solvent system: 1-propanol-methylbenzoate-formic acid-water (7:3:2:5).

Band B

Ultraviolet spectrum in water. - Transparent.

Infrared spectrum in potassium bromide depicted the following bands. 3400(b,s), 2925(sh), 2525(b), 1640(b,s), 1400(b,s), 1280(b,w), 1220(w), 1120(b,m), 800(w) and 715(m) cm^{-1}.

Nuclear magnetic resonance spectrum in deuterium oxide. - Compound did not show any signal.

Calcd for $C_4H_6O_9$:

C, 24.26; H, 3.05; O, 72.69 %.

Found: C, 21.83, 22.07; H, 2.89, 3.02; N, 0.0 and 0.00 %.

The ether extract (5 mg) was subjected to analytical thin layer chromatography over silica gel H using chloroform-methanol (70:30, Rf 0.857), chloroform-methanol (80:20, Rf 0.749), benzene-2-butanone (80:20, Rf 0.196), benzene-2-butanone (50:50, Rf 0.333) as the

developers. The ether extract was then esterified by diazomethane.

Infrared spectrum in chloroform.

3250(sh), 2900(s,b), 2750(sh,b), 1700(s,b), 1650(sh), 1550(b,s), 1400(s,b), 1200(s,b), 1190(m,b), 1040(m,b), 900(b) cm^{-1}.

Gas chromatography of the esterified ether extract over SE30 5% on a Chromosorb W column (1/8" x 10'), using a column temperature of 120°, detector temperature 200°, indicated the presence of seven compounds.

Rt 0'.40", 2'.00", 3'.00", 4'.45", 5'.45", 7'.00" and 8'.00".

6. Modified Kuhn Roth Oxidation of Palytoxin

Palytoxin (10 mg) was reacted with chromium trioxide reagent (1 ml) in water (5 ml), at 90° for 30 min. The reaction mixture was steam distilled. The steam distillate (25 ml) was collected and extracted with ether (50 ml) in a liquid-liquid extractor. The ether layer was dried over sodium sulfate, concentrated to a small volume using a 1' long fractionating column, and reacted with diazomethane. The mixture was examined for the products by analytical gas chromatography over SE30, 30%, on a Chromosorb W, column, 1/8" by 9', column temperature 150°, detector temperature 250° and injector temperature 200°.

Retention Times

2'18"	4'00"	6'48"	10'30"	18'20"
2'42"	5'03"	8'00"	11'20"	19'24"
3'06"	5'36"	9'30"	16'00"	20'04"
25'48"	36'42"	13'20"		

A retention time of 3'06" corresponds to that of a C_5 dicarboxylic acid.

A retention time of 5'03" corresponds to that of a C_{10} carboxylic acid.

A retention time of 8'00" corresponds to that of a C_{11} carboxylic acid.
A retention time of 13'20" corresponds to that of a C_8 dicarboxylic acid.
A retention time of 20'04" corresponds to that of a C_{12} carboxylic acid.

7. Oxidation of Palytoxin by Dimethyl sulfoxide in Acetic Anhydride

Acetic anhydride (0.4 ml) was added to a solution of palytoxin (5.9 mg) in dry dimethyl sulfoxide (9.6 ml). The mixture was left at room temperature for 24 hr, evaporated to a brown gum under vacuum at a water bath temperature of 70°. The gummy mass was mixed with ethanol (4 ml), and evaporated to dryness. The process was repeated thrice. Water (5 ml) was added to the residue and the mixture partitioned between chloroform (10 ml) and water (5 ml). The chloroform extract was backwashed with two portions of water (5 ml each), dried over anhydrous potassium carbonate, filtered and evaporated to a brown gum. The gum was positive to 2,4-dinitrophenyl-hydrazine reagent.

The gum (ca. 4.0 mg) was decolorized by Darco G (20 mg) in acetone (5 ml). The decolorized filtrate was evaporated to dryness, dissolved in chloroform (0.15 ml) and precipitated as a pale solid from petroleum ether (10 ml). The solid on analytical thin layer chromatography over silica gel H indicated the presence of at least two compounds (chloroform-methanol-acetic acid 75:20:5, Rf 0.84; dichloromethane-methanol and dimethylformamid 80:18:2, Rf 0.791 and 0.000). Preparative thin layer chromatography using the former solvent

system furnished a band corresponding to Rf 0.84.

Infrared spectrum in chloroform depicted the following signals. 3350(b,w), 2950(s), 2800(sh), 1750-1700(s,b), 1600(sh), 1450(b,w), 1370(m), 1200(b,w), 1050(b,s) cm^{-1}.

8. Potassium Permanganate Oxidation of Palytoxin

Palytoxin (16.3 mg) was stirred with potassium permanganate (200 mg) in distilled water (20 ml) at room temperature for 10 hr. The excess permanganate was destroyed by dropwise addition of sodium bisulfite. The mixture was then filtered, the residue washed with water (5 ml). The filtrates were extracted with three portions of 1-butanol (10 ml each). The butanol extract on evaporation furnished a gum (2.4 mg) which depicted tailing on silica gel H and alumina H thin layer chromatography (solvent system, methanol). The aqueous solution from butanol extraction was freed of n-butanol and dialyzed against distilled water. The retentate on evaporation furnished a glass, which on paper chromatography indicated the presence of at least 4 compounds (solvent system, ethanol-water-ammonium hydroxide 8:1:1, indicator silver nitrate in acetone). The glass was triturated with ethanol. The ethanol extract was preparatively chromatographed over Whatman No. 1 paper (solvent system, ethanol-water-ammonium hydroxide 8:1:1) and the band corresponding to Rf 0.23 (positive to bromophenol blue) was collected as a white solid (0.4 mg). The solid melted at 50°, solidified and turned brown at 180°. The solid left after ethanol trituration was found to be negative to 2,4-dinitrophenylhydrazine and aniline phthalate sprays. It showed the presence of at least two compounds on cellulose thin layer chromatography.

9. Small Scale Oxidation of Palytoxin with Sodium Metaperiodate

To a solution of palytoxin (2.5 mg) in distilled water (0.4 ml) was added a solution of sodium metaperiodate (15.0 mg) in water (0.5 ml). The mixture was diluted with 1.0 ml water and shaken well. Excess periodate was determined periodically by back titration of the arsenite with standard iodine--potassium iodide solution. The results are presented in Fig. 11.

a. Determination of volatile aldehydes formed during sodium metaperiodate oxidation of palytoxin

Palytoxin (2.1 mg) was dissolved in sodium metaperiodate (18.3 mg) solution in water (1 ml), and sulfuric acid (10N, 14µℓ). After a reaction time of 80 min, the solution was assayed for formaldehyde by complexation with chromotropic acid, and measuring the intense purple colored product by its visible absorption spectrum at 570 nm. The solution was found to be negative for formaldehyde.

b. Determination of total acids formed during sodium metaperiodate oxidation of palytoxin

Palytoxin (2.6 mg) in water (5.0 ml) was diluted with a solution of recrystallized sodium metaperiodate (57 mg) in water. The reaction mixture was heated in a boiling water bath for 30 min. The solution was cooled and ethylene gylcol (200µℓ) was added. After 10 min, 25µℓ of bromothymol blue indicator solution was added and the solution titrated against sodium hydroxide (0.01004 M). The reaction mixture required 2.062 ml (20.7µ moles/2.6 mg of toxin or 8µ moles of base/1 mg toxin) of the standard solution.

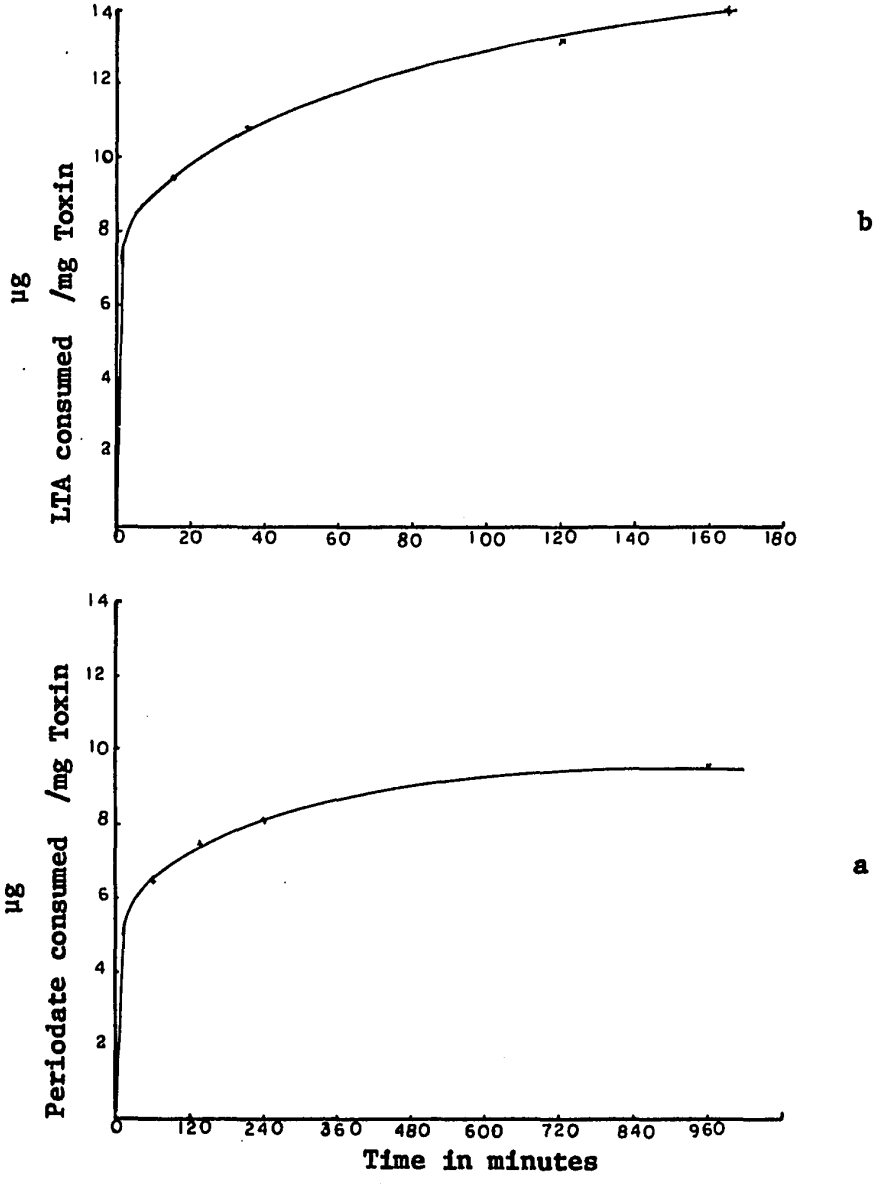


Fig. 11. Kinetics of Oxidation of Palytoxin:

a. Sodium metaperiodate
b. Lead Tetraacetate.

10. Large Scale Sodium Metaperiodate Oxidation of Palytoxin

Sodium metaperiodate (500 mg) in water (12 ml) was added dropwise with stirring to a cooled solution of palytoxin (240 mg) in twice distilled water (8.0 ml), under nitrogen. The mixture was stirred at ice-water temperature for 9 hr. It was then extracted with two 25 ml portions of chloroform. The chloroform extract was backwashed with water (10 ml), dried over sodium sulfate and evaporated to a yellow gummy mass (ca. 80.0 mg). The gummy mass was reduced with sodium borohydride (500 mg) in distilled 2-propanol (8 ml) for 9 hr. The mixture after evaporation to dryness was acidified by 5N-hydrochloric acid to pH 1.0, dissolved in water (5.0 ml), saturated with sodium chloride and extracted with two portions of chloroform (15 ml, each). The chloroform extract after drying over sodium sulfate was evaporated to a pale gummy mass (63.0 mg). The gummy mass was reacted with acetic anhydride (0.5 ml) in pyridine (1.0 ml) at room temperature for 24 hr. The reaction mixture was evaporated to dryness and dissolved in chloroform (10 ml). The chloroform layer was washed with two portions of 1N-hydrochloric acid (5 ml each), saturated sodium carbonate and water. It was then dried over sodium sulfate and evaporated to an acetate mixture (45 mg). The acetate mixture was chromatographed over a Sephadex LH 20 column (2.0x50 cm, flow rate 40 ml/hr, fraction size 5.6 ml). The fractions were combined as follows:

Fractions 10-14	C	18.5 mg
Fractions 15-20	D	26.5 mg

Both C and D on analytical thin layer chromatography over silica gel H, using cyclohexane-ethyl acetate (65:35) as the developer indicated the

presence of more than four compounds in each case.

C Rf's 0.656, 0.531, 0.413, 0.280 and 0.094.

D Rf's 0.600, 0.350, 0.313, 0.280 and 0.094.

Preparative thin layer chromatography of both C and D over silica gel H using cyclohexane-ethyl acetate (65:35 and 50:50) and proper combination of the various fractions yielded:

C-104-D (0.6 mg), C-107-A (2.5 mg), C-109-A (1.9 mg), C-109-B (4.0 mg), C-109-C (1.0 mg), C-107-C (7.0 mg), and C-111-A (1.6 mg).

The reaction sequence and isolation procedure are schematically presented in Fig. 12. C-107-C was crystallized from ethyl acetate-petroleum ether as needles (4.0 mg), mp. 71-75°. C-109-C and C-111-A were found to be mixtures while C-104-D, C-107-A, C-107-C, and C-109-B appeared to be homogeneous on thin layer chromatography (Rf 0.350, 0.690, 0.114 and 0.192 respectively).

a. Compound C-104-D. Attempts to crystallize compound C-104-D from ethyl acetate-petroleum ether failed. The compound is a colorless oil.

Ultraviolet spectrum in methanol. - Transparent.

Infrared spectrum in chloroform.

2940(s), 2850(sh), 1730(s), 1460(b,w), 1370(s), 1275(b,s), 1125(w) and 1050(b,w) cm^{-1}.

Nuclear magnetic resonance spectra (HA100) in deuterochloroform and pyridine-d_5 are presented in Table XIV and Figure 13.

Mass spectra. - 20 and 70 EV Mass Spectra are reproduced in Figures 14 and 15, and Tables XV and XVI.

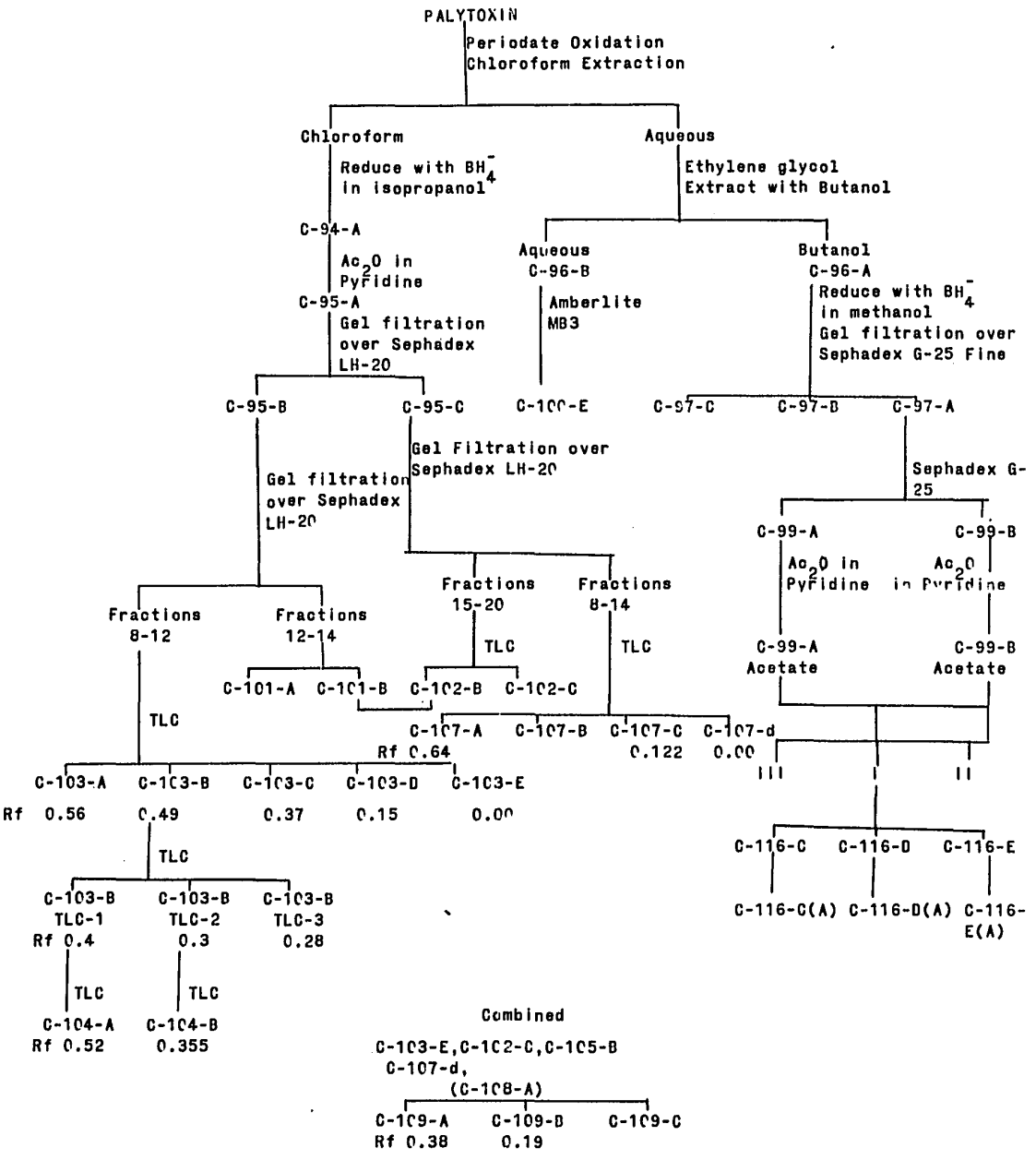


Fig. 12. Isolation Scheme for Periodate Oxidation Products

TABLE XIV

100 MHz NMR SPECTRA OF C-104-d IN DIFFERENT SOLVENTS

Deuterochloroform	Pyridine-d_5	
Chemical Shift	Relative Proton Ratio	Chemical Shift
0.90δ (d, J=7.0 Hz)	6 H	0.810δ (s)
1.21δ (s)	3 H	0.875δ (s)
1.32δ (s,b)	---	0.945δ (s)
1.63δ (s,b)	6-10 H	1.005δ (s)
2.00, 2.01 and 2.02δ (s)	9 H	1.300δ (b)
2.03δ (s,b)	1-2 H	1.600δ (b)
4.00δ (c)	6-8 H	1.92, 1.96
5.00δ (c)	1 H	2.00, 2.02 and 2.05δ (s)

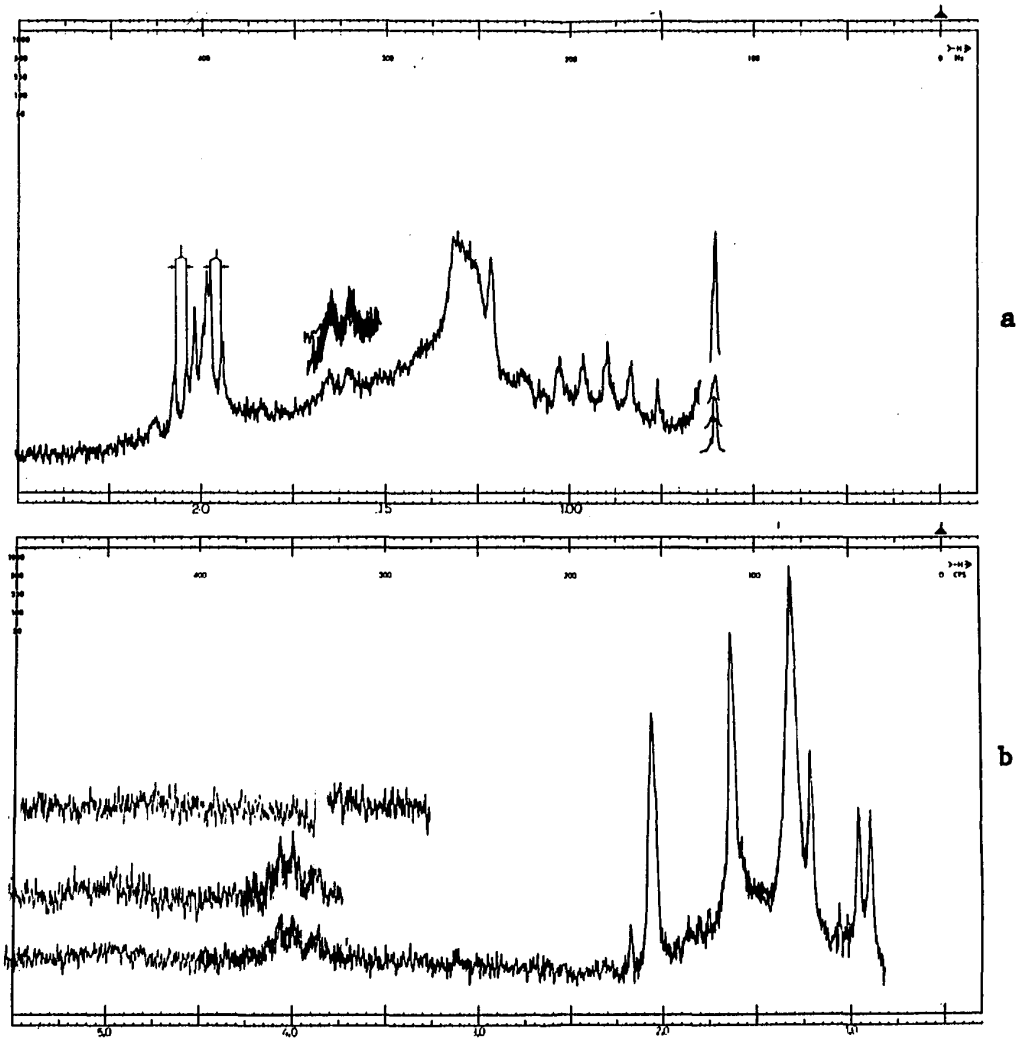


Fig. 13. 100 MHz NMR Spectra of C-104-d in:

a. Deuteropyridine
b. Deuterochloroform

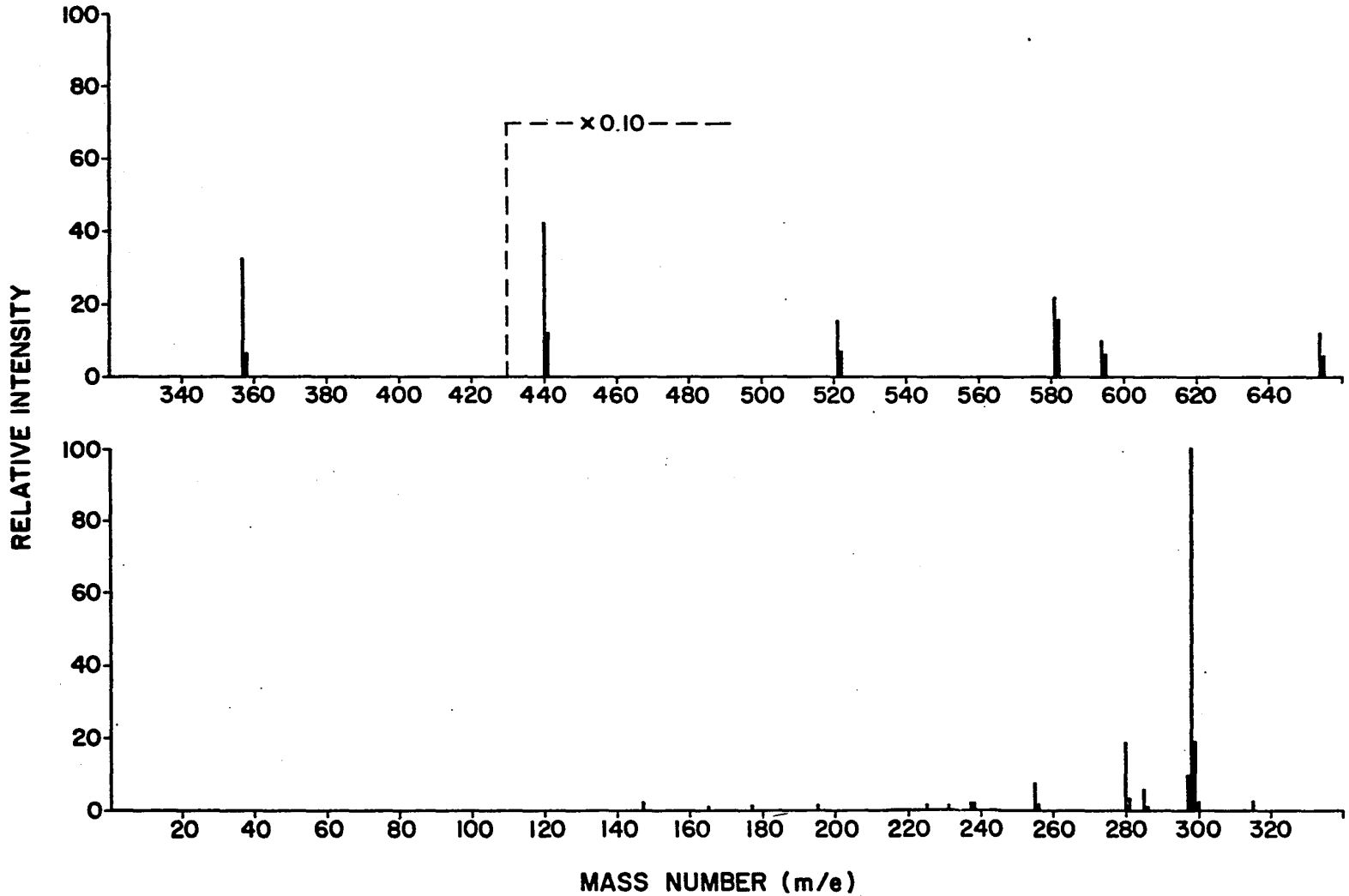


Fig. 14. 20 EV Mass Spectrum of C-104-d

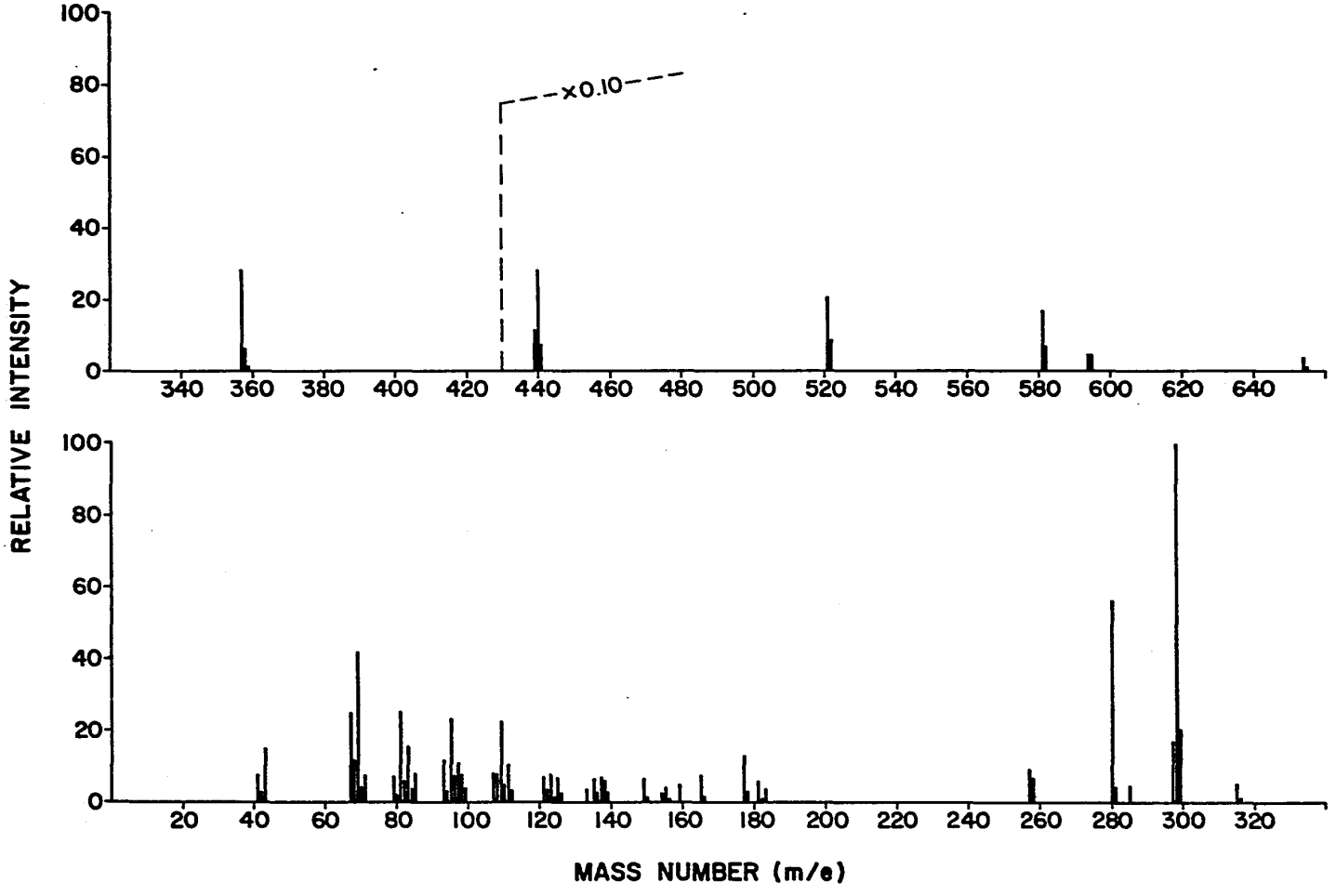


Fig. 15. 70 EV Mass Spectrum of C-104-d

TABLE XV

20 EV MASS SPECTRUM OF C-104-d

m/e	%	m/e	%	m/e	%	m/e	%
654	1.2	534	0.60	255	44	95	24
636	0.24	440	4.2	237	8	81	25
612	0.12	357	32.5	195	22	69	42
594	2.0	315	4.0	177	12	67	25
581	2.2	298	100	155	4	55	26
552	0.24	280	20	109	22	43	15

TABLE XVI

70 EV MASS SPECTRUM OF C-104-d

m/e	%	m/e	%	m/e	%	m/e	%
654	0.4	521	2	298	100	109	22
639	0.03	503	0.30	280	20	95	24
636	0.06	479	0.20	255	44	81	25
612	0.06	461	0.46	237	8	69	42
594	0.5	440	2.8	195	22	67	25
581	1.7	357	27	177	12	55	26
552	0.11	315	4	155	4	43	15
534	0.30						

High resolution measurements on the following peaks were obtained.

m/e	Actual Composition		Measured Mass	Calculated Mass
654	$C_{36}H_{62}O_{10}$		654.43422	654.43427
581	$C_{33}H_{57}O_8$		581.40563	581.40532
521	$C_{31}H_{53}O_6$		521.38435	581.38419
440	$C_{24}H_{40}O_7$		440.27709	440.27738
357	$C_{18}H_{29}O_7$		357.19170	357.19130
298	$C_{18}H_{34}O_3$	(96%)	298.25087	298.25078
280	$C_{18}H_{32}O_2$		280.23999	280.24022
	$C_{14}H_{23}O_4$	(13%)	255.25962	255.25962
255	$C_{15}H_{27}O_3$	(87%)	255.19601	255.19601
	$C_{12}H_{19}O_2$	(65%)	195.13816	195.13850
195	$C_{13}H_{23}O$	(35%)	195.17472	195.17488
	C_4H_5O	(39%)	69.03405	69.03404
69	C_5H_9	(61%)	69.07041	69.07042

Bromination of Compound C-104-d in chloroform.-C-104-d (ca. 0.5 mg) was reacted with bromine (5 mg) in chloroform (0.5 ml). After 12 hr standing at room temperature, the mixture was evaporated to dryness. The dry mass on analytical thin layer chromatography over silica gel H, using cyclohexane-ethyl acetate (65:35) as the developer did not indicate any change in Rf value.

Test for olefin with tetranitromethane.-To one drop of C-104-d (1 mg) solution in chloroform (0.3 ml) was added 2 drops of tetranitromethane (1 drop) solution in chloroform (5 ml). No color other than that of tetranitromethane was observed.

b. Compound C-107-A

Compound C-107-A is a light colorless oil. It fluoresces blue on silica gel HF plates and turns yellow on prolonged exposure to air.

Ultraviolet spectrum in methanol depicted a maximum at 226.5 nm.

Infrared spectrum in chloroform depicted the following bands. 3005(w), 2957(w), 1723(s), 1642(v,w), 1450(w), 1390(w), 1370(m), 1257(s,b), 1210(s), 1047(m), 910(w), and 825(w,b) cm^{-1}.

Nuclear magnetic resonance spectrum is presented in Table XVII and Figure 16.

TABLE XVII

100 Mc NMR SPECTRUM OF C-107-A IN DEUTEROCHLOROFORM

Nature of Signal	Chemical Shift	Nature of Signal	Chemical Shift
singlet (b)	1.64δ	quartet (c)	2.51δ
broad	1.75δ	sextet (2t)	4.10δ
singlet	2.03δ	singlet	4.92δ
singlet	5.00δ	doublet (J=13 Hz)	5.87δ
broad	5.50δ		

Double Resonance Experiment

(i) When irradiated at 1.75δ sextet at 4.10δ collapses to a triplet.

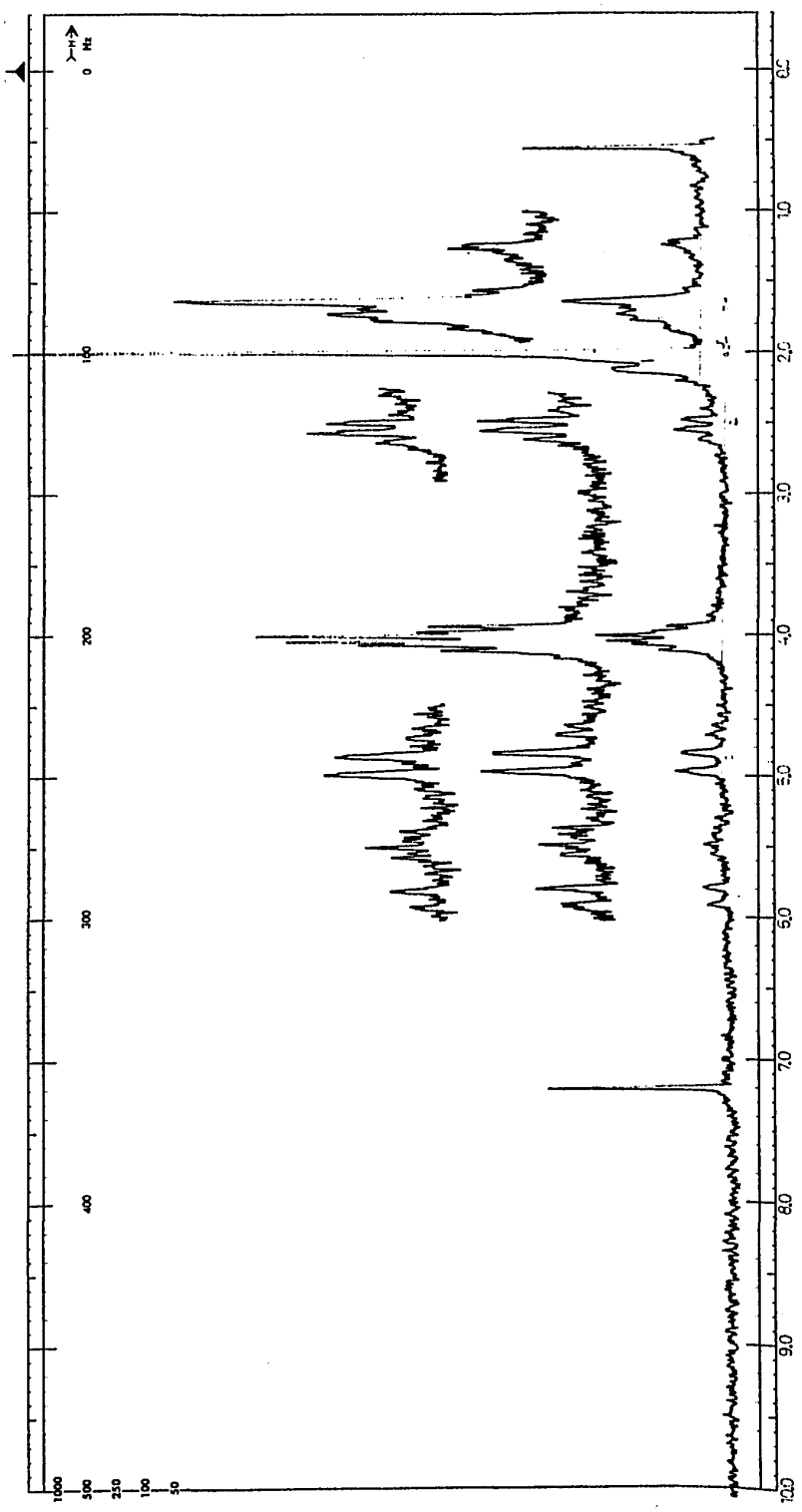


Fig. 16. 100 MHz NMR Spectrum of C-107-A in Deuterochloroform

(ii) When irradiated at 4.95δ no change is observed in the spectrum.

(iii) When irradiated at 5.90δ, no change in signals at 4.92δ, 5.00δ and 4.10δ, however the signal at 2.51δ became a sharp quartet.

Broad signal at 1.75δ appears more like a triplet.

Mass spectrum of compound C-107-A.

22 EV Mass spectrum depicted the following ions.

m/e	%	m/e	%
194	9.5	108	9.0
180	10.0	107	19.0
137	5.0	105	19.0
136	6.0	94	100.0
134	7.0	92	27.0
120	68.0	79	16.0
108	9.0	67	3.0

Bromination of Compound C-107-A. - C-107-A (ca. 0.75 mg) treated with bromine (ca. 5.0 mg) in chloroform (0.5 ml). After 12 hr standing at room temperature, the mixture was evaporated to dryness and the residue was examined by analytical thin layer chromatography over silica gel HF, using cyclohexane-ethyl acetate (65:35) as the developing solvent. The thin layer chromatography indicated the presence of two spots in addition to that of the starting material. Preparative thin layer chromatography using the same solvent system furnished the two bands A and B.

Band A.-70 EV mass spectrum of the band A depicted the following peaks.

m/e	%	m/e	%	m/e	%	m/e	%	m/e	%
401	0.50	321	0.50	277	6.00	155	30.0	95	44.0
399	1.00	319	1.00	258	16.0	137	62.0	85	35.0
397	0.50	317	0.70	197	93.0	128	60.0	81	40.0
382	0.90	307	1.50	191	28.0	123	82.0	73	31.0
368	1.70	281	6.00	189	28.0	119	100.0	55	77.0
341	1.00	279	14.0	162	83.0	109	54.0	43	83.0
339	1.70								

c. Compound C-107-C

C-107-C is a nonfluorescent crystalline compound mp 71-75°.

Ultraviolet spectrum in methanol.-Transparent.

Infrared spectrum in potassium bromide depicted the following bands (Fig. 17).

3250(m,b), 2900(s), 2840(sh), 1728(s), 2450(w,b), 1370(m), 1240(s), 1170(w), 1140(w), 1070(m), 1040(m), 955(w), 900(w), 720(w) cm^{-1}.

Nuclear magnetic resonance spectra.-Nuclear magnetic spectra of C-107-C in deuterochloroform, in pyridine-d_5 and benzene-d_6 are presented in Table XVIII and Figures 18 and 19.

Mass spectra.-70 and 20 EV Mass spectra are presented in Figures 20 and 21 and Tables XIX and XX.

Anal. Calcd for $C_{34}H_{60}O_9$:

C, 66.72; H, 20.21%.

Found: C, 66.64, 64.81; H, 10.19, 10.24; N, 0.0, 0.0%

Qualitative Tests.-The compound is negative to tetranitromethane and also to bromine in chloroform (Rf of the product is the same as that of the starting material).

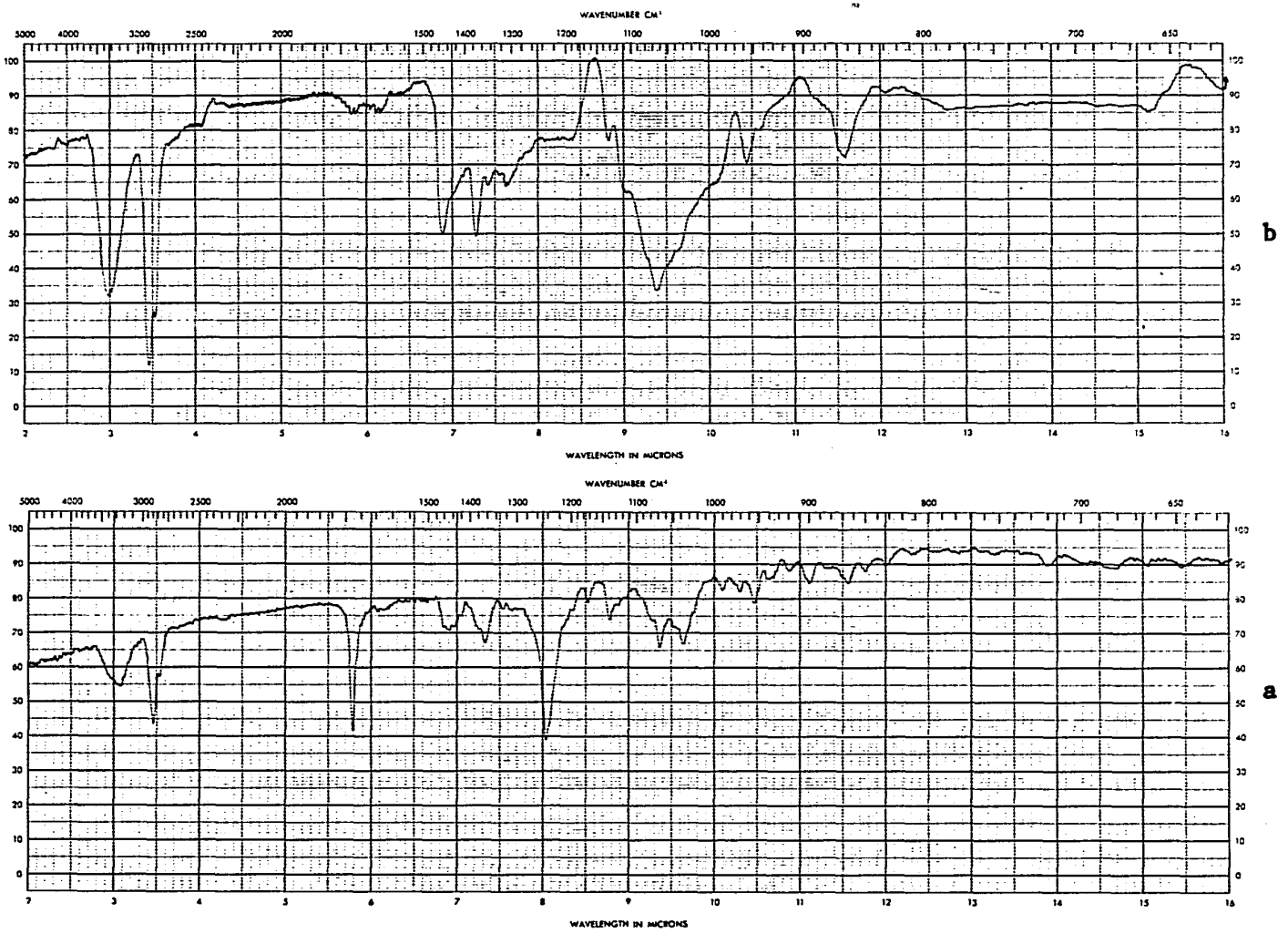


Fig. 17. Infrared Spectra of C-107-C:

a. in Potassium Bromide
b. after Saponification

TABLE XVIII

100 MHz NMR SPECTRA OF C-107-C, δ VALUES IN DIFFERENT SOLVENTS

Deuterochloroform	Benzene-d_6	Pyridine-d_5	No. of Protons
0.840 (s)	0.830 (d, J=7Hz)	0.815 (s)	3
0.900 (s)	1.02 (d,J=7Hz)	0.880 (s)	
1.190 (s)	1.12 (s)	0.95 (s)	3
1.280 (s,b)	1.320 (s,b)	1.015 (s)	
1.600	1.700 (s,b)	1.200 (s)	3
2.000 (s)	3.725 (c)	1.290 (s,b)	16-18
2.040 (s)	3.830 (c)	1.600 (c,b)	14
2.050 (s)	4.000 (t)	1.900 (s)	3
2.500 (s,b)	4.550 (c)	1.970 (s)	6
3.650 (d)		3.920 (c,b)	4
3.820 (d)		4.075 (t)	
4.000 (t)		4.350 (t)	2
5.020 (b)			

s = Singlet; d = Doublet; t = Triplet; c = Complex; b = Broad

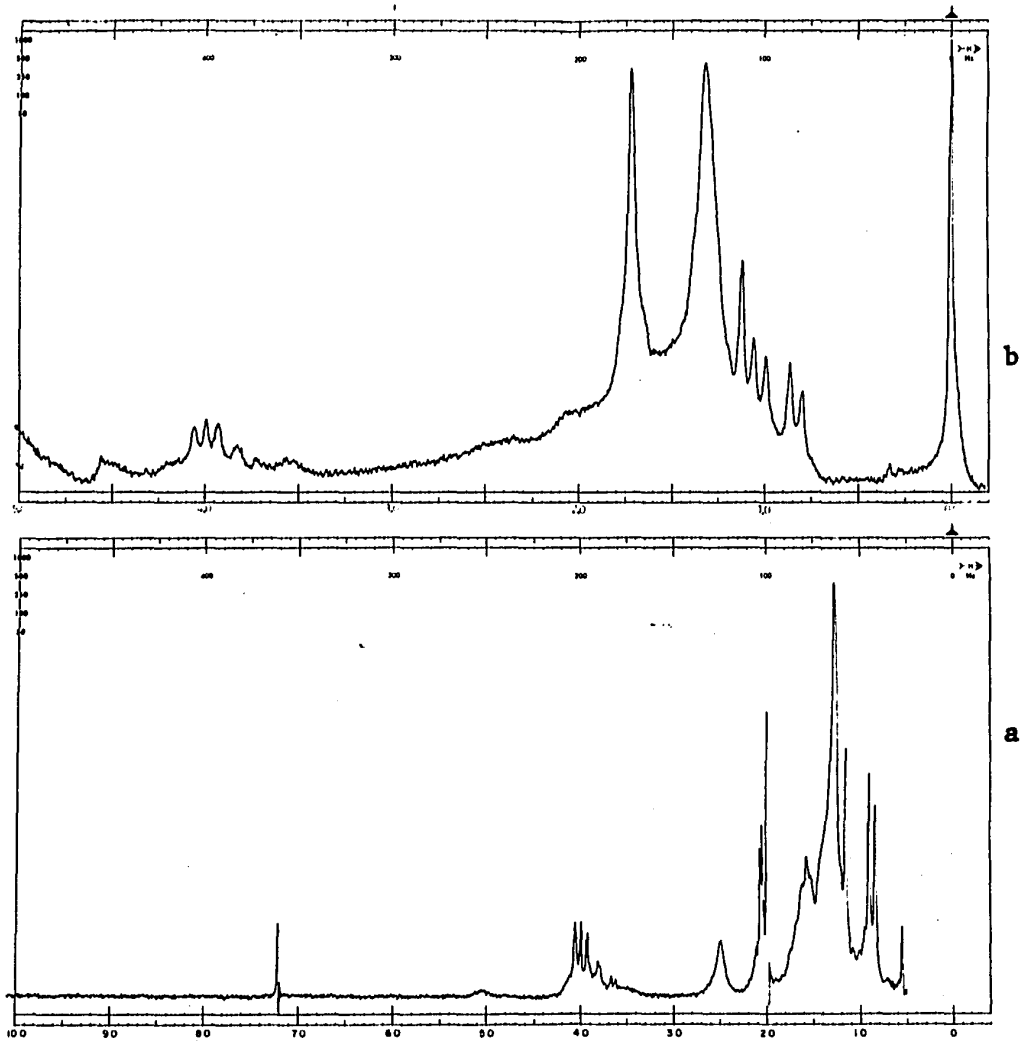


Fig. 18. 100 MHz Nuclear Magnetic Resonance Spectra of C-107-C in:

a. Deuterochloroform
b. Deuterobenzene

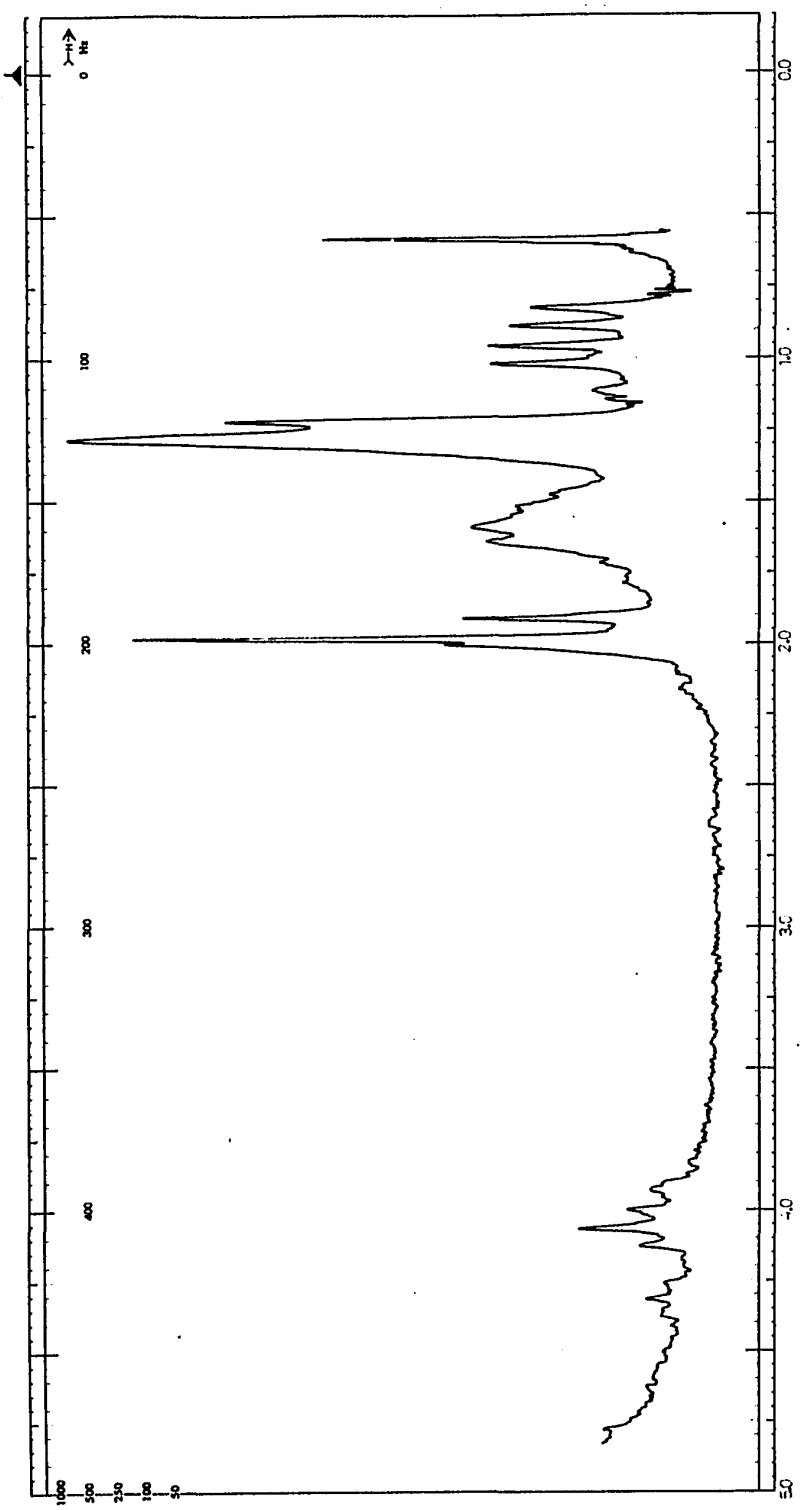


Fig. 19. 100 MHz NMR Spectrum of C-107-C in Pyridine-d_5

TABLE XIX

70 EV MASS SPECTRUM OF C-107-C

m/e	%	m/e	%	m/e	%	m/e	%	m/e	%
612	0.11	510	0.23	337	0.66	209	7	123	8.5
597	0.05	479	1.33	315	2.4	195	36	109	31.5
594	0.10	461	1.0	298	67	177	8	95	36
582	0.1	453	1.7	280	16	155	11	81	38
570	0.16	435	1.23	255	100	149	7	69	52
552	0.16	407	0.6	238	8	135	15	55	48
539	0.46	398	1.03	213	16	125	8.5	43	85
521	0.86	356	1.23						

TABLE XX

20 EV MASS SPECTRUM OF C-107-C

m/e	%	m/e	%	m/e	%	m/e	%	m/e	%
612	0.2	521	0.6	435	0.8	298	100	238	3
581	0.3	497	1.1	424	0.8	280	18	213	6.5
570	0.4	479	1.6	398	0.6	273	5	195	21
552	0.28	461	0.7	357	2.1	255	45	138	13
539	1.7	453	2.8	315	2.5				

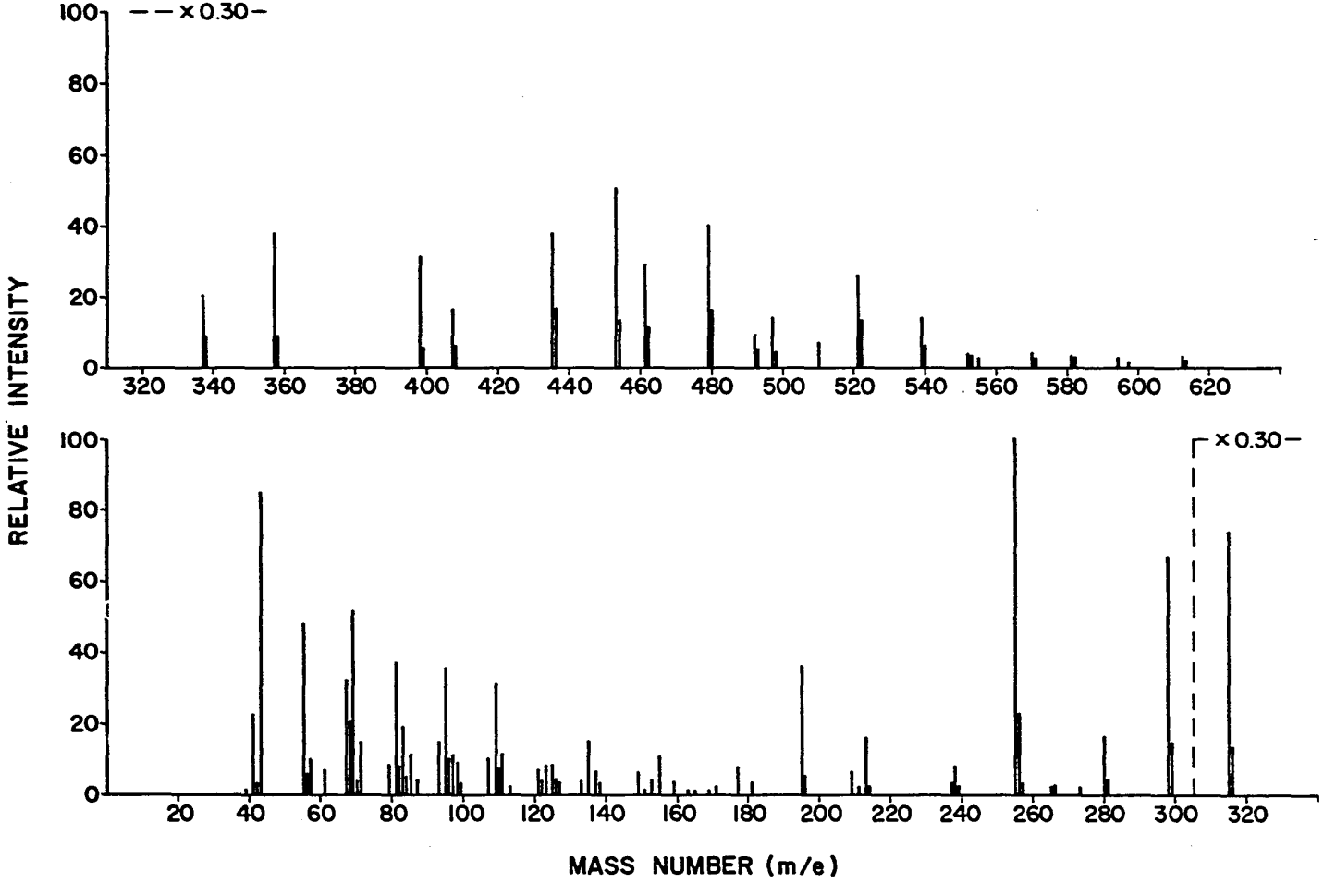


Fig. 20. 70 EV Mass Spectrum of C-107-C

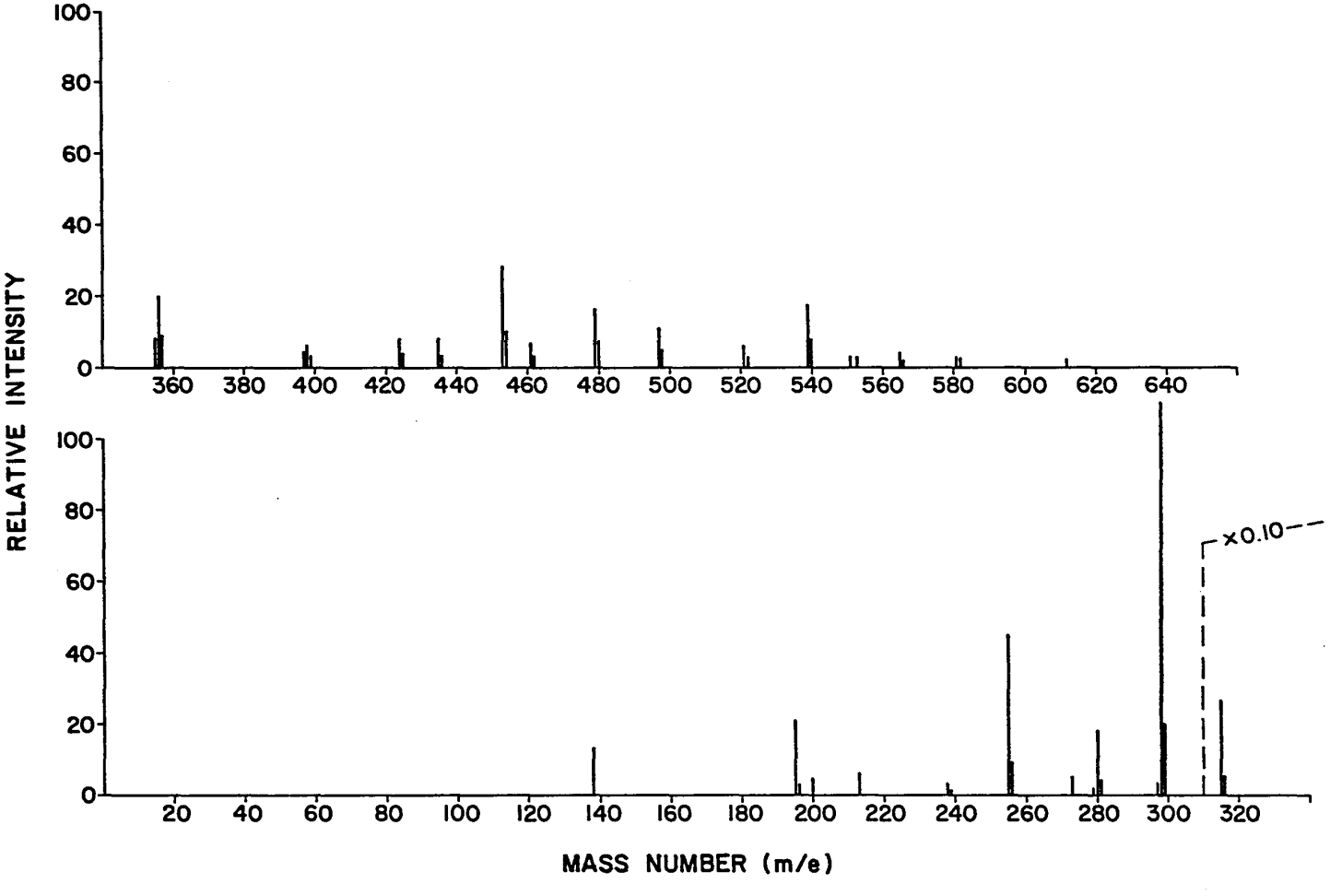


Fig. 21. 20 EV Mass Spectrum of C-107-C

High Resolution Measurements for Compound C-107-C are presented as follows:

m/e	Actual Composition		Measured Mass	Calculated Mass
612	- - -			
581	- - -			
539	$C_{31}H_{55}O_7$		539.39507	539.39478
479	$C_{29}H_{51}O_5$		479.37408	479.37365
453	$C_{27}H_{49}O_5$		453.35803	453.35800
424	$C_{26}H_{48}O_4$		424.35551	424.35526
356	$C_{20}H_{36}O_5$		356.25656	356.25628
298	$C_{18}H_{34}O_3$		298.25087	298.25078
	$C_{14}H_{23}O_4$	(6%)	255.15960	255.15962
255	$C_{15}H_{27}O_3$	(94%)	255.19611	255.19601
	$C_{12}H_{19}O_2$	(43%)	195.13834	195.13850
195	$C_{13}H_{23}O$	(57%)	195.17472	195.17488
280	$C_{18}H_{32}O_2$		280.23999	280.24022
138	$C_{10}H_{18}$		138.14077	

Saponification of C-107-C. - A mixture of C-107-C (7.5 mg), ethanol (95%, 0.5 ml) and aqueous 2 M sodium hydroxide (0.5 ml) was heated under nitrogen on an oil bath at 85° for 4 hr. The mixture was cooled, acidified by 6N hydrochloric acid and extracted with chloroform (30 ml) in a liquid-liquid extractor. The chloroform extract was dried over sodium sulfate and evaporated to a white solid (5.0 mg). The white solid was dissolved in alcohol-free acetone (0.1 ml). Dry ethyl acetate (0.3 ml) was added. The solution on cooling in a refrigerator for 36 hr deposited white needles. On centrifugation the

needles conglomerated to an opaque white solid, mp 79-82°.

Ultraviolet spectrum in methanol. - Transparent.

Infrared spectrum in potassium bromide.

3350(s), 2900(s), 2850(sh), 1650(w,b), 1455(b,m), 1375(m), 1130(w), 1060(s,b), 955(w), and 865(w,b) cm^{-1}.

Nuclear magnetic resonance spectrum in deuterochloroform (HA100, Fig. 15) depicted the following signals.

Nature of Signal	Chemical Shift	Relative Proton Ratio
Doublet (J = 6.0 Hz)	0.910δ	6
Singlet	1.190δ	3
Singlet (broad)	1.290δ	24
Complex (broad)	1.540δ	-
Complex (broad)	3.560δ	4
Complex	3.840δ	3

4-Bromobenzoylation of saponified C-107-C. - Saponified C-107-C (ca. 5.0 mg) was reacted with 4-bromobenzoyl chloride (50 mg) in dry pyridine (2.0 ml) at 80° for 18 hr. The mixture was evaporated to dryness. Water (1.0 ml) was added to the residue, the mixture was thoroughly scrubbed and extracted with chloroform (10 ml). The chloroform extract was backwashed with 10% potassium carbonate solution (two portions of 10 ml each) and saturated sodium chloride solution (5 ml). The chloroform layer was dried over sodium sulfate, filtered and evaporated to a gummy mass (ca. 7.5 mg). Analytical thin layer chromatography over silica gel HF using cyclohexane-ethyl acetate (80:20) as the developer indicated the presence of three major fluorescent and sulfuric acid positive spots (Rf 0.675, 0.580, and

0.540). Preparative thin layer chromatography over silica gel HF using the same solvent system furnished the three bands I (Rf 0.675), II (0.580) and III (0.540). These bands were further purified by repeated thin layer chromatography.

Band I.

Attempts to crystallize band I from chloroform-methanol, ethyl acetate-cyclohexane failed.

Ultraviolet spectrum in methanol.

λ_{max} 243 nm

λ_{min} 218 nm.

Infrared spectrum in chloroform.

3400(w), 2900(s), 2850(sh), 1710(s,b), 1580(s), 1450(w,b), 1385(m,b), 1270(s,b), 1200(s,b), 1115(s,b), 1100(s,b), 1068(s), 1040(b), 1010(s), 960(w,b), 915(w,b), 845(m,b) cm^{-1}.

Mass spectrum 70 EV mass spectrum depicted the following peaks.

m/e	%	m/e	%	m/e	%	m/e	%	m/e	%
480	3	281	5.5	185	54	96	35	55	85
452	2	256	10.0	183	60	83	61	43	94
440	6	229	13	149	53	71	53	41	64
438	6	202	32	111	29	69	68	36	32
397	2.5	200	32	109	25	57	100	32	32
395	2.5								

Band II.

Ultraviolet spectrum in methanol.

λ_{max} 243 nm.

λ_{min} 218 nm.

Infrared spectrum in chloroform depicted the following bands.

3450(w), 2900(m), 2850(sh), 1710(s,b), 1580(s), 1450(b,w), 1385(m), 1270(s,b), 1200(s,b), 1120(s,b), 1100(s,b), 1070(m), 1010(s), 960(w, b), 845(w) cm^{-1}.

Band III.

Ultraviolet spectrum in methanol.

λ_{max} 243 nm

λ_{min} 218 nm.

Infrared spectrum in chloroform depicted the following bands.

3400(w), 2900(m), 2830(sh), 1710(s,b), 1580(s), 1450(b,w), 1390(w), 1270(s,b), 1210(s,b), 1115(s), 1100(s), 1065(s), 1010(s), 960(w,b), 845(w) cm^{-1}.

d. Compound C-109-A

Compound C-109-A was viscous white mass. Compound gave negative bromine and tetranitromethane tests but positive benzidine periodate test. Compound was transparent in the ultraviolet.

Infrared spectrum depicted the following bands.

3400(w), 2900(s), 2825(sh), 1725(s), 1450(m), 1370(s), 1220(b), 1040(s,b), 875(w,b) cm^{-1}.

Mass spectra.

70 and 20 EV mass spectra are presented in Figures 22 and 23 and Table XXI.

TABLE XXI

MASS SPECTRA OF C-109-A

RELATIVE INTENSITIES OF VARIOUS FRAGMENTS

20 EV		70 EV		20 EV		70 EV	
m/e	%	m/e	%	m/e	%	m/e	%
642	0.6	642	0.30	298	100.0	267	8.0
624	0.65	624	0.30	285	5.5	255	95.0
611	0.40	611	0.20	280	19.0	238	6.0
608	0.30	582	0.25	267	3.5	225	9
582	0.40	581	0.25	255	39	213	10.0
581	0.30	569	0.35	238	2	207	15.0
570	0.40	565	0.25	225	2.5	195	19.0
569	0.50	551	1.20	213	3.5	181	7.0
565	0.40	539	2.05	199	3.0	165	4.0
551	1.05	521	0.90	197	3.0	155	10.0
539	1.90	497	1.40	191	2.5	147	6.0
521	0.65	479	2.00	185	2.5	135	12.5
497	1.20	462	0.6	165	1.5	125	10.0
479	1.60	461	1.2	128	3.0	123	11.0
461	0.75	453	4.3	116	1.5	121	8.5
453	3.70	435	1.65	99	1.5	109	32
435	0.80	428	1.90	86	1.5	95	34
428	1.90	370	2.2			81	39.5
424	1.05	345	3.40			69	59.5
410	0.60	327	12.0			55	43.0
340	3.20	298	100.0			43	97.0
327	11.0	285	8.0			29	3.5
						28	3.5

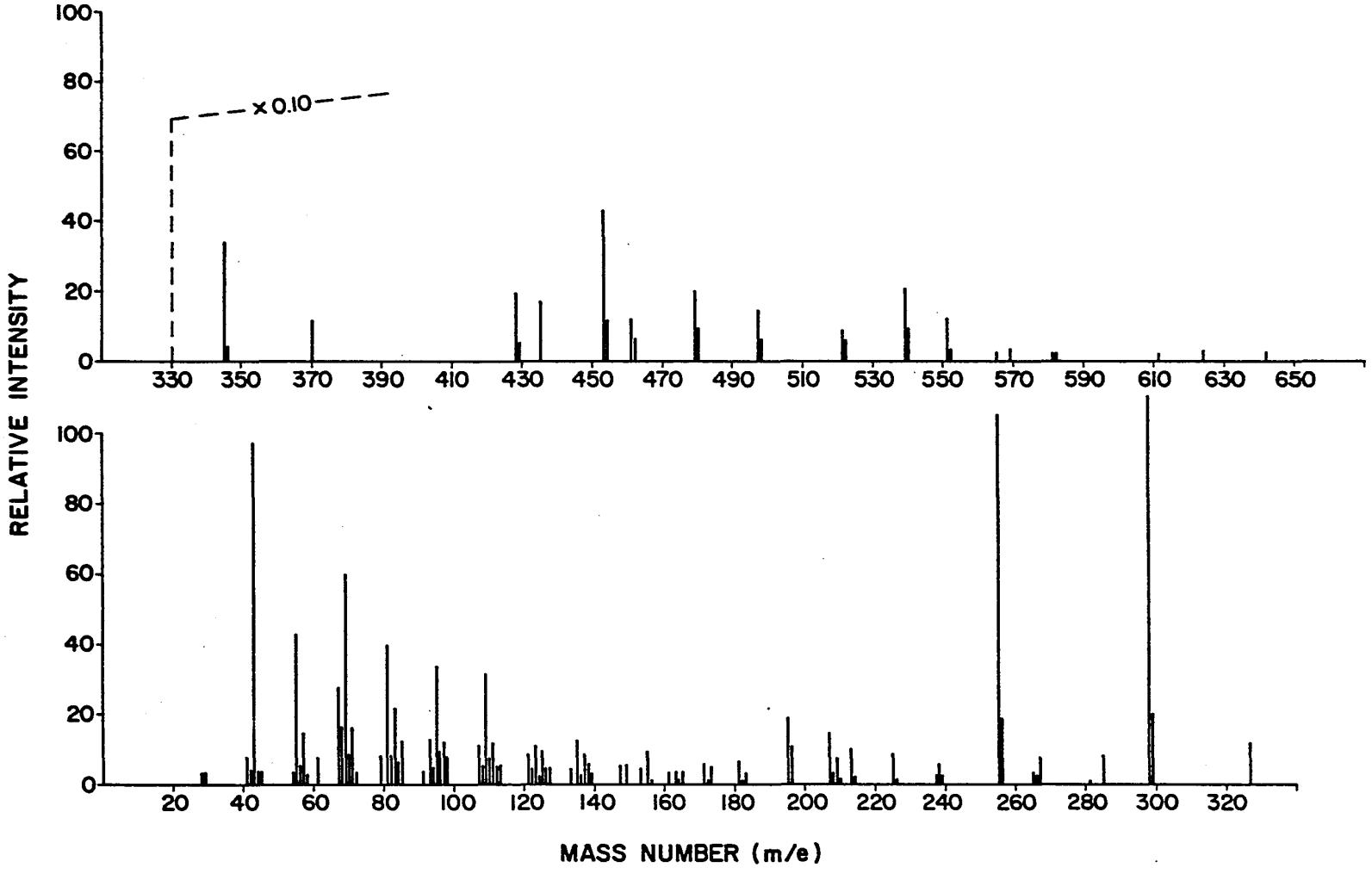


Fig. 22. 70 EV Mass Spectrum of C-109-A

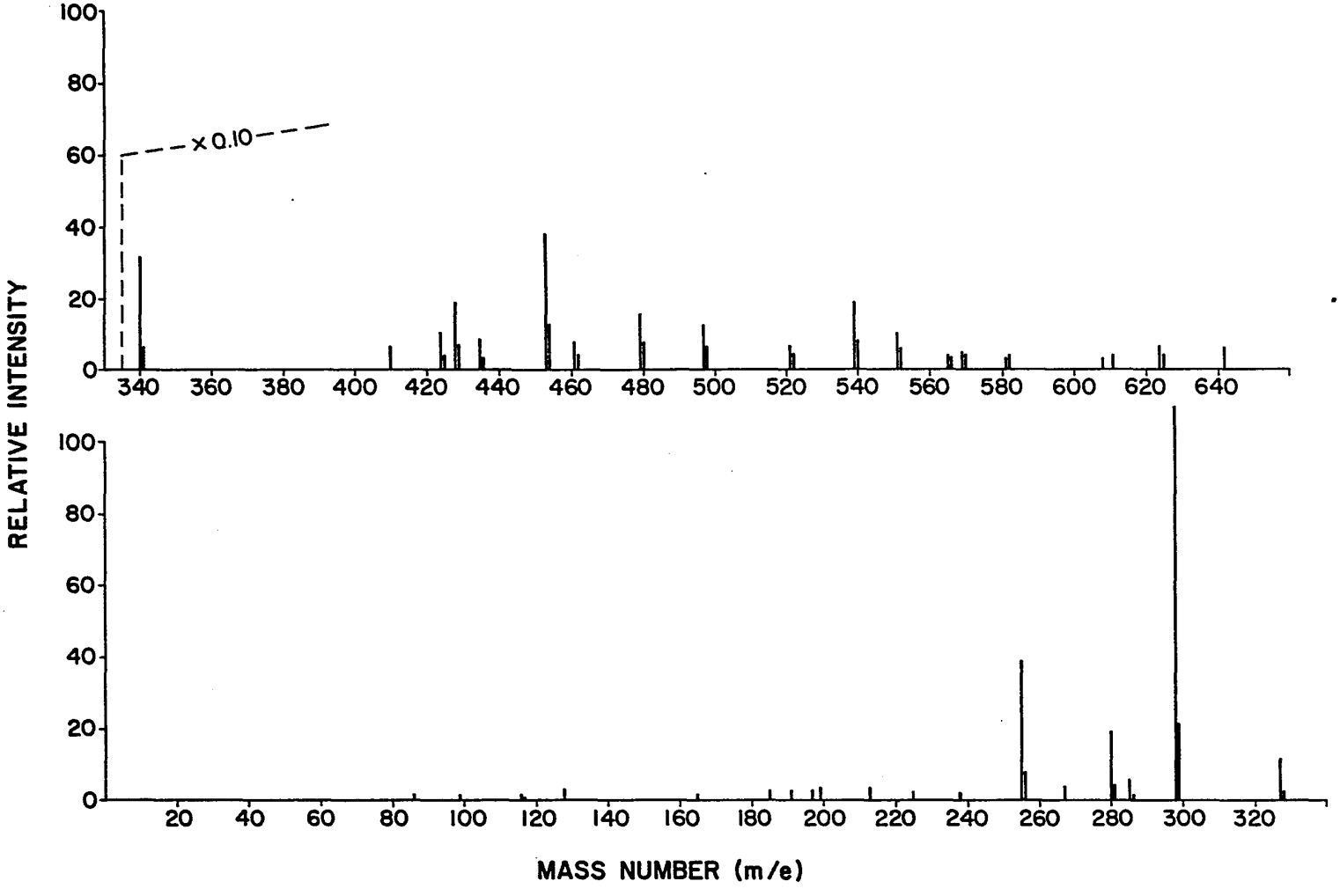


Fig. 23. 20 EV Mass Spectrum of C-109-A

e. Compound C-109-B

Compound C-109-B is a colorless non fluorescent gum at room temperature, but it freezes to a white granular solid at low temperature.

Ultraviolet spectrum in methanol. - Transparent.

Infrared spectrum in chloroform.

3350(w,b), 3000(sh), 2950(s), 2850(sh), 1725(s), 1450(b,w), 1372(m), 1250-1200(s,b), and 1040(b,m) cm^{-1}.

Nuclear magnetic resonance spectra. - Nuclear magnetic resonance spectra in deuterochloroform, pyridine-d_5 and benzene-d_6 are presented in Table XXIV and Figure 24.

Double Resonance Experiment. - On irradiation of peak at 1.32δ triplet at 3.75δ collapsed. The multiplet at 4.0δ could not be observed because of bead nodes.

Mass spectra. -

70 and 20 EV Mass Spectra are presented in Fig. 25 and 26 and Tables XXII and XXIII.

f. Mixture C-111-A

C-111-A was a mixture of three compounds as was indicated by thin layer chromatography over silica gel H.

C-111-A (ca. 2.0 mg) was heated in a sealed tube with red phosphorus (50 mg), hydroiodic acid (0.5 ml) in acetic acid (0.5 ml), at 110° for 4 hr. The mixture was cooled, neutralized with sodium hydroxide and extracted with ether in a liquid-liquid extractor. The ether extract after drying over sodium sulfate was evaporated to a small volume and subjected to analytical gas chromatography over 3% OV

TABLE XXIV

100 MHz NMR SPECTRA OF C-109-B

δ VALUES IN DIFFERENT SOLVENT SYSTEMS

Deuterochloroform		Benzene-d_6	Pyridine-d_5	No. of Protons
0.850δ (s)		0.850δ (D,J=7Hz)	0.795δ (s)	3
0.910δ (s)		1.020δ (D,J=7Hz)	0.860δ (s)	
1.160δ (s)		1.110δ (s)	0.905δ (s)	3
1.280δ (s,b)		1.370δ (s,b)	0.970δ (s)	
1.600δ (b)		1.630δ (s,b)	1.190δ (s)	3
2.000δ (s)		2.420δ (s,b)	1.290δ (b)	
2.140δ (s)		3.500δ (b)	1.660δ (b)	
3.600δ (T)	2	3.900δ (b)	1.850δ (s)	3
3.840δ (s,b)	1	4.200δ (b)	1.930δ (s)	3
3.950δ (s,b)	1		1.940δ (s)	3-5
4.200δ (D,J)	2		3.750δ (T,J=Hz)	2
			3.900δ (c)	2-3
			5.500δ (b)	3-4

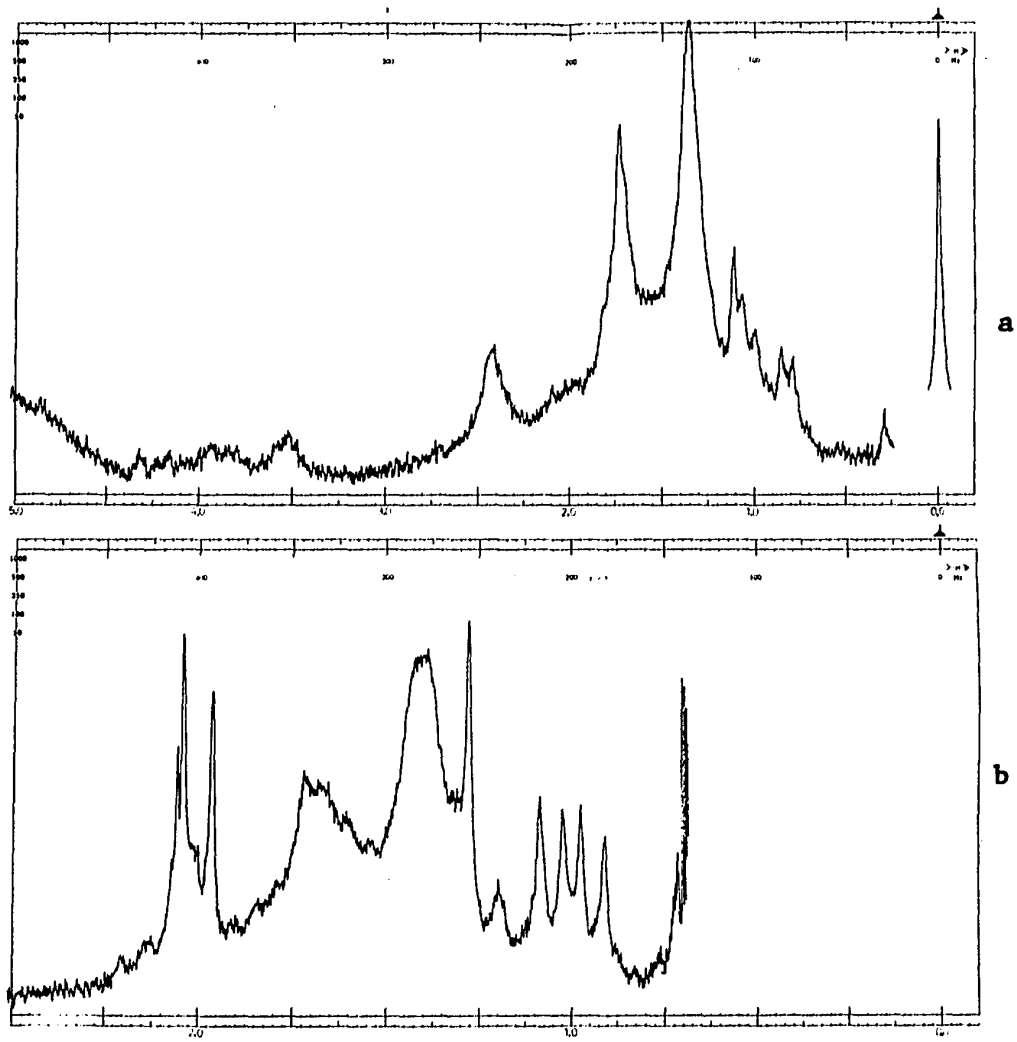


Fig. 24. 100 MHz NMR Spectra of C-109-B in:

a. Deuterobenzene
b. Pyridine-d_5

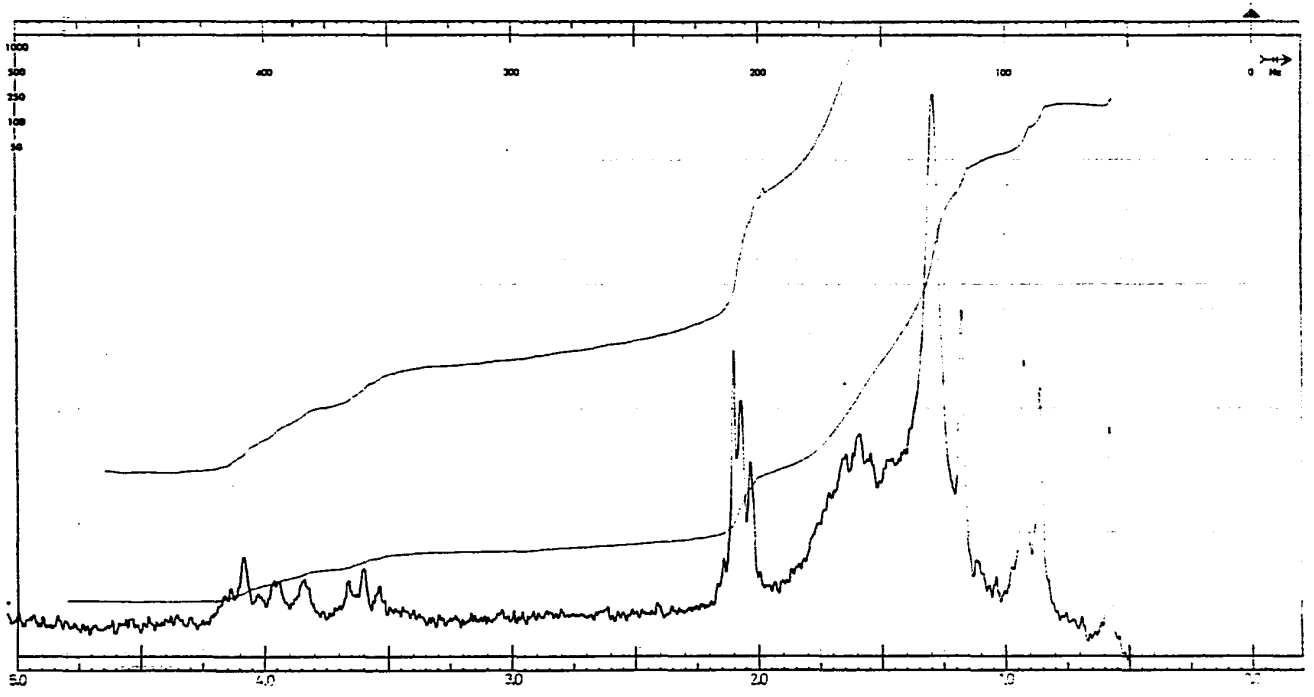


Fig. 24 (continued). 100 MHz NMR Spectrum of C-109-B in Deuterochloroform

TABLE XXII

70 EV MASS SPECTRUM OF C-109-B
RELATIVE INTENSITIES OF VARIOUS FRAGMENTS

m/e	%	m/e	%	m/e	%	m/e	%	m/e	%
672	0.1	509	0.7	367	0.713	238	10.03	125	7
654	0.15	497	1.8	345	1.46	225	7.0	111	10
624	0.2	479	2.33	327	3.3	213	14	95	38
608	0.4	419	3.03	300	4.0	207	9.3	81	32
600	0.3	411	1.03	298	4.0	195	10.6	69	52
582	1.7	401	0.7	285	5.0	138	12	55	44
540	1.1	393	1.0	267	6.0	134	7	43	100
522	1.0	382	0.5	256	13.3				

TABLE XXIII

21 EV MASS SPECTRUM OF C-109-B
RELATIVE INTENSITIES OF VARIOUS FRAGMENTS

m/e	%	m/e	%	m/e	%	m/e	%	m/e	%
672	0.66	509	7.33	401	9	285	33	214	44
654	2.0	497	22.3	393	6	280	9	207	20
624	1.66	491	4.6	382	4.3	273	9	195	17
608	3.66	479	23.6	345	16.6	267	32	138	45
600	3.66	437	5.6	327	28	256	100	126	7
582	25.3	428	12.6	310	46	238	66	109	10
551	4.3	419	25.66	298	46	225	22	96	8
540	14								

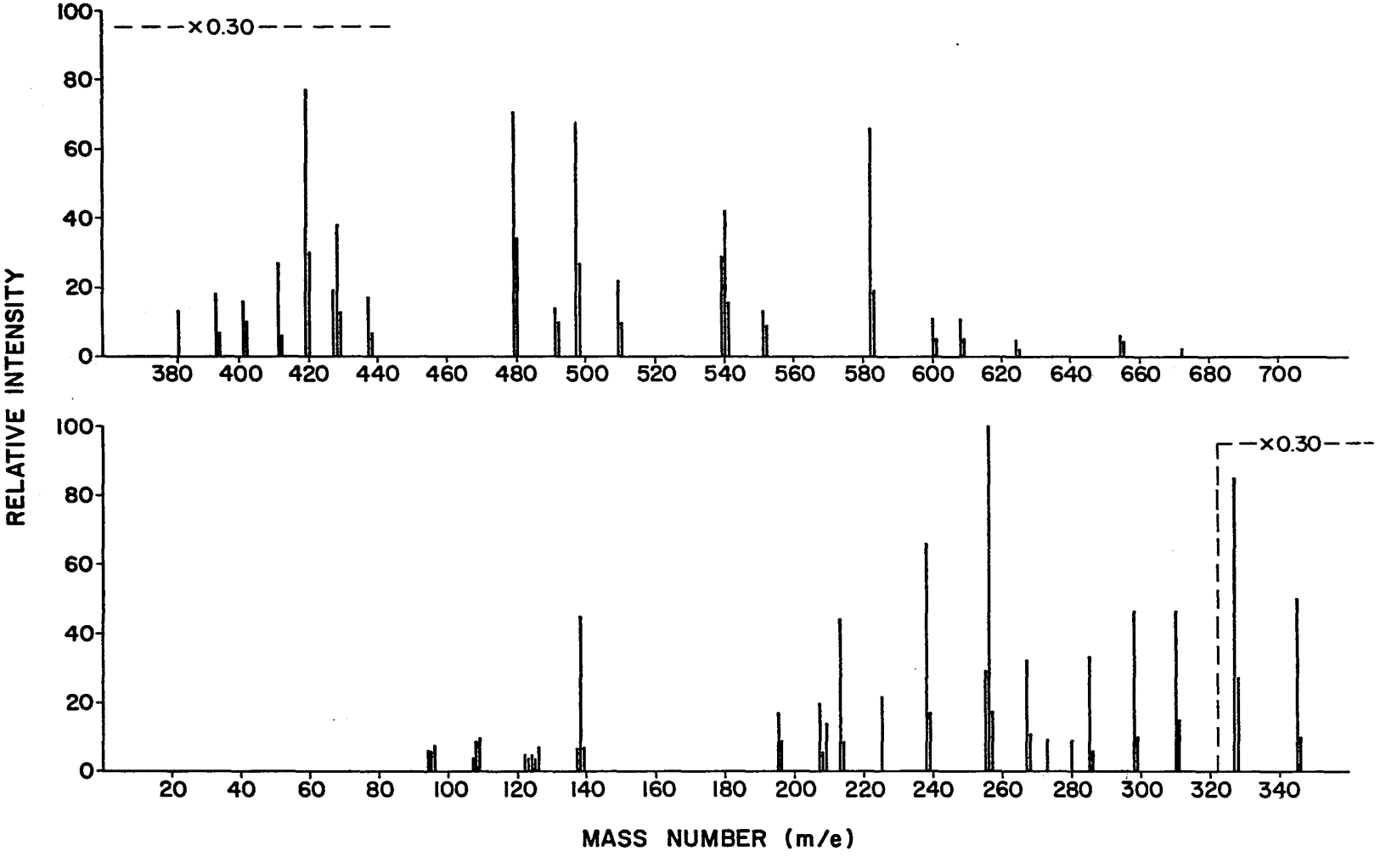


Fig. 25. 21 EV Mass Spectrum of C-109-B

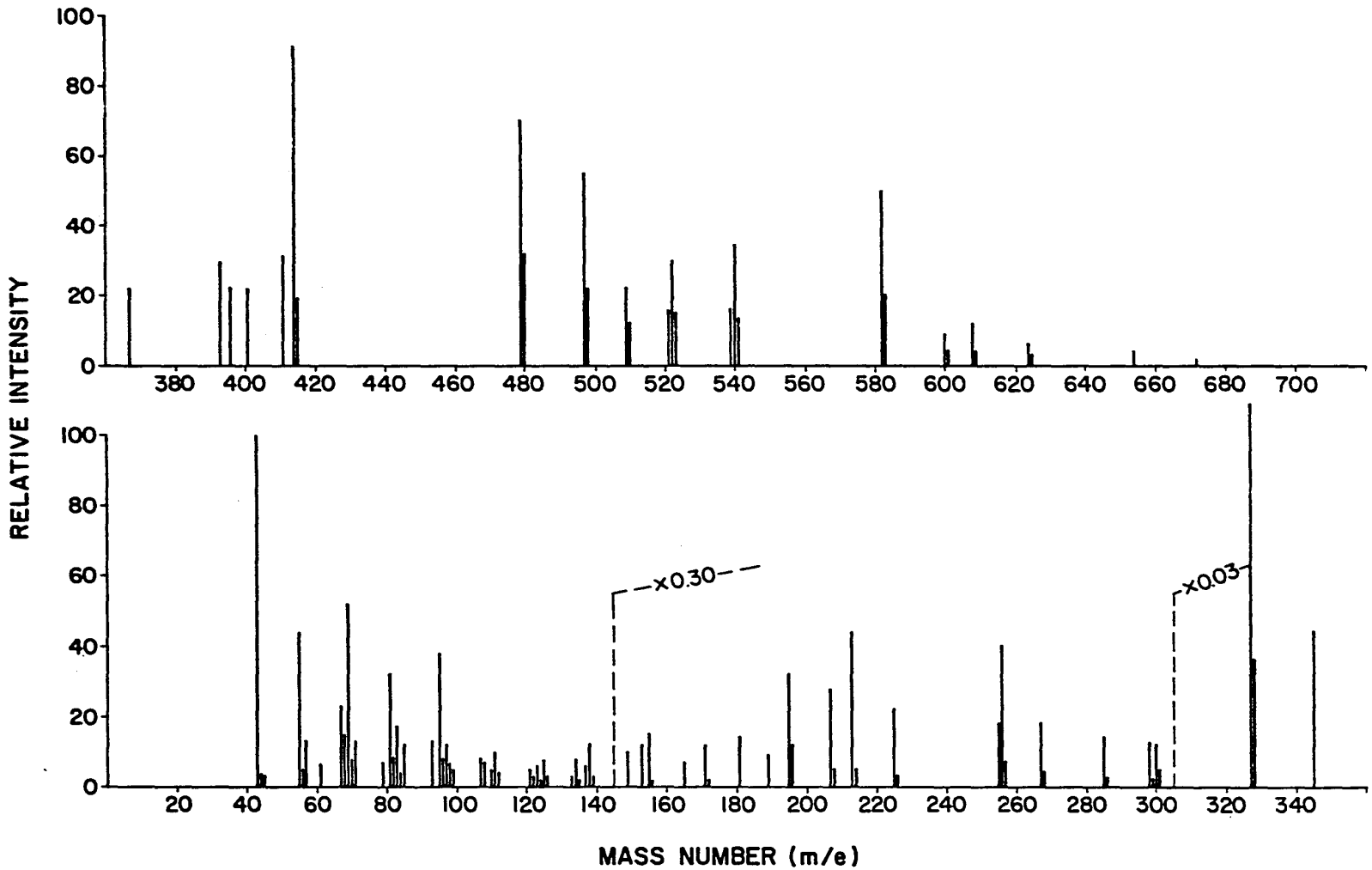


Fig. 26. 70 EV Mass Spectrum of C-109-B

1 on a column of chromosorb w (80 - 100 mesh) (1/8" x 5', column temperature 120°, detector temperature 200°). The gas chromatography indicated the presence of two major compounds Rt's, 6'48", 9'00". Preparative gas chromatography over the same column was effected and the peak corresponding to a retention time of 9'00" was collected and its mass spectra examined.

70 EV mass spectrum

m/e	%	m/e	%	m/e	%	m/e	%
218	34	141	12	71	14	43	40
203	5	135	12	69	21	41	27.5
167	9.5	101	12.5	57	34	28	100
151	11	85	20	55	22		

22 EV mass spectrum

m/e	%	m/e	%	m/e	%	m/e	%
218	100	140	10	96	12	70	13
204	9	119	14	93	14	56	18
154	11.5	111	12	84	16	28	12

g. Aqueous Portion from Periodate Oxidation of Palytoxin

The aqueous portion from the chloroform extraction of the periodate oxidation of palytoxin was freed of chloroform, mixed with ethylene glycol (0.15 ml) and allowed to stand at 7° for 10 hr. The mixture was extracted with 1-butanol (three 50 ml portions each).

The butanol extract after evaporation to a yellow gum was reduced in methanol (10 ml) with sodium borohydride (100 mg) at room temperature for 2.5 hr. The mixture was acidified, evaporated to a white solid and chromatographed over a Sephadex G 25 (Fine) column

(1.5x93.5 cm., fraction size 3.8 ml) and assayed by dichromate oxidation. Fractions 23 to 50 were combined and rechromatographed over the same Sephadex G 25 (Fine) column. Fractions 23 to 31 on combination and evaporation furnished a white viscous mass (A). Fractions 32 to 44 on combination and evaporation furnished a white solid (112 mg, B).

Both A and B were acetylated by acetic anhydride (0.75 ml) in pyridine (2.0 ml) for 48 hours. The acetates were worked up as usual.

A gave 21.8 mg acetate mixture.

B gave 70.0 mg acetate mixture.

Both A and B on gas chromatography over SE30, 5% on a chromosorb w column (1/8" x 10') appeared to be similar in composition and depicted the presence of four major and six minor components.

Retention times 1'48", 2'36", 4'24", 6'18", 11'06", 25'36", 46'48", and 57'48" (column temperature 210°). Preparative gas chromatography of band B was affected on a SE30, 5% on a chromosorb w column (1/8" x 8'), column temperature 230°, detector temperature 250° and injector temperature 275°. The peaks corresponding to retention times 2'00", 3'45" and 8'00" were collected. It was found that the three peaks on reinjection on the same column regenerated the same gas chromatographic pattern.

Both bands A and B were preparatively chromatographed over silica gel HF using benzene-methanol (95:5) as the developers. The plates after 1 hr standing in the developing chamber were divided into three sections.

Section I upper 60% of the plate area.

Section II next 30% of the plate area.

Section III next 10% of the plate area.

The gas chromatogram of the sections I and II under the above conditions are reproduced in Fig. 27. Section I was preparatively rechromatographed over silica gel HF, using cyclohexane-ethyl acetate (1:1) as the developer. The plates were allowed to stand in the developing chamber for 4 hr. The fluorescent band was marked and the bands corresponding to the area above and below the fluorescent band were scraped and designated C-116-C, C-116-D and C-116-E. The three bands on repeated thin layer chromatography and combination of the proper fractions furnished C-116-C (A) (Rf 0.517), C-116-D (A) (Rf 0.414) and C-116-E (A) (Rf 0.306).

C-116-C (A) was a colorless liquid positive to benzidine-periodate and DNP sprays.

Ultraviolet spectrum in methanol. - C-116-C (A) was transparent in the ultraviolet.

Infrared spectrum in chloroform depicted the following bands (Fig. 33). 3400(w), 2940(m), 1730(s,b), 1450(w,b), 1360(s), 1240(b,s), 1075(sh), 1035(s), 980(w), 955(w), 905(w), 840(b), 755(w) cm^{-1}.

Nuclear magnetic resonance spectra in deuterochloroform and benzene-d_6 depicted the following signals.

in deuterochloroform (Fig. 28)	in benzene-d_6 (Fig. 28)	No. of Protons
Triplet (J=6Hz) 0.93δ	Complex 0.89δ	6
Singlets 2.00, 2.10, 2.45 and 2.50δ	Singlets 1.70, 1.72, 1.75, 1.78, and 1.80δ	21

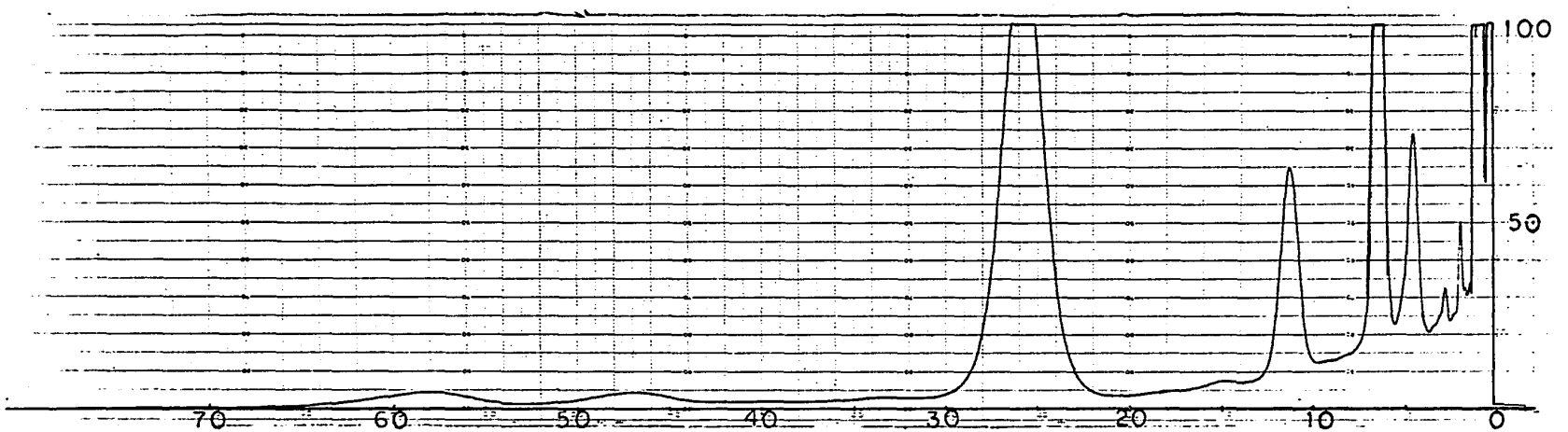


Fig. 27. Gas Chromatogram of the Acetate Mixture from the Periodate Oxidation of Palytoxin (Aqueous portion)

in deuterochloroform (Fig. 28)	in benzene-d_6 (Fig. 28)	No. of Protons
	broad 2.0δ	2
Complex 3.98δ	Complex 3.96δ	4
Complex 4.50δ	Complex 4.50δ	
Complex 5.60δ	Complex 5.00δ	5-6
	Complex 5.60δ	3

Decoupling Experiment.

(i) When irradiated at 4.52δ, the complex doublet at 4.01δ and triplet at 5.55δ collapsed.

(ii) When the signal at 3.855δ was irradiated, the spectrum beyond 4.0δ remained unchanged.

Mass spectra.

70 EV mass spectrum (Fig. 29) indicated the following peaks.

m/e	%	m/e	%	m/e	%	m/e	%	m/e	%
472	0.05	310	2.5	201	53	139	14	81	52
412	0.33	301	8.5	199	30	125	12	69	28
370	1.166	292	16.5	185	50	111	11	55	22
352	4.50	250	20	159	19	99	33	43	100
328	10	237	6	141	17	83	55		

70 EV spectrum on MS9 using heated inlet system depicted the following ions.

m/e	%	m/e	%	m/e	%	m/e	%	m/e	%
357	1.2	250	1.7	199	2.5	91	3.5	69	6.5
301	0.7	237	0.5	185	12.5	85	9	55	6
294	0.5	201	7	99	6	83	13	43	100

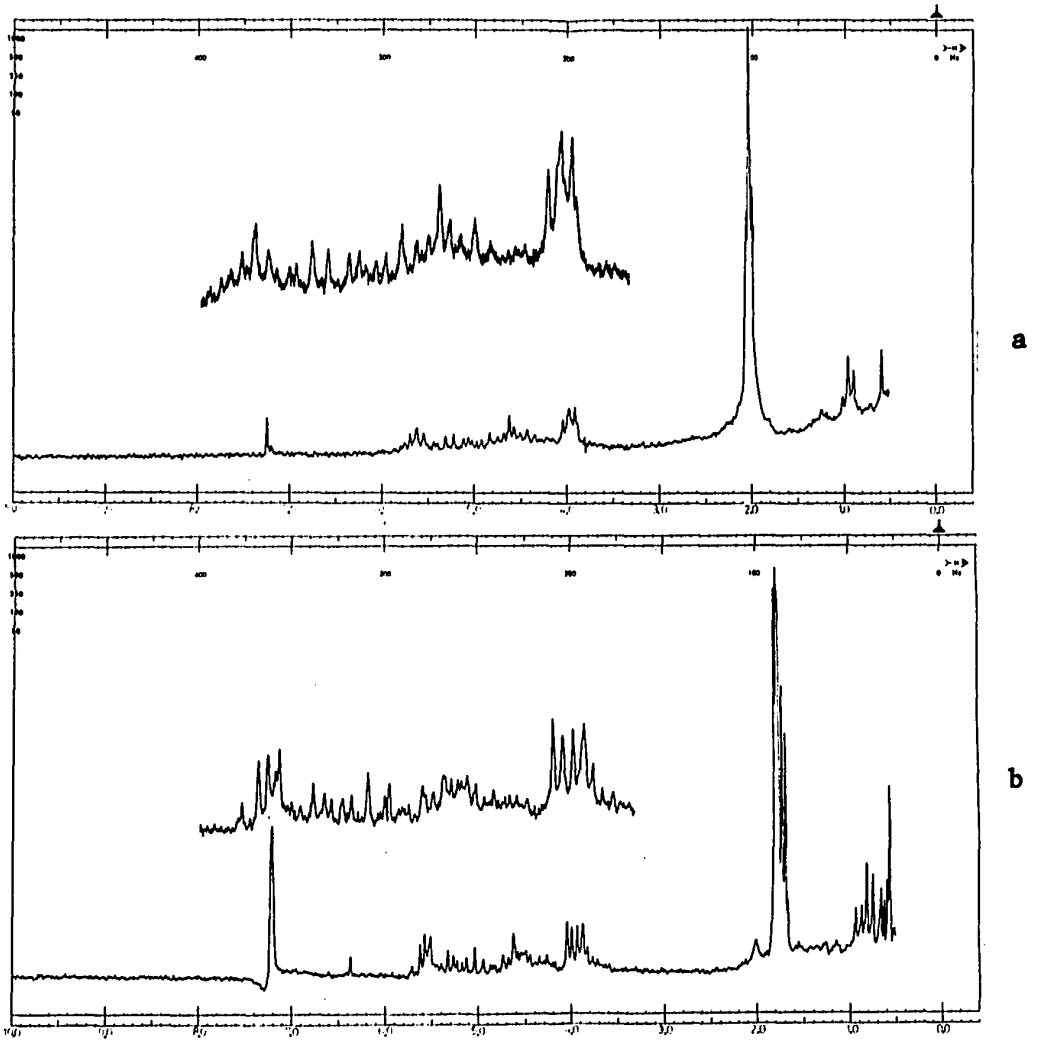


Fig. 28. 100 MHz NMR Spectra of C-116-C(A) in:

a. Deuterochloroform
b. Benzene-d_6

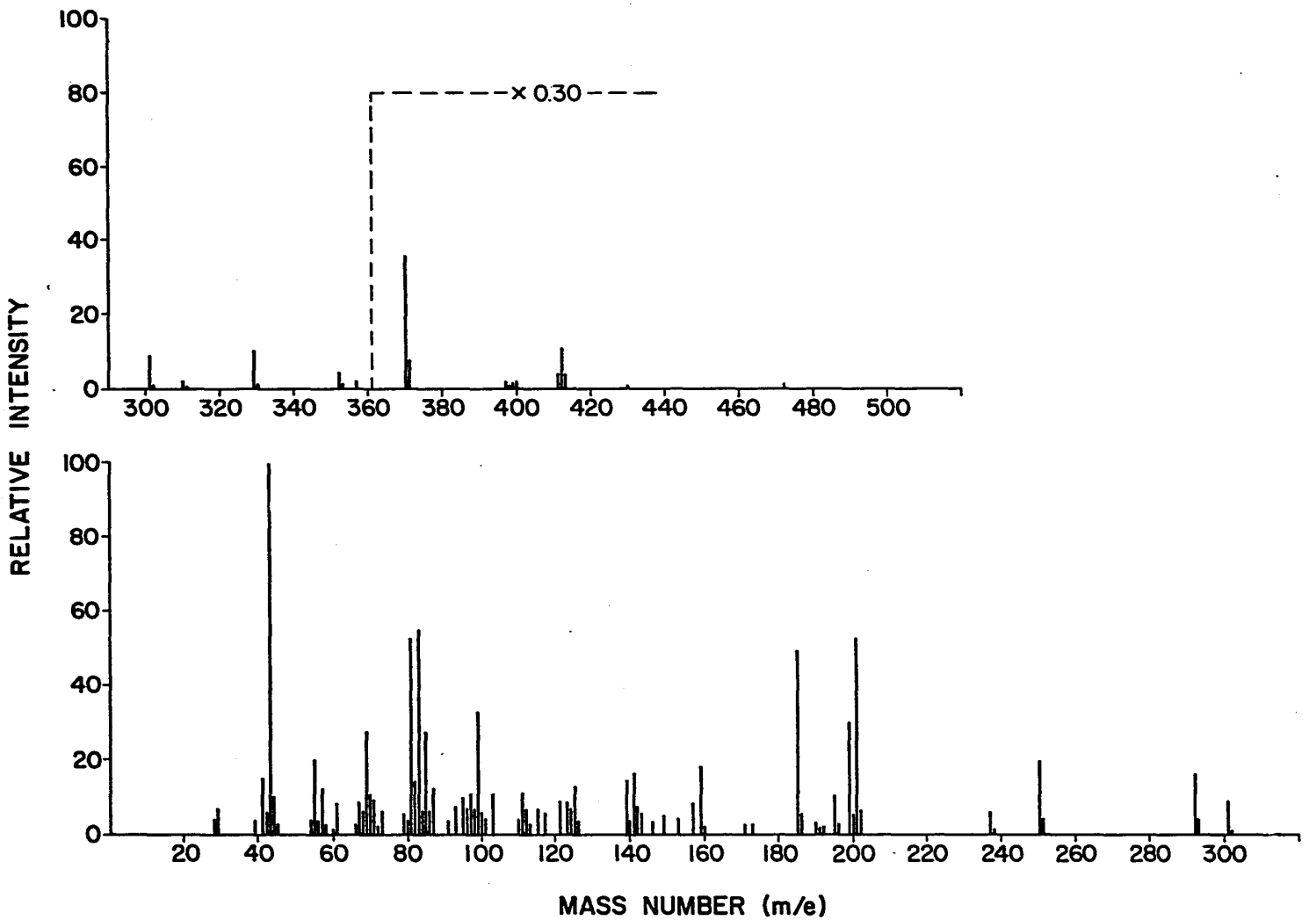


Fig. 29. 70 EV Mass Spectrum of C-116-C(A)

20 EV mass spectrum (Fig. 30) depicted the following ions.

m/e	%	m/e	%	m/e	%	m/e	%	m/e	%
412	1.8	352	32	292	100	237	31	173	4
397	0.4	310	16	268	5	199	32	166	7
370	7	301	46	250	82	185	11	157	4

High Resolution Measurements. - High resolution measurements for the compound C-116-C (A) are presented in Table XXXI.

Elemental Analysis on a 0.28 mg sample

Anal.

Found C, 51.3; H, 10.3; N, 0.00 %.

Compound C-116-D (A).

Ultraviolet spectrum in methanol. - λ_{max} 283 and 229.5 nm

ratio 229.5/283 = 1.222.

Infrared spectrum in chloroform depicted the following bands, 3300(w), 2920(w), 1725(s), 1600(m), 1450(w,b), 1365(s), 1230(b,s), 1195(sh), 1040(b,s) cm^{-1}.

Nuclear magnetic resonance spectrum (100 MHz) in deuterochloroform depicted the following signals.

Nature of Signal	Chemical Shift
Complex	1.80δ
Singlets	2.04 and 2.05δ
Doublet (J=16 Hz)	2.36δ
Complex	3.80δ
Complex	4.10δ

Mass spectra. - 20 and 70 EV mass spectra are presented as follows.

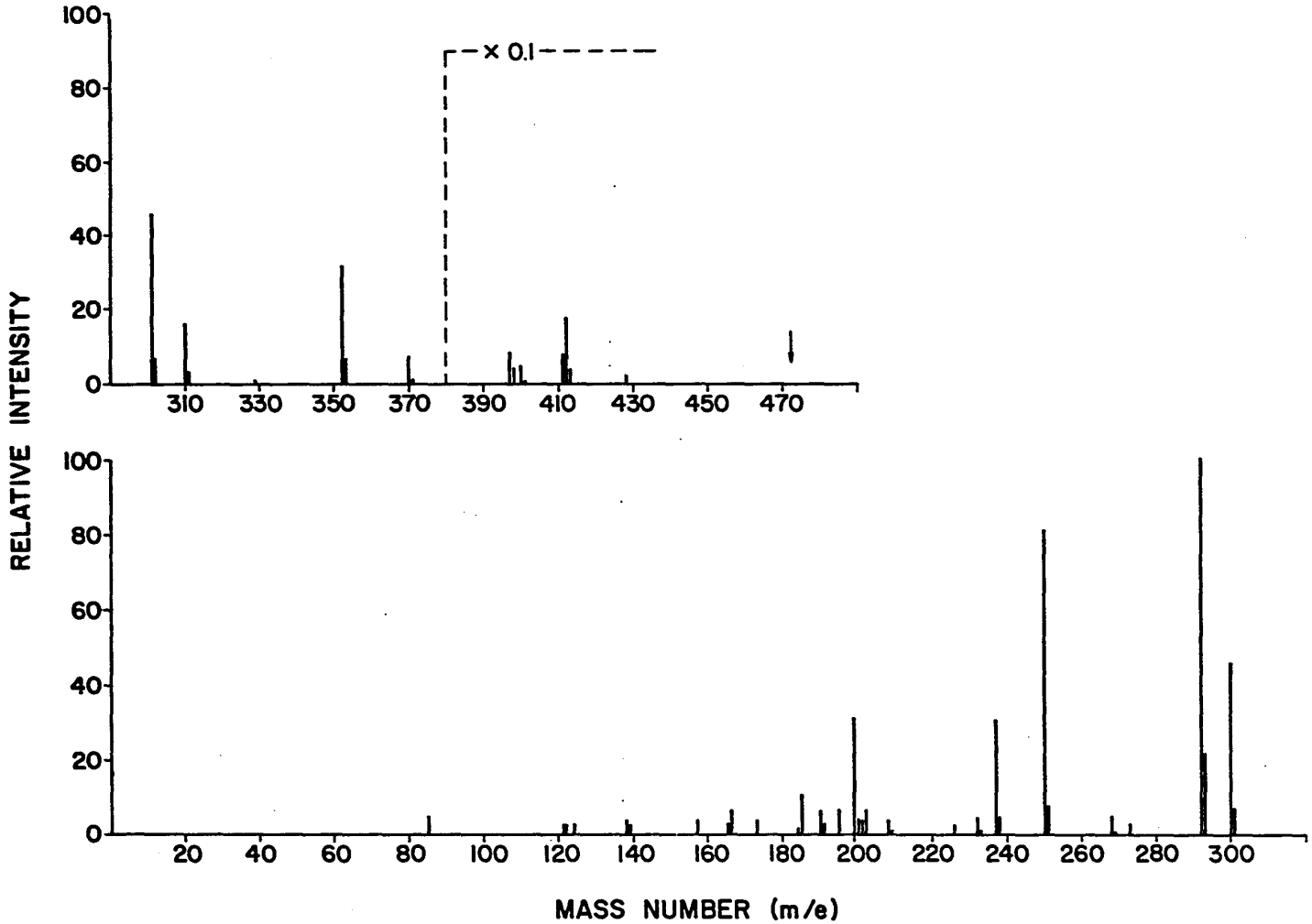


Fig. 30. 20 EV Mass Spectrum of C-116-C(A)

TABLE XXXI

HIGH RESOLUTION MEASUREMENTS FOR THE COMPOUND C-116-C (A)

m/e	Actual Composition	Measured Mass	Calculated Mass
357	$C_{14}H_{29}O_{10}$	357.17033	357.17608
301	$C_{14}H_{21}O_{7}$	301.12843	301.12873
250	$C_{14}H_{18}O_{4}$	250.11994	250.12051
201	$C_{10}H_{17}O_{4}$	201.11273	201.11269
199	$C_{10}H_{15}O_{4}$	199.09675	199.09706
	$C_{10}H_{11}O_{4}$ (50%)	195.06577	195.06574
195	$C_{11}H_{15}O_{3}$ (50%)	195.10179	195.10210
185	$C_{9}H_{13}O_{4}$	185.08152	185.08139

20 EV mass spectrum at a sample temperature of 120° had the following peaks.

m/e	%	m/e	%	m/e	%	m/e	%	m/e	%
342	0.1	296	1	173	23	117	100	75	2.5
328	1.3	233	8	128	10	103	7.5	57	4

70 EV mass spectrum at a sample temperature of 120° depicted the following ions.

m/e	%	m/e	%	m/e	%	m/e	%	m/e	%
374	0.7	310	0.3	275	2.5	159	14	70	27
352	0.3	301	0.5	273	1.3	131	18	57	10
345	0.3	292	1.9	229	24	129	21	43	100
324	0.8	289	6	173	98	113	29		

70 EV mass spectrum at a sample temperature of 220° depicted the following peaks.

m/e	%	m/e	%	m/e	%	m/e	%	m/e	%
448	0.2	352	0.3	275	1.0	213	0.9	173	3
396	0.25	324	1.5	273	1.0	199	2.8	113	6
382	1.6	295	1.4	250	1.6	195	1.4	71	21
380	0.7	292	1.0	229	1.6	185	3.0	43	100

Compound C-116-E (A). - Compound C-116-E (A) is a colorless liquid.

Ultraviolet spectrum in methanol. - λ_{max} 280.0 nm and 230 nm ratio 230/280 = 10.0.

Infrared spectrum in chloroform depicted the following bands (Fig. 33). 3450(b,w), 2900(m), 1725(s), 1600(b,w), 1430(b,w), 1360(s), 1230(s,b), 1120(sh), 1030(b,s), and 760(w) cm^{-1}.

Nuclear magnetic Resonance spectrum (HA 100) in deuterochloroform.

Nature of Signal	Chemical Shift	No. of Protons
Complex	1.66δ	
Singlet	2.04δ	26-28 H
Broad	4.06δ	7-8 H
Complex	5.10δ	1 H

Mass spectra. - 70 and 20 EV mass spectra are presented in Figures 31 and 32 and Tables XXV and XXVI.

High Resolution Measurements on the following ions were obtained.

m/e	Actual Composition	Measured Mass	Actual Mass
396	$C_{21}H_{24}N_4O_4$	396.17951	396.17976
347	$C_{16}H_{19}N_4O_5$	347.13291	347.13555
345	$C_{17}H_{21}N_4O_4$	345.15460	345.15628
303	$C_{14}H_{15}N_4O_4$	303.10779	303.10933

11. Sodium metaperiodate Oxidation of Palytoxin, Oxidative Work-up

Sodium metaperiodate (75 mg) in water (4 ml) was added to a cooled solution of palytoxin (30 mg) in water (1.2 ml). The mixture after 6 hr stirring at ice water temperature was allowed to heat up to room temperature. The mixture was extracted with ethyl acetate (50 ml). The extract after drying over magnesium sulfate was evaporated to a colorless gummy mass (12 mg). The gummy mass was oxidized by freshly prepared silver oxide (silver oxide was prepared by reacting silver nitrate (1 g) with sodium hydroxide, the precipitate was thoroughly washed with distilled water and ethanol respectively) in 10 ml methanol-water (75:25). The mixture was refluxed for 6 hr, acidified

TABLE XXV

70 EV MASS SPECTRUM OF C-116-E (A)

RELATIVE INTENSITIES OF VARIOUS FRAGMENTS

m/e	%	m/e	%	m/e	%	m/e	%
498	0.1	345	1.0	268	1.0	173	1.53
485	0.1	340	0.46	245	0.8	123	6
456	1.7	310	0.96	229	1.1	113	8
396	2.6	303	2.1	225	2.0	107	5
382	0.9	295	0.6	206	2.93	71	20
347	1.0	275	2.9	191	0.8	43	100

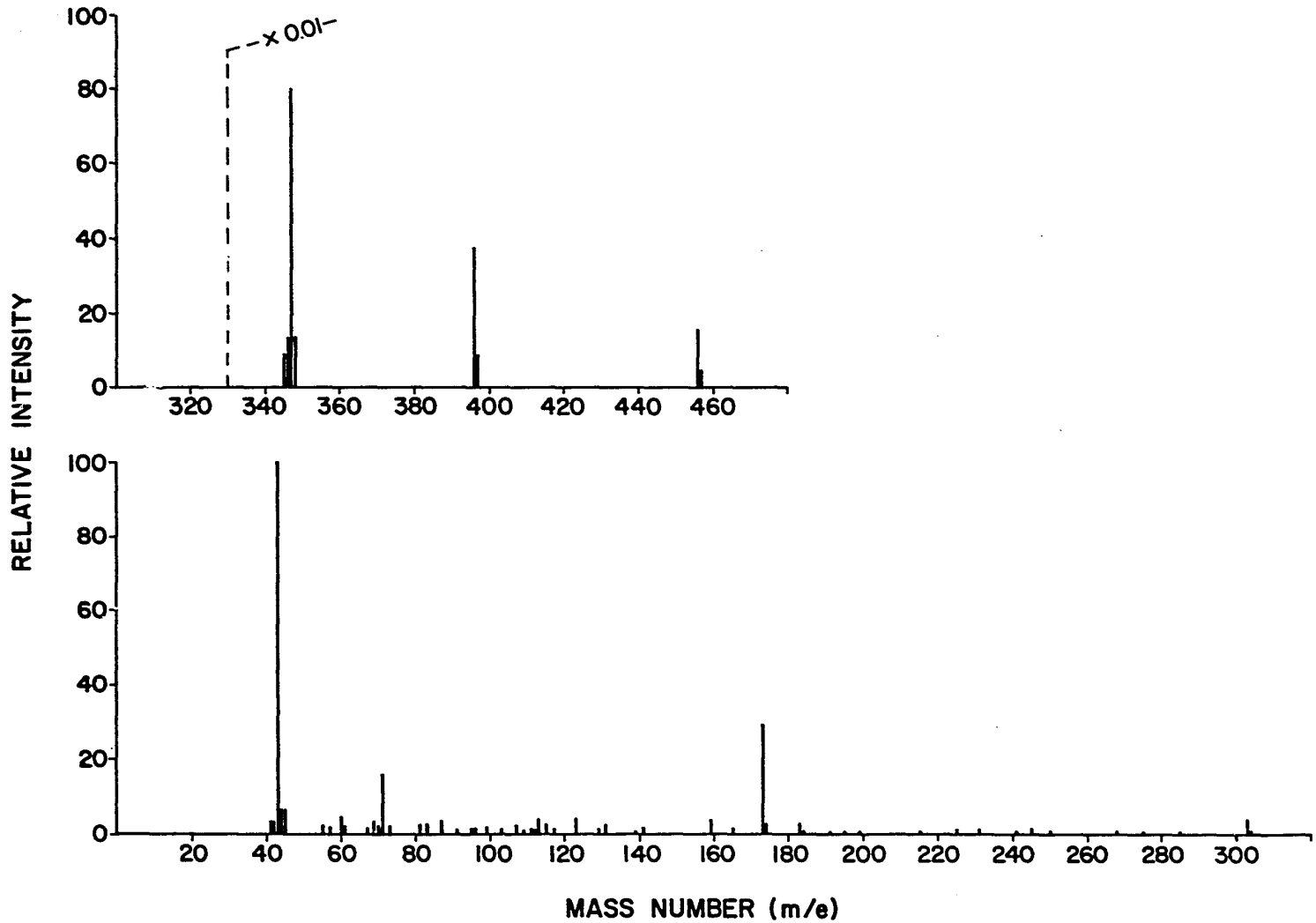


Fig. 31. 70 EV Mass Spectrum of C-116-E(A) (MS 9)

TABLE XXVI

20 EV MASS SPECTRUM OF C-116-E (A)

RELATIVE INTENSITIES OF VARIOUS FRAGMENTS

m/e	%	m/e	%	m/e	%
456	20.3	298	2.3	173	100
438	1.0	275	17	167	4
414	1.3	266	5.3	146	9
396	22	250	2.3	138	15
382	5	229	2.3	98	4
345	4.66	225	5.3	89	7
308	5.3	206	16.3	82	3

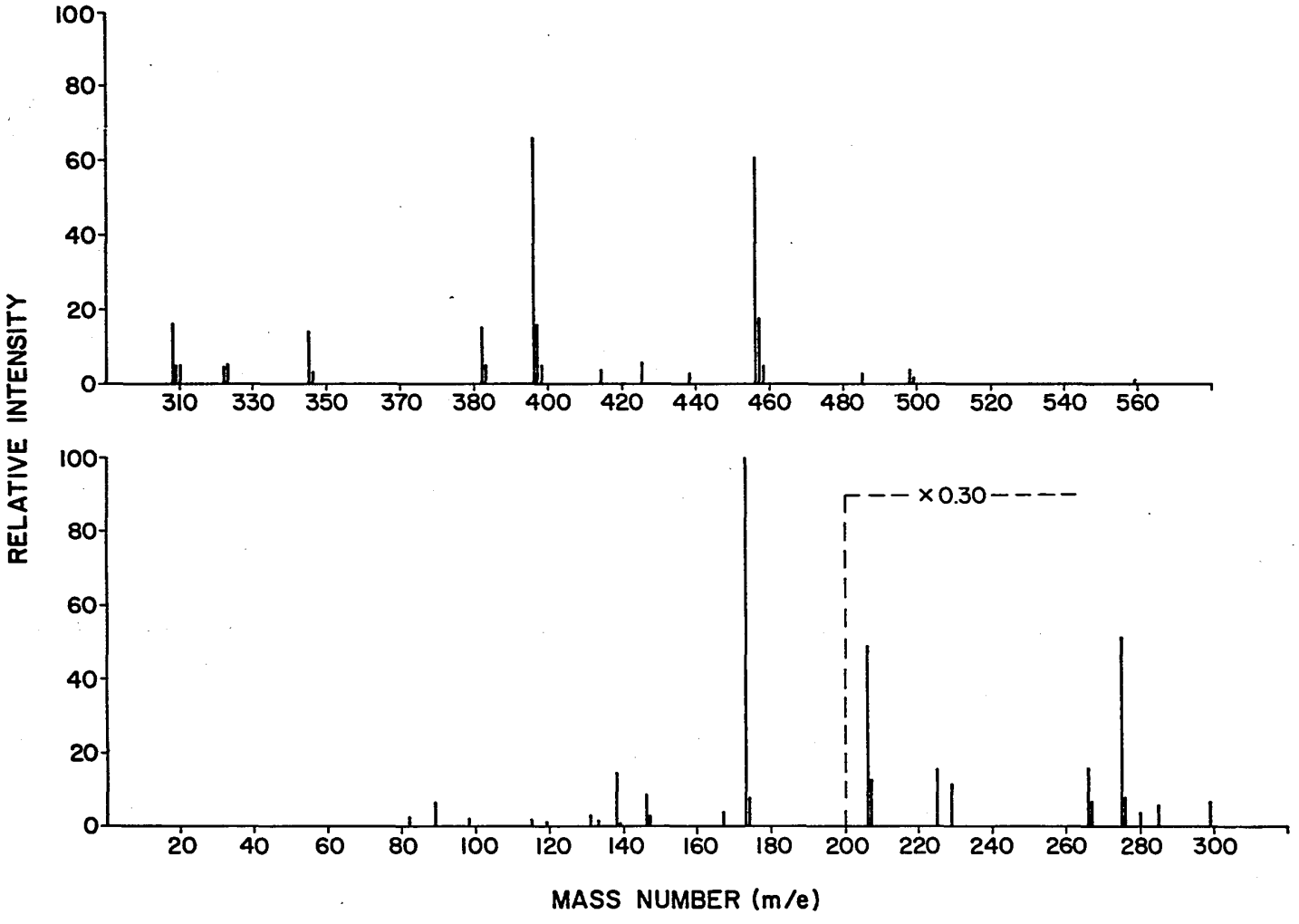


Fig. 32. 20 EV Mass Spectrum of C-116-E(A)

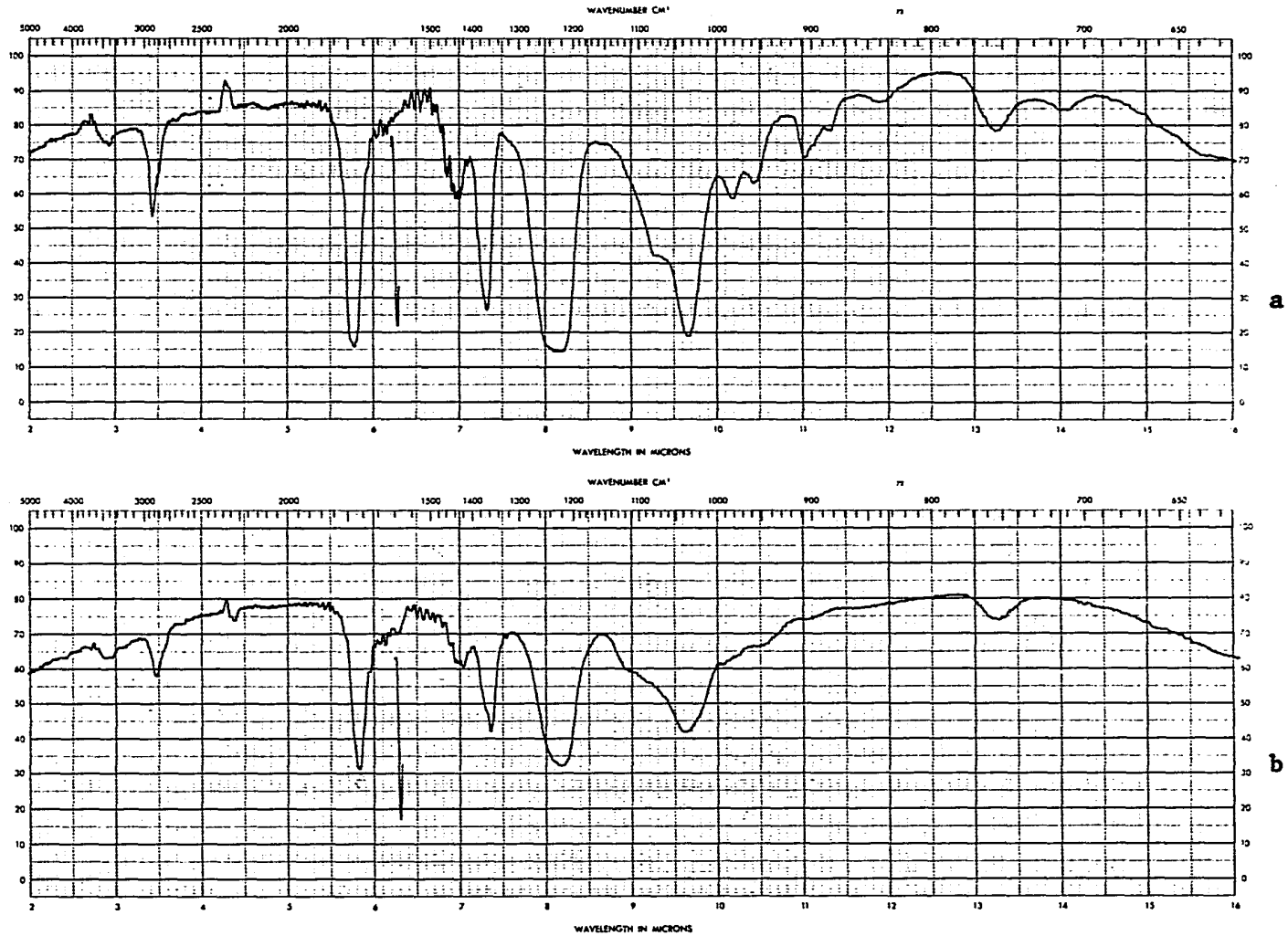


Fig. 33. Infrared Spectra of:

a. C-116-C(A) (Thin film)
b. C-116-E(A) (Thin film)

by hydrochloric acid and freed of solids by centrifugation. The centrifugate was evaporated to a small volume and partitioned between water (10 ml) and chloroform (10 ml). The chloroform layer was dried over sodium sulfate, evaporated to dryness, and reacted with ethereal diazomethane. After standing at room temperature for 2 hr, the mixture was evaporated to a brown gummy mass (4 mg). The brown mass was silylated with hexamethyl disilazane (0.2 ml) and chlorotrimethylsilane (0.1 ml) in pyridine (0.5 ml), for 24 hr. The mixture was freed of solids by centrifugation, the centrifugate was evaporated to dryness, and the residue was extracted with chloroform (0.2 ml). The chloroform extract could not be gas chromatographed over SE30 on a Chromosorb w column (1/8" x 5').

Thin layer chromatography of the silylated mixture over silica gel H (chloroform) indicated the presence of a fast moving material (Rf 1.0) and a slow moving material (Rf 0.000).

12. Sodium Metaperiodate Oxidation of Palytoxin (Jamaican)

Sodium metaperiodate (17 ml, 3 g/50 ml) was reacted with palytoxin (140 mg) in water (5 ml) at ice water temperature for 18 hr. The mixture was extracted with chloroform (3x15 ml), the chloroform extract after drying over sodium sulfate was evaporated to a pale gummy mass. The gummy mass was reduced with sodium borohydride (600 mg) in 2-propanol (5 ml), for 6 hr. The mixture after acidification was extracted with chloroform in a liquid-liquid extractor. The chloroform extract after drying over sodium sulfate, was evaporated to a gummy mass (43 mg). The gummy mass was dissolved in ethyl acetate (0.5 ml) and petroleum ether (3 ml) was added. The mixture on cooling

in a refrigerator overnight furnished a dirty white gelatinous precipitate I and a solution II.

On analytical thin layer chromatography over silica gel HF ethyl acetate, I remained at the starting point while II showed at least two major components.

II after drying under vacuum was reacted with freshly prepared 4-bromobenzoyl chloride (180 mg) in dry pyridine (1.0 ml) for 24 hr. The mixture after work-up in the usual fashion furnished a pale gummy mass.

The gummy mass on recrystallization from chloroform furnished plates, mp 217-219°. The supernatant on thin layer chromatography over silica gel HF indicated the presence of three major fluorescent and sulfuric acid positive compounds (solvent system cyclohexane-ethyl acetate, 60:40; Rf's 0.565, 0.320 and 0.206). Preparative thin layer chromatography over silica gel HF using the same solvent system furnished three bands:

band A - RF 0.565; band B - Rf 0.320; and band C - Rf 0.206.

Band A (Rf 0.565). - Band A was further purified by rechromatography over silica gel HF plates using the same solvent system, followed by precipitation as a colorless white solid from n-hexane. The white solid was twice crystallized from aqueous methanol as white needles; mp 84.5°.

Infrared spectrum in chloroform depicted the following bands. 3450(b,w), 2920(s), 2850(w), 1710(m), 1580(m), 1450(w), 1370(w), 1275(s), 1200(b,s), 1120(s), 1105(s), 1065(w), 1015(m), 960(b,w). 845(b,w) cm^{-1}.

Nuclear Magnetic Resonance Spectrum. 100 MHz NMR in deuterochloroform depicted the following signals.

Nature of Signal	Chemical Shift	Nature of Signal	Chemical Shift
Singlet	0.83δ	Broad	1.60δ
Singlet	0.89δ	Complex	3.84δ
Singlet	1.11δ	Complex	4.26δ
Singlet (broad)	1.26δ	Quartet (A_2B_2)	7.54δ

Mass spectrum. - 70 EV mass spectrum of Band A depicted peaks even above m/e 440.

m/e	%	m/e	%	m/e	%	m/e	%	m/e	%
440	49	360	7.5	200	14	125	9	81	35
438	49	317	6	195	9	123	6	71	22
422	7.5	281	5.5	185	53	109	22	69	59
420	7.5	213	5	183	56	105	37	55	56
397	30	211	5	149	25	95	29	44	65
395	30	202	14	135	12	83	37	28	100

Band B and C.

Infrared spectra in chloroform depicted the following bands. 3450(w,b), 2910(s), 2840(sh), 1710(s), 1580(m), 1450(w), 1380(w), 1275(s), 1200(b,s), 1120(s), 1105(s), 1068(w), 1015(m), 960(b), 848(w,b) cm^{-1}.

14. Quantitative Lead Tetraacetate Oxidation of Palytoxin

a. Palytoxin (3.96 mg) in water (0.39 ml) was reacted with lead tetraacetate (96.185 mg) in acetic acid (0.8 ml). An aliquot of 0.05 ml was periodically removed and titrated against sodium thiosulfate (0.0004464N). A parallel blank was run simultaneously. The results

are graphically presented in Fig. 10 and indicate a consumption of 13.2μ mole/mg of toxin.

b. In a second experiment, palytoxin (0.984 mg) was reacted with lead tetraacetate solution in acetic acid (0.75 ml). A blank was run simultaneously. Both the reaction mixture and the blank were titrated against standard sodium thiosulfate after 24 hr. The amount of lead tetraacetate consumed corresponded to the difference in thiosulfates.

49.75 ml equivalent of 2.2208×10^{-4}N sodium thiosulfate.

c. In a third experiment, palytoxin (0.855 mg) consumed lead tetraacetate equivalent to 47.0 ml of 2.2208×10^{-4}N sodium thiosulfate.

14. Preparative Lead Tetraacetate Oxidation of Palytoxin

Lead tetraacetate (96 mg) in acetic acid (4 ml) was added to a solution of palytoxin (13.0 mg) in water (1.3 ml) and allowed to react for 5 hr at room temperature. Oxalic acid dihydrate (150 mg) was added. The mixture after being freed of lead oxalate by centrifugation, was diluted to 10 ml with distilled water and extracted thrice with chloroform (10 ml each). The chloroform layer after drying over sodium sulfate was evaporated to a small volume. Infrared spectrum in chloroform depicted the following bands. 3400(m), 2930(s), 2730(w), 1725(s), 1700(sh), 1450(m), 1365(m), 1180(s), 1100-950(b) cm^{-1}.

The aqueous layer from the chloroform extraction of the reaction mixture was evaporated to dryness. The residue was reacted with freshly prepared 2,4-dinitrophenyl-hydrazine reagent (5 drops),

in water. After standing overnight the mixture was extracted with an ethyl acetate-benzene (1:2) mixture. The extract on paper chromatography furnished two bands (Rf 1.0 and 0.00).

Band I (Rf 1.0).

70 EV mass spectrum depicted the following ions.

m/e	%	m/e	%	m/e	%	m/e	%	m/e	%
386	1.1	310	2.1	185	7	111	21	69	66
340	2.8	308	2.1	167	8	97	38	60	29
321	0.9	284	7.7	149	21	81	28	43	100
320	1.30	255	8.0	129	16	71	60		

Band II (Rf 0.000).

EV mass spectrum depicted the following ions.

m/e	%	m/e	%	m/e	%	m/e	%	m/e	%
381	12	185	34	141	27	111	21	71	62
353	11	169	12.5	129	12	97	26	69	64
316	4	167	13.5	125	13	85	37	57	100
298	5	149	16	113	17	83	41		
285	7	142	27	112	20	81	28		

One fourth of the chloroform extract from the lead tetra-acetate oxidation of palytoxin was evaporated to dryness and reduced by sodium borohydride (17.0 mg) for 12 hr. The mixture after evaporation to dryness was extracted with chloroform. The chloroform extract could not be gas chromatographed over SE 30, 5% on a Chromosorb w column (1/8" x 5').

The remaining 3/4 of the chloroform extract after evaporation to dryness was reacted with 2,4-dinitrophenyl-hydrazine reagent (3 drops)

in methanol (0.5 ml) for 5 hr. The reaction mixture was extracted with benzene containing a few drops of ethyl acetate. The benzene extract was backwashed with two portions of distilled water (7 ml). The analytical thin layer chromatography of the benzene extract on silica gel H using benzene as developer indicated the presence of at least 6 compounds (Rf's 0.695, 0.400, 0.266, 0.209, 0.066, and 0.000).

In another run, palytoxin (51.0 mg) in water (3.0 ml) was reacted with lead tetraacetate (480 mg) in acetic acid (10 ml), under nitrogent for 5 hr. The mixture was warmed to 70° for 5 min. A stream of nitrogen was bubbled through the solution, passed through a fractionating column (1') and finally bubbled through cool methanol (5 ml). The methanol solution was reacted with freshly prepared 2,4-dinitrophenylhydrazine reagent. The mixture did not indicate the presence of 2,4-dinitrophenylhydrazones by thin layer chromatography.

Oxalic acid dihydrate (600 mg) in acetic acid (5 ml) was added to the above reaction mixture. The mixture after 20 min stirring was freed of lead oxalate by centrifugation, diluted with water (15 ml) and extracted with chloroform (40 ml), in a liquid-liquid extractor. The chloroform extract after removal of chloroform was reduced with lithium aluminum hydride (400 mg) in ether (150 ml). After 5 hr the product was worked up in the usual fashion to give a yellow mass. Infrared spectrum in chloroform depicted the following bands. 3450(w), 2950(s), 2850(sh), 1725(s,b), 1600(w), 1575(w), 1450(m), 1370(m,b), 1280-1200(s,b), 1160(s,b), 1070(s), 960(m,b) cm^{-1}. Mass spectrum.

70 EV mass spectrum depicted an ascending pattern. Some of the more important peaks are as follows.

m/e 420, 414, 400, 386, 372, 340, 299, 284, 279, 270, 263, 256, 242, 238, 228, 224, 213, 199, 195, 185, 171, 167, 157, 143, 136, 134, 132, 129, 123, 97, 95, 83, 81, 71, 70, 59, 57, 56

15. Lead Tetraacetate Oxidation of Palytoxin in Pyridine

Palytoxin (160 mg) in pyridine (60 ml) was added dropwise to a stirred solution of lead tetraacetate (1.5 g) in pyridine (60.0 ml) at 0°. The mixture was stirred at 0° for 30 min and then at room temperature for 40 min. Solid oxalic acid dihydrate (3.0 g) and five drops of water were added and the reaction mixture stirred at room temperature for 1 hr. The reaction mixture was then filtered and the residue washed with pyridine (20 ml). The filtrates were combined and evaporated to a brown crystalline mass. The brown mass was leached with boiling chloroform (120 ml). The chloroform extract after drying over sodium sulfate, was evaporated to a gum. The residue left after leaching with chloroform was dissolved in water (7.5 ml), stirred with calcium carbonate (2.0 g) for 2 hr, filtered and evaporated to a small volume.

a. Chloroform Extract

The chloroform extract depicted streaking on thin layer chromatography over silica gel H, and kieselguhr G using a variety of solvent systems. The chloroform extract was reduced by sodium borohydride (0.8 g) in methanol (25 ml). The mixture on work-up in the usual fashion provided a light yellow mass (25 mg).

A part of this yellow mass was silylated by a 1 to 1 mixture of hexamethyldisilazane and dimethylformamide for 24 hr at room temperature. The silylated mixture on gas chromatography over SE 30 5% on a Chromosorb W column (1/8" x 6', column temperature 280°, detector temperature 350°) indicated the presence of four major (Rt 3'48", 5'36", 10'52" and 16'36" and two minor (Rt 1'12" and 2'00") components. The remainder of the borohydride reduced product was acetylated by acetic anhydride (0.5 ml) in pyridine (1.0 ml), for 48 hr at room temperature. The mixture was evaporated to dryness, dissolved in chloroform (20 ml). The chloroform solution after backwashing with two portions each of 1N-hydrochloric acid (10 ml) saturated sodium chloride (5 ml) was dried over sodium sulfate and evaporated to a gum. Analytical thin layer chromatography over silica gel H using cyclohexane-ethyl acetate (70:30) as the developing solvent indicated the presence of at least five compounds (Rf's 1.00, 0.692, 0.430, 0.323 and 0.053). The mixture was subjected to preparative thin layer chromatography using the same solvent system, and the bands corresponding to the above mentioned Rf values were collected.

Band A	Rf 1.00	2.0 mg	
Band B	Rf 0.692	3.0 mg	(C-107-A)
Band C	Rf 0.430	3.5 mg	(C-104-d)
Band D	Rf 0.053	6.0 mg	(C-107-C and C-109-B)

Band C corresponded to band C-104-d of the periodate oxidation while band B corresponded to C-107-A, and band D to a mixture of C-107-C and C-109-B of the periodate oxidation.

b. Aqueous Portion

The aqueous solution was desalted over an AG 11 AX ion retardation resin column (1.5x33 cm, fraction size 2.5 ml, flow rate 15 ml/hr). The desalted material was acetylated by acetic anhydride (3.0 ml) in pyridine (3 ml) for 24 hr. The acetate mixture on analytical gas chromatography over SE 30 5% on a Chromosorb W column (1/8" x 5') indicated the presence of at least 8 compounds.

L. Lipids of Toxic Palythoa

1. Fatty Acid Analysis

The benzene extract from 4 kg of toxic Palythoa was evaporated to a viscous brown mass (20 g). The brown mass was saponified by refluxing with 20% potassium hydroxide in 50% methanole-water (150 ml) overnight. The reaction mixture was poured into water (100 ml), filtered and extracted with two portions of ether (500 ml each). The aqueous layer after acidification by conc hydrochloric acid was extracted with two portions of ether (250 ml each). The ether layer was decolorized with Darco G, dried over sodium sulfate and evaporated to a brown gummy mass. The gummy mass was esterified by reacting with methanol saturated with hydrogen chloride for 12 hr. The product mixture was freed of hydrogen chloride and decolorized with Darco G. A portion of the product mixture was catalytically hydrogenated over catalytic platinum. Both fatty acid and reduced fatty acid ester mixtures were subjected to analytical gas chromatography over SE 30 3% and 5% and EGSS-X 15% on Chromosorb W columns (1/8" x 6'). Standard fatty acid methyl ester mistures were gas chromatographed simultaneously.

Peak to peak matching, reading from the plots of log Rt and carbon chain length and also the comparison with the reduced fatty acid ester gas chromatogram indicated the presence of the following fatty acids; caproic acid C_6, caprylic acid C-8, capric acid C_{10}, myristic acid C_{14}, isopentadecanoic acid C-15, pentadecanoic acid C-15, palmitoleic acid C-16-2H, palmitic acid C-16, isoheptadecanoic acid C-17, heptadecanoic acid C-17, linolenic acid C-18-4H, linoleic acid C-18-2H, stearic acid C-18, eicosadienoic acid C-20-4H, arachidic acid C-20, 19-methyl eicosanoic acid C-21 and iso C-22 acid. The gas chromatograms of the fatty acid methyl esters and reduced fatty acid methyl esters are reproduced in Fig. 34.

2. Isolation of Polyolefinic or Polyacetylenic Lipids

Toxin Palythoa (wet weight 1 kg) after drying under vacuum at a temperature of 60-65° (dry weight 415 g) was extracted in a Soxhlet with the following solvents in the given order; n-hexane, benzene, chloroform, absolute methanol (500 ml each).

n-Hexane Extract. - The n-hexane extract was expected to contain polyacetylenes. It was therefore chromatographed over a silica gel column (1.8x23 cm) using a stepwise elution in the following order; n-hexane (250 ml) extract gave a green pigment (Pg-1), 10% chloroform in n-hexane (750 ml) gave a white solid (S-10), 20% chloroform in n-hexane (1000 ml) gave a colorless liquid (L-20), 20% chloroform-n-hexane (2 to 3 litre) gave a green pigment (Pg-2), and chloroform in a Soxhlet gave an orange pigment (Po-1). The chromatographic behavior of S-10, S-20 and L-20 is presented in Table XXVII. Phosphomolybdic acid, 10% in ethanol, was used for visualization.

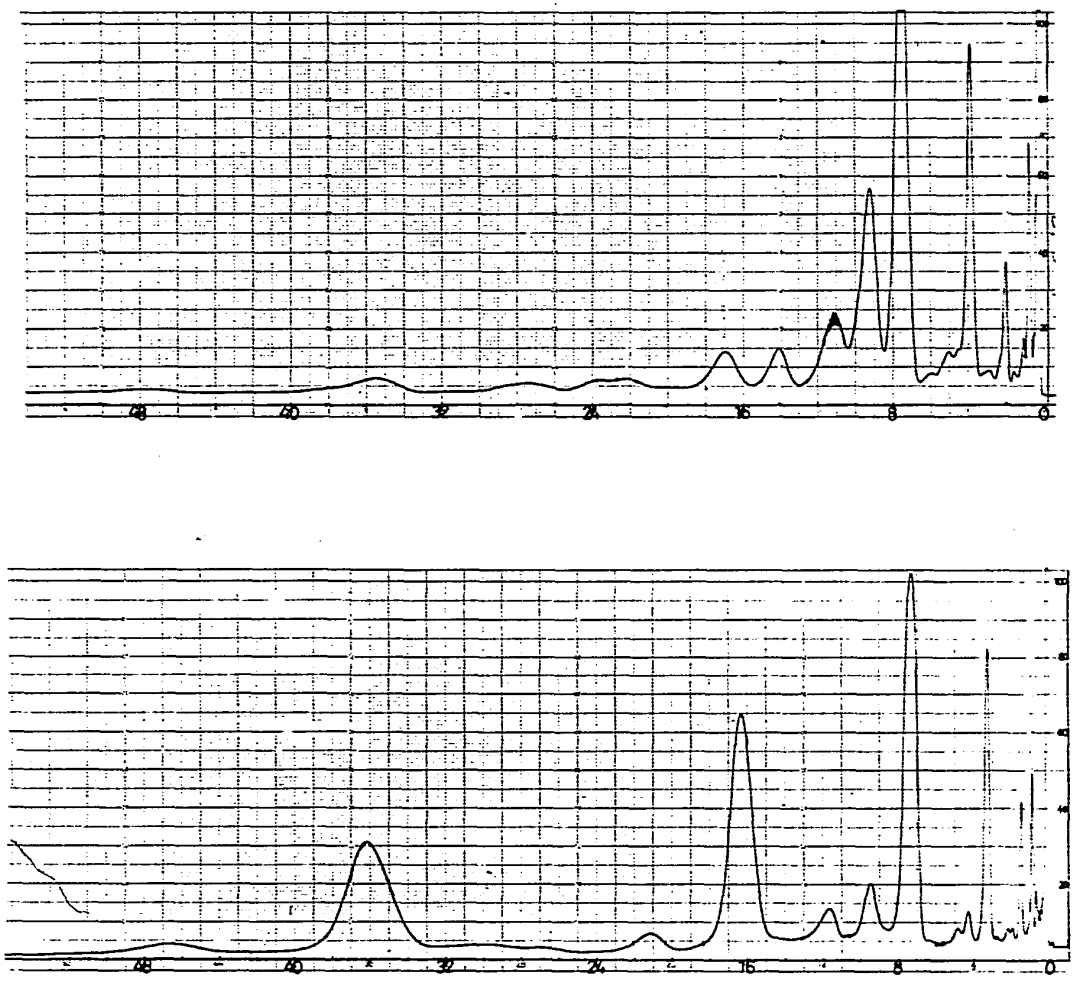
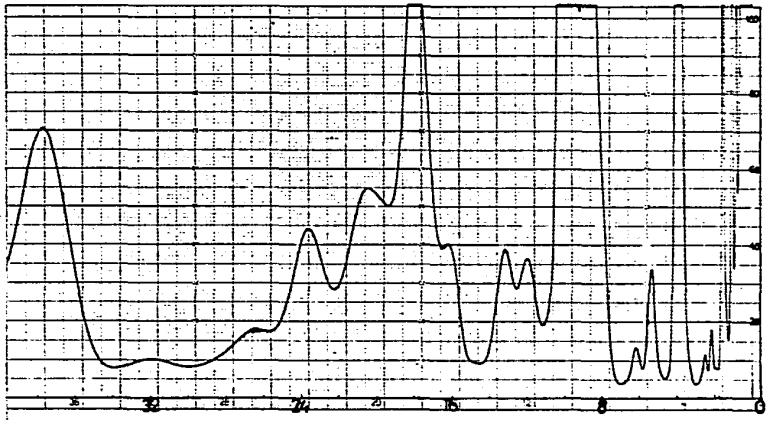


Fig. 34. Gas Chromatograms of

a. Fatty Acid Mixture
b. Hydrogenated Fatty Acid Mixture, of Toxic Palythoa over EGSS-X 15% on Chromosorb W Column (1/8" x 6')



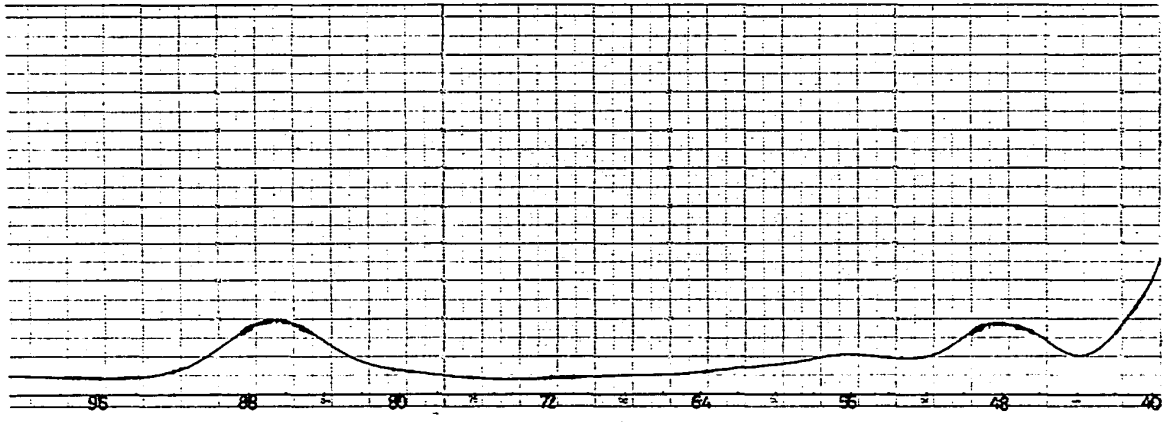


Fig. 34 (continued). Gas Chromatogram of Fatty Acid Mixture from Toxic Palythoa over SE 30 3% on Chromosorb W Column (1/8" x 6')

Compound S-10. - S-10 was a white waxy solid, mp 47.5°, transparent in the ultraviolet.

Infrared spectrum in potassium bromide depicted the following peaks. 2925(s), 2850(s), 1725(s), 1460(s), 1380(w), 1340(w), 1310(w), 1295(w), 1270(w), 1240(w), 1220(m), 1200(s), 1180(s), 730(s), 720(s) cm^{-1}.

Mass spectrum. - 70 EV mass spectrum depicted the following ions.

m/e	%	m/e	%	m/e	%	m/e	%	m/e	%
480	27	258	100	185	11	110	37	71	76
452	6	228	21	138	14	96	66	55	71
285	9	223	53	124	23	83	65	43	87
268	9	195	17						

Fraction L-20. - L-20 was found to be a mixture of three compounds by thin layer chromatography over silica gel H and silica gel H impregnated by silver nitrate. Gas chromatography over SE 30 20% on a Chromosorb W column (1/8" x 10', column temperature 248°) also indicated the presence of three compounds Rt, 1'00", 4'7", 7'00", and 11'7". No further work was done on this fraction.

Pg-2. - The fraction Pg-2 was chromatographed on a Microcel C column (2.1x35 cm) using a stepwise elution in the following order; n-hexane (250 ml), 2% acetone-n-hexane (250 ml), 5% acetone-n-hexane (500 ml), 10% acetone-n-hexane (1000 ml), and 20% acetone-n-hexane (250 ml). 10% acetone-n-hexane eluent was found to have the green pigment. The green pigment depicted a characteristic ultraviolet absorption spectrum λ_{max} 670, 610, 535, 500, and 410 nm.

TABLE XXVII

THIN LAYER CHROMATOGRAPHY OF n-HEXANE SOLUBLE

LIPID FRACTIONS FROM TOXIC PALYTHOA (Rf VALUES)

Fraction	Chloroform	n-Hexane	Benzene	Benzene-Hexane (30-70)	Benzene-Hexane (10-90)
S-10	Solvent Front	0.169	0.882	0.912	0.379
S-20	Solvent Front	0.169	0.882	0.910	0.379
L-20	0.000 to 0.160 (Three spots)				0.000 0.09 to 0.369 (Three spots)

Po-1. - The fraction Po-1 was chromatographed over a Microcel C column (2.1x32 cm) using a stepwise elution, in the following order; 4% acetate in n-hexane (250 ml), 5% acetone in n-hexane (250 ml), 8% acetone in n-hexane (750 ml), 10% acetone in n-hexane (250 ml), and finally 15% acetone in n-hexane (250 ml).

The 10% acetone in n-hexane eluant depicted ultraviolet and visible spectra identical with that of β-carotene λ_{max} 460, 431.5 and 410 nm.

M. Water Soluble Constituents of Toxic Palythoa

1. Isolation of UV-Active Material from Tahitian Palythoa

The aqueous effluents (ca. 10 l) from Tahitian Palythoa (10 kg) were concentrated to a volume of 1000 ml and divided into four equal parts.

a. Isolation Procedure I.

The concentrated aqueous effluents (250 ml) were extracted with 1-butanol (4x250 ml). The butanol extract after evaporation to dryness under vacuum was partially desalted by dissolving in methanol (200 ml). The methanol extract was evaporated to dryness, the residue was dissolved in water (15 ml) and extracted with chloroform (100 ml) in a liquid-liquid extractor for 24 hr.

(i) Purification on Amberlite IRC. 50 resin (hydrogen form)

After chloroform extraction the aqueous solution (made up to 50 ml) was passed through an Amberlite IRC. 50 (H^+) column (2x20 cm). The flow rate was 1 ml/min. The column was then washed

with water (50 ml) and the effluents which contained the λ_{270} material were combined and evaporated *in vacuo*.

The purpose of this step was to remove amino acids. The adsorbed bases could be eluted from the column by 1N ammonium hydroxide (500 ml).

(ii) Purification of Dowex 50W-X4 resin (mercuric form)

After the pass through IRC 50, the material was dissolved in 1N hydrochloric acid (5 ml) and introduced onto a Dowex 50W-X4 ion exchange resin (mercuric form, 2x55 cm). The column was washed with 0.1N hydrochloric acid (500 ml) and then the mercury complex of the 270 nm compound was eluted with 1.5ℓ of 3N hydrochloric acid. The effluent was concentrated to 1ℓ *in vacuo*, the concentrate was saturated with hydrogen sulfide for 20 min at room temperature and the mixture was filtered by gravity. After washing the precipitate with 0.1N hydrochloric acid (2x100 ml) the filtrate and the washings were heated to expel excess hydrogen sulfide gas and the solution was evaporated *in vacuo*.

The mercuric form of the resin was generated in the following manner: Dowex 50W-X4 was successively washed with 6N hydrochloric acid, water, 10% ammonium hydroxide, 3N hydrochloric acid and finally with water. The resin was then soaked in a 10% solution of mercuric acetate containing a small amount of acetic acid for about 1 hr. The resin was packed in a column and washed with 5% acetic acid until the effluent gave only a weak coloration with diphenylcarbazone (0.05M in 95% ethanol). Finally the resin was washed thoroughly with water.

(iii) Purification on Dowex 50W-X4 resin (Hydrogen form)

The residue obtained above was dissolved in 2N hydrochloric acid (5 ml) and the solution was introduced onto a Dowex 50W-X4 column (Hydrogen form, 2x30 cm). It was then eluted with 1N hydrochloric acid (400 ml) followed by 2N hydrochloric acid (1500 ml). Fractions (10 ml) were collected and assayed spectrophotometrically for 270 nm material. Fractions 90-110 containing most of 270 nm material were combined and evaporated in vacuo to a light yellow mass (ca. 250 mg). The yellow mass was dissolved in 1N hydrochloric acid (4 ml) and rechromatographed over a Dowex 50W-X4 column (Hydrogen form, 2.0x32.5 cm, 2N-hydrochloric acid as the eluant, fraction size 10 ml). Fractions 100-120 were combined and evaporated in vacuo to give a 270 nm hygroscopic pale oil, which slowly crystallized on standing (150 mg).

Dowex 50W-X4 (Hydrogen form) was prepared as described above. Washings were continued until the effluents did not show absorption in the 210 to 400 nm region.

(iv) The yellow oil from the Dowex 50W-X4 column chromatography was finally purified by preparative paper chromatography over Whatman No. 1 paper, using butanol-acetic acid-water (4:1:5) as the developer.

A band corresponding to Rf 0.49 was cut, eluted with water and evaporated to a pale oil (130 mg, Z-1).

Ultraviolet spectrum of compound Z-1.

In water. - λ_{max} 281, 278.5, and 274.5 nm

In 2N sodium hydroxide. - λ_{max} 291.75 and 240 nm

In 2N hydrochloric acid. - λ_{max} 360 and 300 nm

280.5, 276.8, 272.5, 217 nm,

214, 211.1 and 200.5 nm.

Nuclear Magnetic Resonance - Spectra are presented in Table XVIII.

Infrared spectrum in potassium bromide depicted the following bands.

3330(s,b), 2959(s,b), 2899(sh), 1587(s), 1495(s,b), 1473(s),

1337(w), 1269(m), 1224(m), 1152(m,b), 1010(sh), 1000(sh), 943.3(m),

927.6(m), 862.1(m), 828.5(m), 778.2(m), 752(m), and 694.4(m) cm^{-1}.

Mass spectrum. - 70 EV mass spectrum depicted the following ions.

m/e	%	m/e	%	m/e	%	m/e	%	m/e	%
137	58	107	43	45	40	37	95	30	100
108	98	91	17						

Acetylation of Compound Z-1. Compound Z-1 (3 mg) was added to a 2 to 1 mixture of pyridine and acetic anhydride (0.5 ml). After 24 hr standing at room temperature, the mixture was evaporated to dryness. The dry mass was freed of pyridine and acetic anhydride by repeated evaporation with absolute ethanol. The residue was partitioned between chloroform (10 ml) and water (5 ml). The chloroform layer was evaporated to dryness. The dry mass on analytical thin layer chromatography over silica gel H using chloroform-methanol (70:30) as the solvent system indicated one spot (Rf 0.83).

Ultraviolet spectrum in methanol. - λ_{max} 272 nm.

In methanolic hydrochloric acid. - λ_{max} 272 nm.

TABLE XXVIII

60 MHz NMR SPECTRA COMPOUND Z-1

in Deuterium oxide	No. of Protons	in Trifluoro acetic acid	No. of Protons	in Dimethyl sulfonide
		2.70 (t, J=6Hz)	2	2.80 (s,b)
3.00 (m, a_2b_2)	4	3.15 (c)	2	4.95 (s,b)
6.75 (s)	1	6.60 (d, J=6.5Hz)	2	6.50 (s,b)
6.88 (c)	2	6.90 (d, J=6.5Hz)	1	6.85 (s,b)
7.20 (c)	1	7.00 (c)	1	

Chemical shifts in δ units.

Nuclear magnetic resonance spectrum

in deuterochloroform depicted the following signals

2.10δ(s), 2.30δ(s), 2.90 to 3.50δ(c), 6.80 to 7.30δ(c)

Mass spectrum. - 70 EV mass spectrum depicted the following ions.

m/e	%	m/e	%	m/e	%	m/e	%	m/e	%
221	18.5	149	52	120	100	69	33	56	50
179	32	141	17	107	20	58	62	30	39
162	26								

Methylation of Compound Z-1. - The compound Z-1 (1.3 mg) was reacted with ethereal diazomethane solution. The solution was evaporated and examined for its NMR.

Nuclear magnetic resonance spectrum. - Nuclear magnetic resonance spectrum in deuterium oxide depicted two peaks around 4.70δ.

Synthesis of Compound Z-1. - m-Hydroxy-ω-nitrostyrene. -

A cool solution of sodium hydroxide (42 g) in water (100 ml) was dropwise added to a vigorously stirred solution of m-hydroxybenzaldehyde (61 g, 0.5m), and nitromethane (35.0 g, 0.5m) in methanol (150 ml). The mixture after stirring for 1 hr was poured into 2N hydrochloric acid (500 ml) with vigorous stirring whereby a yellow solid separated. The solid was filtered, thoroughly washed with cool distilled water. The yellow solid was crystallized from 95% ethanol as yellow needles mp 136-137° (64 g, 89%)

Ultraviolet spectrum. - In water λ_{max} 310 and 245 nm.

Nuclear magnetic resonance spectrum

in deuterochloroform depicted the following signals.

Nature of Signal	Chemical Shift	Relative Proton Ratio
Singlet	6.78δ	3
Complex	7.10δ	1
Doublet	(J=14Hz) 7.45δ	1
Doublet	(J=14Hz) 7.88δ	1

Mass spectrum. - 70 EV mass spectrum exhibited the following ions.

m/e	%	m/e	%	m/e	%	m/e	%	m/e	%
165	100	136	5	110	16	89	26	65	19
148	5.5	118	74	107	12	77	21	55	44

Lithium aluminum hydride reduction of m-hydroxy-ω-nitrostyrene. -

m-hydroxy-ω-nitrostyrene (16.5 g) in dry ether (50 ml) was dropwise added to a slurry of lithium aluminum hydride (4.5 g) in dry ether (50 ml). The mixture was stirred at room temperature for 6 hr. The mixture was worked up in the usual manner, and the crude product was purified by column chromatography over Dowex 50W X-4 resin using 1N hydrochloric acid as the eluant, to give a pale viscous mass.

Infrared spectrum in potassium bromide indicated the following bands. 3333(m,b), 2959(s,b), 2899(s), 1587(s), 1495(s,b), 1473(s), 1337(w), 1269(m), 1224(m), 1152(m,b), 1010(sh), 1000(sh), 934.4(m), 927.6(m), 862.1(m), 929.5(m), 778.2(m), 752(m), 694.4(m) cm^{-1}.

Nuclear magnetic resonance spectrum

in deuterium oxide depicted the following signals.

Nature of signal	Chemical Shift	Relative Proton Ratio
Complex (A_2B_2)	3.00δ	4 H
Singlet	1.70δ	
Complex	1.80δ	4H
Complex	7.10δ	

Mass spectrum. - 70 EV mass spectrum indicated the following ions.

m/e	%	m/e	%	m/e	%	m/e	%	m/e	%
137	41	101	7	77	31	42	25	36	83
118	85	91	14	56	15	39	22	30	100
117	42	85	12	44	22	38	26		

b. Isolation Scheme II

The concentrated aqueous effluents (250 ml) were evaporated to a brown gummy mass. The gummy mass was dissolved in water (25 ml), methanol (25 ml) was added, and the mixture cooled in a refrigerator overnight. The mixture was freed of solids and the process was repeated twice. The salt free gummy mass was then chromatographed over a Dowex 50W-X4 column.

Column Chromatography of the Brown Gummy Mass over Dowex 50W-X4 (H^+) resin.

The brown gummy mass in 2N-hydrochloric acid (5 ml) was applied onto a Dowex 50W-X4 column (2.1x33 cm, Hydrogen form). Elution was continued with 2N-hydrochloric acid. Fractions (6 ml) were collected and assayed spectrophotometrically. Fractions were combined thus:

I	Fractions 20-38	essentially 260 nm material
II	Fractions 50-64	essentially 272 nm material
III	Fractions 80-100	essentially 320 nm material
IV	Fractions 130-229	essentially 310 nm material
V	Fractions 70-78	Both 272 and 330 nm
VI	Fractions 350-400	-----

Fractions 50-64. - Fractions 50-64 were combined and evaporated to a brown mass. The brown mass was further purified by rechromatography over a Dowex 50W-X4 column (2.1x33 cm) using 2N hydrochloric acid as the eluant. Fractions 50-64 as before were combined and evaporated to a pale solid (Z-2). The pale solid was homogeneous on thin layer chromatography over alumina H and silica gel H using a variety of solvent systems. The results of thin layer chromatography are presented in Table XXIX.

Ultraviolet spectrum in distilled water. -

λ_{max} 272 (ε max 6100) nm.

Infrared spectrum as a thin film indicated the following bands. 3333(b,s), 3030(b), 1449(b), 1370(w), 1282(s), 1176(m), 1047(w), 934.6(s), 840.3(m), 787.4(s), 769.2(m), 678(s) cm^{-1}.

Nuclear magnetic resonance spectrum

in deuterium oxide. -

Nature of Signal	Chemical Shift	No. of Protons
Singlet	4.425δ	3
Complex	8.050δ	2
Complex	8.800δ	2

(Both complexes together correspond to a ABCD system)

Mass spectrum. - 70 EV mass spectrum depicted the following peaks.

m/e	%	m/e	%	m/e	%	m/e	%	m/e	%
121	.45	78	4	44	21	37	43	35	38
95	1.0	51	15	38	42	36	100	30	13
79	9	45	14						

TABLE XXIX

THIN LAYER CHROMATOGRAPHY OF THE FRACTIONS FROM DOWEX 50W-X4

	Alumina H				Silica Gel H			
	2-Propanol-Acetic acid-Water 70:5:25	1-Butanol sat'd with Hydrochloric acid	Ethanol-Ammonium hydroxide (4:1)	1-Butanol 1-Propanol-Water (4:2:1)	2-Propanol-Acetic acid-Water 70:5:25	1-Butanol sat'd with Hydrochloric acid	Ethanol-Ammonium hydroxide (4:1	1-Butanol 1-Propanol-Water (4:2:1)
	0.857							
Z-2	0.700 (minor)	0.200	0.7882	0.283	0.716	0.050	0.475	0.0526
							0.800 (maj)	0.2100
	0.647	0.140	0.568	0.0943	1.000	0.05	0.00	0.000
Z-3 (A)			0.7613 (maj)				0.205 (maj)	
Z-3	0.647	0.190	0.571	0.0943	1.000	0.05	0.205	0.000
	0.647		0.623		0.300		0.000	0.000
	0.762	0.214	0.764	0.0943	0.780	0.05	0.205	0.0520
Z-3 (B)	0.780				0.900			0.736
	0.88							

Hydrogenation of Compound Z-2. - Compound Z-2 (4.5 mg) was hydrogenated over catalytic platinum (25 mg) in absolute ethanol (15 ml) at 40 lbs/ $inch^2$ for 5 hr. The reaction mixture after filtration was evaporated to dryness. The residue was examined for physical constants.

Ultraviolet spectrum in methanol. - Transparent.

Infrared spectrum in potassium bromide depicted the following bands.

3300(s,b), 2925(s), 2850(sh), 1650(s), 1570(w,b), 1480(w), 1420(s,b), 1380(w), 1340(w), 1050(w), 1025(w,b)

Nuclear magnetic resonance spectrum

in deuterium oxide indicated the following signals.

1.35δ (c,b), 1.65δ(c,b), 3.15δ(c,b), 4.75δ(s,b)

Synthesis of Homarine. - To a mixture of picolinic acid (2 g), and silver oxide (2 g) in dimethylformamide (15 ml), was added methyl iodide (10 ml). The mixture was stirred at room temperature for 11 hr. The reaction mixture became deep red after an exothermic reaction. The mixture was poured over crushed ice (400 g). The mixture was well stirred, acidified with hydrochloric acid and filtered. The filtrate after concentration to 5 ml was applied onto a Dowex 50W-X4 column (Hydrogen form, 2.1x35 cm). Fractions (17 ml) were collected and assayed spectrophotometerically. Fractions 25 to 50 were combined and evaporated to a pale solid (1 g).

Ultraviolet Spectrum

At pH 7.0 in water. - λ_{max} 276 nm.

At pH 1.0 in aqueous hydrochloric acid. - λ_{max} 271 nm.

At pH 11.0 in aqueous sodium hydroxide. - λ_{max} 274 nm.

Infrared spectrum in potassium bromide depicted the following bands. 3333(b,s), 3030(b), 1449(b), 1370(w), 1282(s), 1176(m), 1047(w), 934.6(s), 840.3(m), 787.4(s), 769.2(m), 768.0(s) cm^{-1}.

Nuclear magnetic resonance spectrum

in deuterium oxide exhibited the following signals

Nature of Signal	Chemical Shift	Relative Proton Ratio
Singlet	4.425δ	3 H
Complex	8.050δ	4 H
Complex	8.800δ	

Both complexes made an ABCD system.

Mass spectrum. - 70 EV mass spectrum

m/e	%	m/e	%	m/e	%	m/e	%	m/e	%
121	0.45	78	4	44	21	37	43	35	38
95	1.0	51	15	38	42	36	100	30	13
79	9	45	14						

Fractions 80 to 100. - Fractions 80 to 100 were combined and evaporated to a pale gummy mass. The analytical thin layer chromatography over alumina H using the solvent system of ethanol-ammonium hydroxide (4:1) indicated the presence of four compounds (Rf 0.647, 0.762, 0.780 and 0.880). Preparative thin layer chromatography provided a band corresponding to Rf 0.647 (Z-3). The homogeneity of the band was indicated by thin layer chromatography over alumina H and silica gel H using a variety of solvent systems (see Table XXIX). The compound is a hygroscopic colorless liquid.

Ultraviolet spectrum in water λ_{max} 214 nm.

Infrared spectrum in potassium bromide depicted the following bands. 3450(b,s), 1630(s), 1572(sh), 1450(b,w), 1389(s,b), 1325(sh), 1200(sh), 1130(m), 1100(s), 940(w), 785(w) cm^{-1}.

Nuclear magnetic resonance spectrum

in deuterium oxide indicated the following signals.

Nature of Signal	Chemical Shift	Relative Proton Ratio
Singlet	3.40δ	3
Singlet	3.80δ	3
Singlet	3.90δ	3
Singlet	4.70δ	3
Singlet	4.90δ	1
Singlet	7.50δ	1

Mass spectrum. - 70 EV mass spectrum

m/e	%	m/e	%	m/e	%	m/e	%	m/e	%
140	100	95	8	70	21	56	9	42	44
125	31	82	9	68	14	44	69	28	8
109	43								

Attempted Acetylation of Compound Z-3. - Z-3 (5.0 mg) was reacted with acetic anhydride (0.1 ml) in dry pyridine (0.3 ml) for 24 hr. The mixture was evaporated to dryness. The residue was freed of acetic anhydride and pyridine by repeated evaporation with absolute ethanol. The crude product was found to have an ultraviolet spectrum and a nuclear magnetic resonance spectrum identical with the starting material.

Ultraviolet spectrum. - λ_{max} 214 nm (not affected by acid and base).

Nuclear magnetic resonance spectrum

in deuterium oxide showed the following signals

Nature of Signal	Chemical Shift	Relative Proton Ratio
Singlet	3.45δ	3
Singlet	3.85δ	3
Singlet	3.95δ	3
Singlet	4.75δ	(HOD)
Singlet	4.92δ	1
Singlet	7.50δ	1

Fractions 350-400. - Fractions 350 to 400 were combined and evaporated to a pale mass. The pale mass was preparatively paper chromatographed with butanol-acetic acid-water (4:1:5), on Whatman No. 1 paper. A band corresponding to Rf 0.10 was eluted with water. The eluate on evaporation furnished a white crystalline solid (Z-4) mp 240°.

Ultraviolet spectrum in water. -

$$\lambda_{max}$$ 212 (ε 5000) nm.

Infrared spectrum in potassium bromide showed the following bands. 3030(b), 1563(s,b), 1471(s,b), 1429(w), 1389(m), 1325(s), 1266(sh), 1227(sh), 1105(b), 1087(m,b), 99.1(b), 939(m,b), 819.7(s,b), 769.2(b), 662.3(m) cm^{-1}.

Nuclear magnetic resonance spectrum

in deuterium oxide depicted the following signals.

Nature of Signal	Chemical Shift	Relative Proton Ratio
Complex (A_2B_2)	3.03δ	4
Singlet	7.03δ	1
Singlet	7.73δ	1

Mass spectrum. - 70 EV mass spectrum

m/e	%	m/e	%	m/e	%	m/e	%	m/e	%
112	26.5	95	6	93	10	81	100	55	93
111	33	94	7	82	100	68	7	54	97
52	25	39	26	30	100	28	98	26	11
41	26								

c. Isolation Scheme III

After extraction with 1-butanol (5x300ml) to remove the 270 nm compound, the aqueous concentrate (250 ml) was evaporated *in vacuo* at *ca.* 50°. The reddish brown gum was dissolved in water (20 ml) and introduced onto a Sephadex G 25 (medium) column (5x28 cm). Fractions (20 ml) were collected and assayed spectrophotometrically. Fractions 12 to 17 were combined and evaporated *in vacuo* at *ca.* 40°.

(i) Purification on Sephadex Anion Exchange Gel

The crude 330 nm material obtained above was dissolved in 0.02M, pH 7.0 sodium phosphate (5 ml) and introduced onto a DEAE-Sephadex A-25 (medium, phosphate form) column (2.0x31.5 cm). The temperature was 7° throughout the gel filtration. Elution was continued with 0.02M, pH 7.0 phosphate buffer; fraction size 5.5 ml. Fractions 25 to 45 containing the bulk of 330 nm material were combined and desalted on a 0.8x50 cm column of AG 11-A8 (50-100 mesh, Bio Rad) resin.

The AG 11-A8 ion retardation resin was prepared or regenerated by washing successively with 10% sodium chloride solution and then thoroughly with water.

(ii) Purification on Sephadex Cation Exchange Gel

The desalted 330 nm material was dissolved in 0.02M sodium dihydrogen phosphate (5 ml) and introduced onto a

CM-Sephadex C-25 column (medium, phosphate form, 0.02M sodium dihydrogen phosphate). Elution with 0.02M sodium dihydrogen phosphate was continued and fractions (4.5 ml) were collected and assayed spectrophotometrically. Fractions 5 to 12 were combined and evaporated to a pale solid. The solid was extracted with hot methanol (50 ml). The extract was filtered and evaporated to dryness.

(iii) Final Purification on Sephadex Anion Exchange Gel

The partially purified 330 nm material was dissolved in 0.02M, pH 7.0 sodium phosphate buffer and the solution was introduced onto a DEAE-Sephadex A-25 (column 2x32 cm). Elution was continued with 0.02M pH 7.0 phosphate buffer. Fractions (5 ml) were collected and assayed spectrophotometrically. Fractions 30 to 50 were combined and concentrated. After desalting by a pass through an AG 11-A8 column (0.80x50 cm), the 330 nm compound was obtained as an amorphous pale yellow hygroscopic solid. The compound was found to be negative to ninhydrin, Dragendorff, isatin and Prochazka's regents. Ehrlich reaction gave a light yellow spot while test for purines gave a brownish yellow spot.

Ultraviolet Absorption Spectrum in distilled water. -

λ_{max} 330 and λ_{min} 270 nm

Nuclear magnetic resonance spectrum in deuterium oxide depicted the following signals.

Nature of Signal	Chemical Shift	Relative Proton Ratio
Singlet	2.650δ	3H
Singlet	2.850δ	9H
Singlet	2.960δ	3H

Nature of Signal	Chemical Shift	Relative Proton Ratio
Singlet	3.25δ	9H
Singlet	3.27δ	
Singlet	3.40δ	15H
Singlet	3.65δ	6H
Singlet	3.725δ	9H
Singlet	4.100δ	9H

Infrared spectrum in potassium bromide showed the following bands. 3200(s,b), 1600(s,b), 1550(s,b), 1395(s,b), 1310(w), 1265(w), 1130(w), 1050(w,b), 1000(w), 950(w,b) cm^{-1}.

Mass spectrum. - The compound did not provide a decent mass spectra.

d. Isolation Scheme IV

The concentrated aqueous effluents were freed of salts as described in isolation scheme II. The desalted effluents were evaporated to a small volume (50 ml) made 10% in trichloroacetic acid, and extracted with ether till the pH of the aqueous solution was 4. The aqueous phase was poured into a saturated ammonium reineckate solution (100 ml). After 1 hr in the cold, the precipitates were centrifuged and washed with saturated choline reineckate. The precipitates were dissolved in a minimum amount of acetone and reacted with 2M silver perchlorate with vigorous stirring until the solution gave a positive test for silver. Aqueous 50% acetone was added (150 ml). The precipitates were centrifuges, the supernatant was evaporated to dryness. The residue was extracted with three portions of ethanol (7 ml each). The ethanol extract was mixed with sodium dihydrogen phosphate (0.1M, 3 ml), reduced to 5 ml and treated with 5 g Dowex

CG 50 (OH) and centrifuged. The supernatant was chromatographed over Dowex CG 50 (phosphate form, 200-400 mesh) column (2.0x27 cm). Elution was continued with 0.1M sodium dihydrogen phosphate. Fractions (5 ml) were collected and assayed spectrophotometrically and by analytical paper chromatography. Fractions 16 to 22 had essentially all the ultraviolet activity at 270 nm. Paper chromatography of the eluted fractions (160 in all) failed to indicate the presence of any choline esters.

2. Isolation of UV-Active Material from Palythoa mammilosa

Palythoa mammilosa (1 kg) was processed according to the isolation scheme II, followed by preparative paper chromatography using butanol-acetic acid-water (4:1:5) to give a white crystalline solid (ca. 100 mg) Rf 0.57

Ultraviolet spectrum in water. -

λ_{max} 280 (ε= 1242), 275 (ε 1258) nm.

pH 13.0 λ_{max} 292.5 (ε 1617) nm λ_{min} 265 nm.

Infrared spectrum in potassium bromide showed the following bands. 3077(s,b), 2941(m), 1613(m), 1587(w), 1493(m), 1460(m), 1385(w), 1351(w), 1252(w), 1220(b), 1174(m), 1139(s), 1111(s), 943.3(w), 837(m), 833(m), 775(w) cm^{-1}.

Nuclear magnetic resonance spectrum in deuterium oxide. -

Nature of Signal	Chemical Shift	Relative Proton Ratio
Complex (A_2B_2)	3.00δ	1
Complex (A_2B_2)	6.80δ	1

Mass spectrum. - A 70 EV mass spectrum depicted the parent ion at m/e 137 and a base peak at m/e 30.

N. UV-Active Constituents of Ophiacoma insularia and Ophiacoma erinaceous

Ophiacoma insularia (500 g) was poured into a large chromatographic column and soaked with acetone. After 2 d the extract was drained off and the animals soaked in 70% acetone for 24 hr. The process was repeated twice. The extracts were combined and evaporated to a small volume (100 ml). The concentrate was extracted with benzene (250 ml) and 1-butanol (1000 ml). The butanol extract was evaporated to a small volume, freed of salts by precipitation with methanol. The partially desalted material was chromatographed over a CM-Sephadex C-25 column.

Chromatography over CM-Sephadex. - A CM-Sephadex medium, C-25 column (2.0x31 cm) was prepared in 0.02M sodium dihydrogen phosphate. The crude desalted material was added to the column and elution continued with sodium dihydrogen phosphate (0.02M). Fractions (5 ml) were collected and assayed spectrophotometrically. Fractions were combined thus:

Fractions 18 to 28 - A

Fractions 38 to 49 - B

Fractions 18 to 28 were combined and evaporated to dryness. The white residue thus obtained was leached with methanol. The methanol extract was preparatively chromatographed over silica gel HF using chloroform-methanol (80:20) as the developer. Fluorescent bands corresponding to Rf values were collected and identified as Ins-A and Ins-B respectively. The physical data for Ins-A and Ins-B are presented in Table XXX.

A similar isolation procedure was adopted for Ophiacoma erinaceous and two compounds Er-A and Er-B were isolated. The physical data for the two compounds are presented in Table XXX.

O. UV-Active Constituents of Echinothrix diadema and Holothuria atra

Echinothrix diadema (200 g) was processed for UV-active constituents following the procedure used in the previous section for Ophiacoma insularia. Compound Ech-A was isolated. Compounds H_A-A and H_A-B were isolated from Holothuria atra (see Table XXX).

TABLE XXX

UV ACTIVE WATER SOLUBLE COMPOUNDS FROM SOME ECHINODERMS

UV	IR	NMR	MS	MP
Ins-A λ_{max} 265 nm (and Er-A)	3200, 3050 (b,m), 1725 (m), 1660 (m), 1430 (w), 1410 (w), 1380 (w), 1240 (m), 1200 (m), 845 (b,w), 815 (w), 765 (w), 740 (w)	1.90δ (D,J=1.5 Hz) 7.400δ (Q) (J=1.5) (3-1)	70 EV m/e (100%), 126 (78%), 97 (10%), 83 (42%), 70 (20%), 55 (100%), 53 (45%), 43 (32%), 39 (38%)	
Ins-B λ_{max} 265 nm (and Er-B)	3200 (s), 2900 (sh), 1710 (s), 1650 (s), 1460 (m), 1420 (s), 1398 (w), 1300 (w), 1235 (s), 1000 (w), 852 (b), 760 (w)	1.95δ (D,J=1.5 Hz) 2.40δ (T, Complex J 6 CPS) 3.79δ (C) 3.85δ (C) 4.03δ (Q) 4.50δ (T) 6.28δ (T,J=7 Hz) 7.65δ (D,J=1.5 Hz)	70 EV m/e (100%), 242 (1%), 126 (45%), 117 (27%), 113 (43%), 83 (18%), 70 (73%), 67 (48%), 54 (58%), 43 (100%)	
Ech-A λ_{max} 265 nm			m/e 126, 97, 83, 70, 55 (base peak), 44, 43, 28	
H_A-A λ_{max}		Doublet 5.85δ (J=8.0 Hz) Doublet 7.60δ (J=8.0 Hz)		
H_A-B λ_{max} 265 nm		Doublet 1.95δ (J=1.5 Hz) Quartet 7.50δ (J=1.5 Hz)		

CHAPTER III

RESULTS AND DISCUSSION

A. Isolation of Palytoxin

Palytoxin was isolated from toxic zoanthid material according to the scheme outlined in Figure 1. The isolation procedure consisted of preliminary work-up, involving maceration of the animals with aqueous ethanol, defatting of the extract with benzene, removal of water soluble pigments by butanol extraction and enrichment of the toxin by column chromatography over DEAE-Sephadex A-25, and CM-Sephadex C-25 using phosphate buffers followed by desalting over a polyethylene column. Relatively high elution volumes on CM-Sephadex and elution almost with the solvent front on DEAE-Sephadex A-25 depicted the basic nature of the toxin. A desalting procedure involving column chromatography over Bio Rad AG11A8 resin proved ineffective. The toxin was irreversibly adsorbed on Darco G and Amberlite MB3.

For an optimum yield of palytoxin the isolation had to be carried out in a minimum amount of time. In a set of two experiments, on a single collection of Palythoa destruction of 20 to 30% of toxin was observed, when the time for isolation was increased from two and one half to four weeks. All evaporations had to be conducted in one batch at a water bath temperature of 50-60°. The Sephadex cation and anion exchange chromatographies, when conducted in a cold room (ca. 7°), increased the elution volumes and furnished better resolution of the toxin. In one experiment destruction of about ten percent

of toxin was observed when toxin adsorbed on polyethylene was eluted after standing overnight at 7°. It was particularly noted that the crude toxin on standing decomposed whereas a sample of pure palytoxin could be stored for over a week in a stoppered container without any noticeable decomposition. The presumed presence of degradative enzymes in the crude preparation could be shown by precipitation with ammonium sulfate of a gummy grey solid from concentrated aqueous effluents.

The crude preparation on mixing with a pure sample of palytoxin resulted in a rapid decrease in toxicity. No quantitative correlation between the amount of crude preparation and the half life of the toxin was obtained. Palytoxin could not be dialyzed through a cellophane membrane.

A sample of pure palytoxin depicted strong retardation on a freshly prepared Sephadex G-25 (fine) column (in distilled water) most probably due to adsorption on the active sites of the gel.

The ratio of 1.7 for ultraviolet maxima at 263 and 233 nm and of 1.14 for the minima at 210 and 255 nm were found to be essential criteria of toxin purity.

Homogeneity of palytoxin was established by column chromatography over Cm-Sephadex C-25 and Sephadex G-25 (Fine). In each case, the column chromatograph depicted only one symmetrical peak. The presence of essentially one peak in the countercurrent distribution curve further substantiated this observation.

Further support for the homogeneity of palytoxin was obtained by paper and thin layer chromatography using a variety of solvent

systems (Tables V and VI). The presence of more than one spot in the toxin chromatogram when visualization was affected by exposing the silver nitrate soaked chromatogram to light, was shown to be due to the presence of sodium salts, in particular sodium phosphate.

Ultracentrifugation of palytoxin at 67,770 rpm furnished a sedimentation constant of 0.46 Svedberg and indicated that the toxin is homogeneous with respect to a material absorbing at 263 nm (44).

B. Toxicity of Palytoxin

Palytoxin is a potent toxin with LD_{50} 0.75 μg/kg mice (see Table III for comparison of toxicity). The great toxicity of the toxin requires that it be handled with great care and respect.

In mice palytoxin poisoning is manifested by paralysis of the hind legs, followed by respiratory difficulty, gasping, convulsions and finally death. Symptoms of palytoxin poisoning in human beings are not known. However, inhalation of fine palytoxin dust results in a strong burning sensation accompanied with bleeding (in some severe cases) of the nasal passages lasting sometimes over a week.

Mucus from toxic Palythoa polyps caused redness and itching of tender parts of the skin.

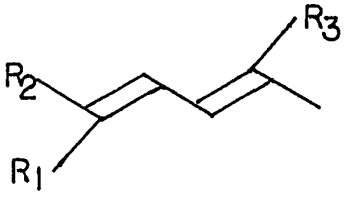
C. Spectral Characteristics of Palytoxin

1. Ultraviolet Spectrum

A quantitative ultraviolet spectrum of palytoxin (Fig. 2) in distilled water exhibited maxima at 263 (ε 8.4xM) and 233 (14.1xM) nm and minima at 255 (ε 8.0xM) and 210 (ε 7.1xM) nm. The different rates of irreversible disappearance of the two maxima in acid and

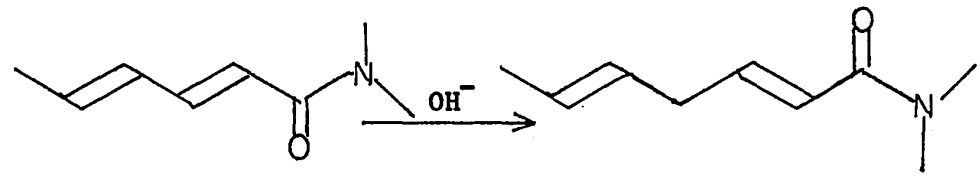
base suggest the presence of at least two independent chromophores (Fig. 6). This observation was confirmed by a complete retention of the 233 nm (ε 14.0xM) maximum in base (0.002 M sodium hydroxide) and a comp.ete retention of the 263 nm (8.4xM) maximum when an aqueous solution of palytoxin was stirred with degassed catalytic platinum.

An aqueous solution of palytoxin, on prolonged stirring with manganese dioxide, suffered a change in the intensity of the 233 nm maximum (14.1xM to 7.0xM). The lack of further change in the intensity of the 233 nm maximum with time or fresh manganese dioxide suggested the presence of two similar chromophores varying only in their environments in the molecule. The great susceptibility of the absorption (233 nm) to metal surfaces strongly suggested the presence of two triply substituted butadiene moieties, which would be expected to absorb at 232 nm.



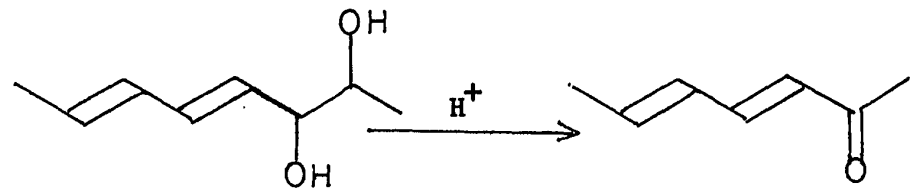
Loss of conjugation on metal surfaces and polymerization in strong acid (see Section E) would explain the UV behavior. Reaction of palytoxin with tetracyanoethylene to furnish a Diels-Alder adduct confirmed the presence of butadiene (45 a).

The rate of disappearance of the 263 nm maximum varies greatly with the concentration of acid or base. The facile destruction of the 263 nm maximum in base suggests the presence of a conjugated chromophore.



Irreversibility of the above reaction in acid is attributed to a fast polymerization step. No direct evidence for the above chromophore is available at the moment.

Generation of a 290 nm chromophore on methanolysis (hydrogen chloride in methanol) of palytoxin suggests the change from a butadiene glycol to a butadienone (46).



2. Infrared Spectrum of Palytoxin

The infrared spectrum of palytoxin (Fig. 3) depicted the presence of hydroxyl (3300 and 1060 cm^{-1}), amide or tetrasubstituted olefin (1650 cm^{-1}), methyl (1370 cm^{-1}), terminal methylene (892 cm^{-1}) and triply substituted double bond (850 cm^{-1}) functions.

3. NMR Spectrum of Palytoxin

A 220 MHz nuclear magnetic resonance spectrum (Fig. 4) is interpreted as follows:

Chemical Shift	No. of Protons	Interpretation
0.863δ (s)	3H	CH_3-C-
1.000δ (s)	3H	CH_3-C-
1.227δ (s)	3H	CH_3-C-O-
1.159δ (b)	18H	$-(CH_2)_n-$ $+CH_3-$
1.369δ (b,c)	10H	$-CH_2-C-OH$ CH_3-C-OH
1.636δ (b,c)	17H	$CH_3-C=C-$
2.386δ (b,c)	4H	$-CH_2-C=C-$
2.772δ (b,c)	6H	$CH_2-C=C-$ $-CH-C=C=$
3.773δ (b,c)	9H	$-CH_2-O-$
4.318δ (b,c)	28H	-CH-OH
5.046δ (b,c)	7H	$C=C<^{H}$
5.9318δ (b,s)	52H	-OH and H_2O

The spectrum clearly indicated the absence of N- methyl or acetyl groups.

4. Elemental Analysis of Palytoxin

Elemental analysis of two samples of palytoxin indicated the absence of phosphorus and sulfur and suggested the following empirical formulas $(C_{30}H_{60}NO_{15})_n$ or $(C_{33}H_{52}NO_{16})_n$.

The discrepancy between the two analyses is easily rationalized by the large size and the hygroscopic nature of the toxin. Also the problem involved in the removal of adsorbed and trapped cations have considerably plagued our experiments. Acetylation of palytoxin (see Section D-1) led to an acetate which provided perhaps a more accurate composition $(C_{30}H_{50}NO_{13})_n$.

Palytoxin was examined for inorganic cations by neutron activation and atomic absorption. Only traces of sodium and manganese were indicated.

5. Molecular Weight of Palytoxin

The molecular weight of palytoxin was determined by vapor pressure osmometry, in water and 80% ethanol. Calibration curves were prepared using sucrose and benzil respectively. Palytoxin gave molecular weights of 1160, 1570 and 1880 for 10.8, 18.4 and 31.2 mg/ml concentration of toxin in water. Molecular weights of 2490 and 2900 respectively were obtained for 13.8 and 23.6 mg/ml 80% ethanol, of toxin. The results evidently show either association of palytoxin in 80% ethanol or dissociation of palytoxin in water (44).

Ultracentrifugation experiments indicated molecular weight range of 1100 to 2000 for palytoxin (44).

Indirect evidence for the molecular formula of palytoxin was furnished by the periodate oxidation (Section G-9). Presence of four nitrogen atoms in a single periodate oxidation fragment strongly suggested the presence of a minimum of four nitrogens in the toxin. Therefore a molecular formula $C_{120}H_{200}N_4O_{52}$ could be written. Support for this formula could be obtained from the integration of the 220 MHz nmr spectrum of palytoxin in pyridine - d_5. The signal at 3.77δ and 4.138δ corresponding respectively to methylene and methines attached to hydroxyl alone represented 9 and 28 protons. Furthermore the signal at 5.932δ corresponded to 52 protons. Additional support for the above formula was obtained from the nmr spectrum of palytoxin acetate (see Section D-1).

6. Chemical Properties of Palytoxin

An aqueous solution of palytoxin was heated to 100° for three hours without any significant decomposition. However, the toxin was found to be very sensitive to acid and base. First order kinetics of decomposition in 0.04N sodium hydroxide and 0.20N hydrochloric acid depicted a half-life of 75 and 77.5 minutes (for 263 nm absorption) respectively (Fig. 6).

Palytoxin was positive to silver nitrate-sodium hydroxide, benzidine-periodate and periodate-permagnate sprays, which are commonly used for vicinal diols, but was negative to Dragendorff, Konig, Paulys, Jaffês, Sakaguchi reagents and Griess test used for quaternary and tertiary amines, pyridinium compounds, phenols and imidazoles, α-guanidins, guanidino derivatives and nitroso compounds. Palytoxin was only questionably positive to triphenyltetrazolium chloride, benzidine, p-anisidine hydrochloride, anthrone and ninhydrin reagents which are employed for reducing compounds, polysaccharide, sugar, primary amines and amino acids in that order.

D. Derivatization

Derivatization of palytoxin was attempted with a twofold purpose; one, to obtain more accurate empirical formulas for the toxin; two, to obtain a crystalline derivative which might be suitable for single crystal x-ray diffraction studies. It appears that we have succeeded in the former but failed in the latter of our objectives.

1. Acetylation of Palytoxin

Palytoxin on acetylation with acetic anhydride in pyridine followed by work-up and purification by column chromatography furnished

white amorphous acetate, mp 100-105°. The acetate was soluble in chloroform, benzene, acetone and methanol but insoluble in water and petroleum ether. Elemental analysis provided an empirical formula of $(C_{42}H_{62}NO_{19})_n$.

Palytoxin acetate had ultraviolet maxima (Fig. 7) at 263 nm (6.5xM) and 233 nm (8.88xM), thereby suggesting a complete retention of the ultraviolet chromophores on acetylation. The infrared spectrum (Fig. 8) showed the presence of tertiary or hydrogen-bonded hydroxyls (3350 cm^{-1}), acetate (1735 and 1235 cm^{-1}) and an amide or tetra-substituted olefin (1660 cm^{-1}). The nuclear magnetic resonance spectrum of palytoxin acetate (Fig. 9) depicted four sets of signals. Signals at 0.68δ (s), 1.00δ (b, 27H), 1.18δ (b,c) and 1.27δ (s, 40H) represented the aliphatic portion of the molecule. Signals at 1.51δ (s, 6H) and 2.025δ (s, 107H) represented olefin and acetate methyl groups. A broad signal at 3.30δ most probably represented allylic methylenes. The signals at 4.200δ (c) and 5.10δ (c) corresponded to 14 and 18 protons, respectively and represented methylenes and methines attached to oxygen.

A Rast molecular weight determination of palytoxin acetate gave a molecular weight of 498, whereas gel filtration on Sephadex LH20 indicated a molecular weight greater than 800. Isopiestic distillation of a palytoxin acetate solution in benzene and 2-butanone gave molecular weights of 4520 and 4128 (45c). A computation based on the weight gained on acetylation utilizing the decrease in extinction coefficient at 263 nm indicated a molecular weight of 2625 for palytoxin and 3675 for the acetate. On the basis of four nitrogen atoms, a molecular formula of $C_{168}H_{248}N_4O_{76}$, M.Wt. 3536, can be written for the

acetate. The considerably lower value for the molecular weight, in the Rast determination is evidently a result of heterogeneous solution.

The chemical properties of palytoxin acetate may be summarized as follows:

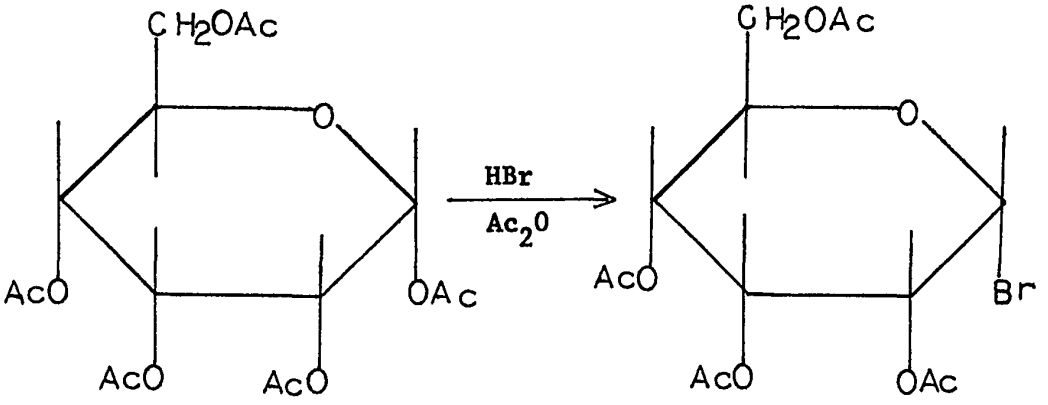
a. Palytoxin acetate rearranged on catalytic platinum or silica gel HF to a compound having only a 263 nm UV maximum and an infrared spectrum identical to that of palytoxin acetate. The disappearance of the 233 nm maximum strongly suggested the rearrangement of the diene moiety on the metal surface or during adsorption on silica gel.

b. Palytoxin acetate could be hydrogenated to a UV inactive hydrogenated palytoxin acetate. Hydrogenated palytoxin acetate was positive to the hydroxamate ester test but negative to Dragendorff and ninhydrin reagents. The infrared spectrum indicated the presence of hydroxyl (3400 and 1020 cm^{-1}), acetate (1740 and 1265-1200 cm^{-1}) and amide or tetrasubstituted olefin (1660 cm^{-1}). A 60 MHz nmr spectrum differed from that of palytoxin acetate only in the presence of a doublet at 0.87δ (J=6 Hz) with a shoulder at 1.03δ. If it is assumed that the signal at 0.87δ represents 9 protons, the acetate signal at 1.98δ corresponds to 72 protons. A 70 EV mass spectrum of hydrogenated palytoxin acetate had peaks as high as m/e 757. The spectrum indicated an ascending pattern from higher to lower mass units thereby suggesting the presence of a long hydrocarbon chain. The spectrum also showed a number of peaks which differed by 28 mass units.

c. Since the ir spectrum of palytoxin (Fig. 3) indicated the presence of an amide function (1650 cm^{-1}), it was desirable to confirm this observation. Hydrolysis of palytoxin acetate was therefore

attempted by triethyloxonium fluoborate according to a procedure by Hanesian (47). The reaction product did not have a primary amine function as shown by a negative ninhydrin test and IR and NMR spectra of the product were almost identical to that of palytoxin acetate.

d. Spot tests had already indicated the presence of vicinal diol and carbohydrate-like moieties in palytoxin. It was therefore expected that dry hydrogen bromide gas would substitute an acetate at C-1 of a sugar by bromine, thereby furnishing a useful bromine derivative of palytoxin.



The product, however, indicated the absence of C-Br stretching signals in the ir.

e. A similar attempt to prepare a bromoacetate of palytoxin by transfer bromoacetylation of palytoxin acetate with bromoacetic anhydride failed.

f. It is obvious that a partially acetylated palytoxin (carrying some vicinal hydroxyls), would be of potential use in periodate oxidation. Therefore, methanolysis of palytoxin acetate was attempted in the hope of obtaining such a compound. However, under

our experimental conditions, palytoxin acetate was not only completely deacetylated but further degraded to three compounds.

g. It was noted that palytoxin acetate gave a pale color with Dragendorff reagent whereas a mixture of palytoxin acetate and methyl iodide gave an orange color suggesting thereby possible presence of a tertiary amine.

2. Benzoylation of Palytoxin in Sodium Hydroxide

Palytoxin could be benzoylated under Schotten Baumann conditions to a white amorphous solid, mp 123-128°. The solid had maxima at 281.8, 273.0 and 227.3 nm and infrared bands at 1710, 1260-1190 and 1600 cm^{-1}, thereby indicating the presence of benzoates. The nmr spectrum further indicated the presence of secondary benzoates (5.50δ). Elemental analysis furnished an empirical formula of $(C_{66}H_{72}NO_{22})_n$ suggesting the introduction of five benzoate units for every nitrogen (parent $C_{31}H_{47}NO_{17}$).

3. Benzoylation of Palytoxin in Pyridine

Palytoxin when reacted with benzoyl chloride in pyridine furnished a white amorphous benzoate, mp 122-126°. The ultraviolet maxima at 282 and 273 suggested the presence of benzoate groups. The infrared spectrum depicted the presence of hydroxyl (3400, 1700 cm^{-1}) and confirmed the presence of benzoate groups (3050, 1595, 708 and 1720 cm^{-1}). A 70 EV mass spectrum had a base peak at m/e 105 (ϕCO^{+}) and a next higher peak at m/e 122 ($\phi COOH$). Elemental analysis furnished an empirical formula of ($C_{60}H_{52}NO_{15}$) corresponding to a tetrabenzoate. The lower hydrogen content of the empirical formula may be a result of a small analytical sample and/or probable dehydration during benzoylation.

4. 4-Bromobenzoylation of Palytoxin in Sodium Hydroxide

4-Bromobenzoylation of palytoxin under Schotten Baumann conditions resulted in an amorphous white solid, mp 131-135°. The compound had an ultraviolet maximum at 246 (ε 56.14xM) and a minimum at 235 (ε 48.81xM) nm. The infrared spectrum depicted the presence of hydroxyl (3400, 1100 and 1070 cm^{-1}) and 4-bromobenzoates (1720, 1240, 1580 and 758 cm^{-1}). Elemental analysis furnished an empirical formula of $(C_{66}H_{66}Br_5NO_{22})_n$ derived from a parent of composition $(C_{31}H_{46}NO_{17})_n$. Comparison with the parent for the Schotten Baumann benzoate ($C_{31}H_{47}NO_{17}$) and the empirical formula for palytoxin $(C_{30}H_{50}NO_{13})_n$ clearly indicated the generation of a derivative containing a lower hydrogen to carbon ratio than palytoxin.

5. 4-Bromobenzoylation of Palytoxin in Pyridine

Palytoxin could be 4-bromobenzoylated in pyridine to an amorphous white 4-bromobenzoate, mp 137-141°. The compound had an ultraviolet maximum at 247 (47.8xM) nm. The infrared spectrum confirmed the introduction of bromobenzoate groups (1710, 1580, 1260 and 752 cm^{-1}). The mass spectrum showed base peaks at m/e 185 and 183 (100% each) and the most abundant peaks at m/e 202 and 200 (80% each). A meaningful elemental analysis could not be obtained because of the small analytical sample.

6. Mesylation followed by Acetylation of Palytoxin.

Palytoxin when reacted with methanesulfonyl chloride in pyridine at room temperature gave a non-crystalline white mesylate, mp 140-145°. The infrared spectrum of the mesylate confirmed the presence of sulfonate (1350 cm^{-1}) and indicated the presence of

hydroxyl (3400, 1050 cm^{-1}) and amide or olefin (1640 cm^{-1}). Palytoxin mesylate could be converted to a mesylate-acetate on reaction with acetic anhydride in pyridine. The mesylate acetate softened on heating to 125°, solidified to a brown mass and melted with decomposition at 140-150°. The infrared spectrum indicated the presence of hydroxyl (3400), acetate (1730 and 1235 cm^{-1}), mesylate (1350 cm^{-1}) and terminal vinyl (910 and 965 cm^{-1}).

Preparation of a mesylate acetate of palytoxin evidently furnishes a procedure for partial acetylation of palytoxin.

7. Tritylation Followed by Acetylation of Palytoxin

Palytoxin could be tritylated in pyridine (48). The trityl derivative on further reaction with acetic anhydride furnished a trityl acetate of palytoxin. The infrared spectrum of the compound indicated the presence of acetate (1720 cm^{-1}), phenyl ring (1600, 1500 cm^{-1}) and hydroxyl (3450 and 1075 cm^{-1}) groups. Thin layer chromatography of the compound on silica gel HF resulted in hydrolysis to triphenyl carbinol and partially acelylated palytoxin.

8. 4-Bromobenzene Sulfonation of Palytoxin

In a continuing effort to introduce a hetero atom into the toxin molecule, palytoxin was 4-bromobenzenesulfonated to an amorphous white solid (IR SO_2 1370 cm^{-1}), which could not be further purified.

9. Attempted Bromoacetylation of Palytoxin

A similar effort to prepare a bromoacetate of palytoxin with bromoacetyl chloride and bromoacetic anhydride failed.

10. Attempted Formation of Isopropylidene Derivative of Palytoxin

Palytoxin could not be reacted with acetone in the presence of anhydrous copper sulfate, to furnish an isopropylidene derivative,

perhaps because of low solubility of the toxin in acetone.

11. Attempted Formation of Trimethylsilyl Ether of Palytoxin

Palytoxin could be converted to a silyl ether by hexamethyl disilazane and trimethyl chlorosilane, in pyridine. However, all attempts to put the silyl ether through a gas chromatograph or a mass spectrometer failed.

12. Formation of Methyl Ether of Palytoxin

In an attempt to prepare a methyl ether of palytoxin for mass spectral study, palytoxin was treated with sodium hydride and methyl iodide in dimethyl formamide (49). Interestingly enough, palytoxin furnished three products which not only differed from one another in degree of methylation but also in functional groups.

Band I (Rf 0.850) had only shoulders at 276 and 230 nm in its ultraviolet spectrum. The infrared spectrum indicated the presence of an oxo function (1725 cm^{-1}) and hydroxyl (3400 and 1100 cm^{-1}). The nmr spectrum depicted signals at 0.875δ (distorted, t, 6H) corresponding to methyl 1.25δ (b,s) and 1.72δ (b,c) corresponding to aliphatic and alicyclic methylene groups (28H in all), 3.30δ (c), and 3.625δ (b,s) corresponding to CH_2OH, CHOH, CHOMe and CH_2OMe (20H in all). The mass spectrum quite expectedly did not furnish a parent peak. The profile of the spectrum in addition to the mass peaks at m/e 43, 57, 85, 99, 113 ... 365 strongly suggested the presence of a long hydrocarbon chain of as many as 28 carbons. Support for this observation was further provided by abundant loss of 28 mass units 364, 336, 308 ... .

Band II (Rf 0.600) had a shoulder at 275 nm and a peak at 228 nm in its ultraviolet spectrum, thereby suggesting the presence of a

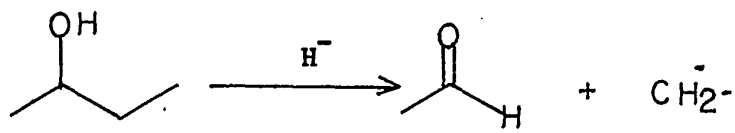
butadiene moiety. The infrared spectrum depicted the presence of methoxy (2800, 1250 and 1090 cm^{-1}) groups. The nmr spectrum had signals at 1.25δ (C, 3H), 1.70δ (C, 7H), 2.00δ (C, 6H) and 3.60δ (C, 24H), corresponding respectively to methyl on a carbon bearing oxygen, alicyclic methylene or methylene next to a carbinol group, methyl on an olefin and methylene, methine and methyls attached to oxygen. The 70 and 25 EV mass spectra had a base peak at m/e 87 (most probably, C_4H_8O Me). A parent ion could not be observed. Peaks at m/e 149, 163 and 122, which were intensified at low electron voltage, suggested that they arose by a primary process.

Comparison of the intensity ratios of C-O-C and C-H stretching frequencies for band II and I clearly indicated a higher order of methylation in band II

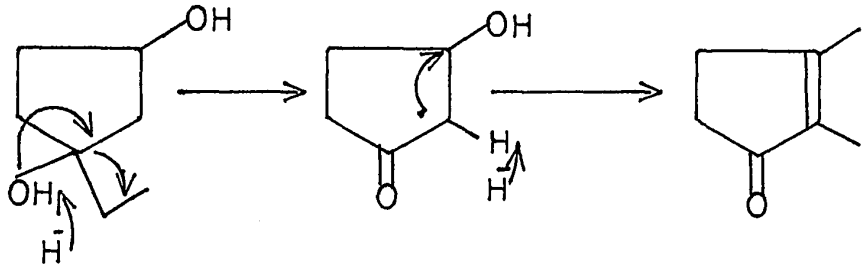
$$\text{I} \quad \frac{\text{C-O-C}}{\text{C-H}} = 0.93 \qquad \text{II} \quad \frac{\text{C-O-C}}{\text{C-H}} = 1.28$$

Band II (Rf 0.511) had ultraviolet maxima at 275 and 228 nm. The ir spectrum in chloroform depicted the presence of a cyclic enone (1750(w), and 1710(s) cm^{-1}). NMR and mass spectra of band III did not prove useful.

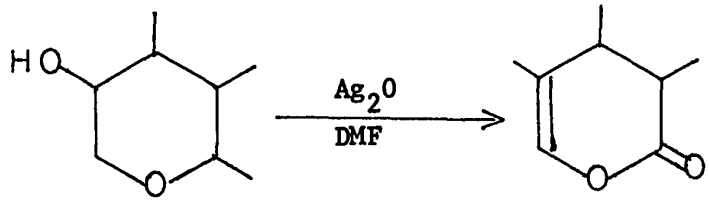
The presence of an oxo function in all three products strongly suggested a retroaldol reaction of the type.



The cyclic enone is generated along the same lines.



b. Methylation of palytoxin with methyl iodide and silver oxide in dimethylformamide was accompanied with oxidation to a lactone as evidenced by the infrared spectrum of the product (1780, 1740 and 1270 cm^{-1}).



The infrared spectrum also indicated the presence of an amide or olefin (1670 cm^{-1}), ether (1110 cm^{-1}) and methyl (1380 cm^{-1}) groups.

It has become apparent that we have succeeded in preparing a number of palytoxin derivatives. Sadly, however, derivatization has failed to provide significant structual information, primarily because of the large size of the toxin. Furthermore, all attempts to crystallize these derivatives have failed.

In a positive sense, derivatization has furnished a more accurate elemental analysis of the toxin and has established beyond any doubt the presence of at least 24 hydroxyls (acetate 24, 4-bromobenzoate 20 for 4N) in palytoxin.

Reproducibility of all reactions has further substantiated the homogeneity of palytoxin.

E. Hydrolytic Studies on Palytoxin

Since the presence of a sugar or a sugar-like constituent in palytoxin had already been indicated, hydrolytic attempts aimed towards the identification and quantitative assay of the presumed sugar moiety were made.

Preliminary studies indicated that the kinetics of disappearance of the 263 nm UV maximum in both 0.20 N hydrochloric acid and 0.04N sodium hydroxide (Fig. 6) were first order and also indicated a half life of 77.5 and 75 minutes respectively.

1. Hydrolysis of Palytoxin by 5N Hydrochoric Acid

Hydrolysis of palytoxin with 5N hydrochoric acid resulted in a brownish black precipitation which was insoluble in most organic solvents. The supernatant on paper and thin layer chromatography indicated the presence of D-ribose (see Table XI). Unfortunately, however, the small quantity of D-ribose and in particular the destruction of D-ribose under the experimental conditions defied all attempts aimed towards quantitative determination. Hydrogenolytic experiments using sodium and liquid ammonia have also been inconclusive (45a).

2. Hydrolysis of Palytoxin with 2N followed by 4N Hydrochloric Acid

Hydrolysis of palytoxin by 2N followed by 4N hydrochloric acid resulted in more than four products. The mixture was not separated.

3. Hydrolysis of Palytoxin by 3N Hydrochloric Acid

Hydrolysis of palytoxin by 3N hydrochoric acid, followed by purification by paper chromatography furnished three spots with Rf's 0.000, 0.350, and 0.72. The three components had almost identical infrared spectra and depicted the presence of hydroxyl (3350 and 1060 cm^{-1}), an amide or tetrasubstituted olefin (1656 cm^{-1}).

4. Methanolysis of Palytoxin

Methanolysis of palytoxin in methanolic hydrogen chloride followed by acetylation resulted in three fragments with Rf 0.700, 0.616 and 0.000.

Compound I (Rf 0.700) had ultraviolet maxima at 290 nm and 245 nm representing most probably an oxo function conjugated with butadiene. The infrared spectrum indicated the presence of hydroxyl (3400 and 1050 cm^{-1}), acetate and oxo (1740 cm^{-1}), amide or tetra-substituted olefin (1670 cm^{-1}) and methyl (1370 cm^{-1}). The nmr spectrum exhibited four sets of signals corresponding to aliphatic proton, (0.85δ (s), 1.22δ (s,b)) acetate units (2.22 and 2.40δ), methylenes and methines attached to oxygen (3.50 and 5.5δ) and olefin (5.80 and 6.30δ). A 70 EV mass spectrum had a base peak at m/e 43 (100%) and abundant loss of 28 mass units: m/e 486, 458, 430, 402 ... .

Compound II (Rf 0.616) had an ultraviolet maximum at 290 nm representing most probably a conjugated enone. The infrared spectrum depicted hydroxyl (3400, 1050 cm^{-1}) acetate (1740 and 1250 cm^{-1}), olefin (1630 cm^{-1}) and a conjugated ketone (1685 cm^{-1}). The nmr spectrum depicted a number of broad bands from 3.0 to 5.2δ and a very small aliphatic portion. Although the small quantity of compound at

hand precluded determination of a meaningful nmr spectrum, the nmr profile suggested a hexose bearing an aliphatic portion. This observation was further supported by the intensity ratios of >C=O and - C-H stretching signals in the three bands (Band I, II and III 1.455, 2.86 and 1.32 respectively). The intensity ratio of C-H and O-C-H nmr signals for the three bands also suggested the presence of far more hydroxyl functions in band II (C-H/O-CH, 2.40, 1.0 and 1.55).

Band III had only end absorption in the ultraviolet spectrum. The infrared spectrum was very similar to that of band II. The nmr spectrum indicated the presence of a large aliphatic portion (0.83, 1.10 and 1.22δ S), small acetate (2.0δ, S) and very small methylene and methines attached to the acetates (4.0δ, b). The mass spectra of bands II and III were inconclusive. On the basis of the above information, it can therefore be said that methanolysis of palytoxin resulted in three compounds, band I arising by simple dehydration of palytoxin, bands II and III both by methanolysis of palytoxin.

5. Photocatalyzed Hydrolysis of Palytoxin

Photocatalyzed hydrolysis of palytoxin in 0.2N hydrochloric acid resulted in the conversion of more than 80% toxin to a fibrous white solid which could not be dissolved in most organic solvents, and had an infrared spectrum similar to that of the toxin. The supernatant on paper chromatography indicated the presence of four components. Two of the compounds Rf 0.28 and 0.094 were positive to ninhydrin spray indicating thereby the presence of a primary amine group. One of the components was positive to dinitrophenylhydrazine, indicating the presence of an oxo function. The formation of a fibrous white solid

suggested that photopolymerization had taken place.

6. Hydrolysis of Palytoxin with 50% acetic acid.

Hydrolysis of Palytoxin with 50% acetic acid, followed by purification of the reaction mixture by paper chromatography furnished one component (Rf 0.307). It had ultraviolet maxima at 275 and 225 nm. Acetylation in pyridine provided an acetate ($C_{45}H_{66}NO_{22}$, octaacetate) which compared very well with palytoxin acetate ($C_{42}H_{62}NO_{19}$, hexaacetate).

7. Attempted Hydrolysis of Palytoxin by 50% Formic Acid.

Hydrolysis of Palytoxin with 50% formic acid provided three spots which could not be separated and examined for their physical characteristics.

8. Hydrolysis of Palytoxin by Sodium Methoxide in Dimethylsulfoxide

Palytoxin could be hydrolyzed by sodium methoxide in dimethylsulfoxide to a fragment which had ultraviolet maxima at 310, 267 and 228 nm. The infrared spectrum depicted hydroxyl (3400, 1075 cm^{-1}) and resembled closely the infrared spectrum of palytoxin. It appeared that palytoxin had only been slightly modified by the reaction.

9. Hydrolysis of Palytoxin by Sodium Hydroxide

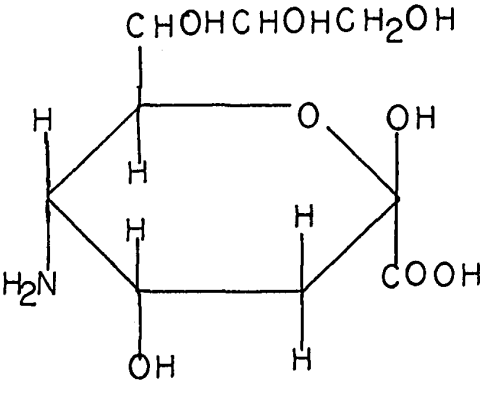
Hydrolysis of palytoxin by sodium hydroxide followed by chromatography over a Sephadex G10 column (2.0x60 cm) furnished two fractions corresponding to elution volumes of 62.4 to 78.0 (A) and 83.2 to 104.0 ml (B). Part A on preparative paper chromatography furnished a white hygroscopic solid ($C_{35}H_{64}NO_{21}$), transparent in the ultraviolet. The compound did not have any significant infrared band. Acetylation of band A furnished an acetate corresponding to an empirical formula of

$C_{42}H_{58}NO_{19}$ (for 4 acetate units, parent $C_{34}H_{50}NO_{15}$). Band B furnished an acetate $C_{54}H_{63}NO_{27}$ (for 10 acetates, parent $C_{34}H_{43}NO_{17}$). Despite the observation that compound B is well separated from A in the column chromatogram, both A and B acetates have similar composition, thereby suggesting hydrolysis of palytoxin in two portions, almost similar in weight but differing in functional groups (hydroxyl or carboxyl) which are responsible for retardation of compound B on a Sephadex G-25 column.

10. Enzymic Hydrolysis of Palytoxin

Attempted hydrolysis of palytoxin by various enzymes, α, and β-glucosidases, β-glucuronidase, glutaminase, hyaluronidase, invertase, lipase (wheat germ), maltase and snail gut enzymes were unsuccessful (45b).

Contrary to our expectation hydrolytic experiments have added little to our knowledge. In general, strong acids (HCl, H_2SO_4, HCOOH) resulted in palymerization while weak acids (50% acetic acid) resulted only in minor modification of the toxin. Since essentially all types of glycosidic linkages are hydrolyzed under one or the other of our experimental conditions (51), the inability of the toxin to furnish smaller fragments, excludes the presence of a glycosidic linkage. It is very likely that sugar moieties in palytoxin if they are present are joined via a -C-C linkage as in neuraminic acid



Neuraminic Acid

Base hydrolysis of palytoxin has resulted in a fragment which is relatively higher in C to O ratio but is very similar to palytoxin in its physical characteristics (Band A). The reaction needs further attention.

F. Reductive Experiments

Reductive experiments were conducted in the hope to (a) hydrogenolyze palytoxin to relatively smaller pieces, (b) determine the molecular weight of palytoxin, and (c) make a more stable hydrogenated palytoxin. As of now we have failed in all of our objectives.

Reduction of palytoxin with sodium borohydride and lithium aluminum hydride proved inconclusive.

Quantitative hydrogenation of palytoxin was attempted over catalytic platinum, palladium on charcoal, palladium on strontium carbonate and ruthenium oxide. The results were found to be inconsistent (45b). With platinum as a catalyst, the apparent molecular weight varied anywhere from 233 to 600 for one double bond, whereas when palladium on strontium carbonate was used as a hydrogenation catalyst, the apparent molecular weights were found to be between 589 and 740 for every double bond. The apparent inconsistency in results is attributed to hydrogenolysis and partial hydrogenation.

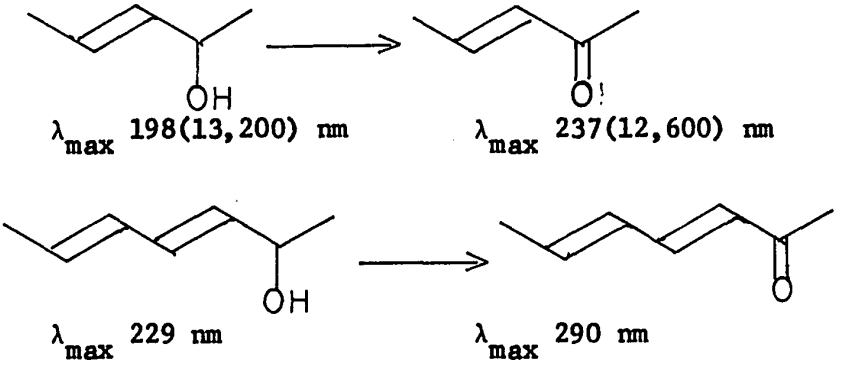
G. Oxidative Reactions

It should be recalled that reductive and hydrolytic experiments on palytoxin failed to provide smaller fragments. Oxidative reactions were therefore explored. A variety of oxidizing agents were selected and arranged in an ascending order of oxidative power. In the first

phase of the work, reaction conditions as mild as catalytic oxidation and oxidation by dimethyl sulfoxide in acetic anhydride were employed in the hope of obtaining an oxopalytoxin which could be converted to some crystalline derivative. In the second phase of the work, more vigorous oxidizing agents such as nitric and chromic acid were used. And in the final and most informative phase of the work, selective oxidizing agents for vicinal diols were employed.

1. Oxidation of Palytoxin by Active Manganese Dioxide

Active manganese dioxide oxidizes allylic alcohols to enones (51). It was hoped that if palytoxin contains one or more allylic alcohols, active manganese dioxide would oxidize it to an enone and the formation of an enone would be very easily detected by changes in the ultraviolet spectrum.



The final reaction product however had UV maxima at 263 and 233 nm with a ratio of 1.22 (palytoxin 0.580). The infrared spectrum and spot tests failed to indicate the presence of an oxo function.

2. Oxidation of Palytoxin by Fuming Nitric Acid

Palytoxin on oxidation with fuming nitric acid did not leave any residue.

3. Oxidation of Palytoxin by Molecular Oxygen

Molecular oxygen oxidizes primary alcohols to carboxylic acid and secondary alcohols to ketones in the presence of catalytic platinum (52). Palytoxin on oxidation with molecular oxygen at 65° at pH 9.2, followed by purification by column chromatography furnished a yellow granular solid. The solid was positive to bromocresol green, benzidine-periodate and DNP reagents indicating thereby the presence of keto and carboxylic acid functions. This observation was further substantiated by the infrared spectrum (1720 cm^{-1}, b). The spectrum also indicated the presence of hydroxyls (3350, 1100 cm^{-1}). The nmr spectrum depicted only broad bands. Potentiometric titrations did not reveal the presence of a titrable group; however, on allowing the sample to sit at pH 12.0 for several hours a group representing only a small quantity of the sample or indicating a huge molecular weight was generated. Attempted formation of a β-napthylhydrazone and a dinitrophenylhydrazone resulted in more than six compounds in each case. The catalytically oxidized palytoxin on reaction with acetic anhydride in pyridine furnished an acetate which had mass peaks as high as m/e 750. The mass spectral profile clearly suggested the presence of a long hydrocarbon chain.

A similar oxidation of palytoxin at pH 7.0 resulted only in minor modification of the toxin. The product on examination by infrared spectroscopy and spot tests did not show the presence of oxo or carboxylic acid groups.

4. Oxidation of Palytoxin by Concentrated Nitric Acid

Oxidation of palytoxin by concentrated nitric acid followed by work-up in the usual fashion provided an aqueous potion and an ether extract. The aqueous portion after purification over a Sephadex G25 column followed by preparative paper chromatography furnished a pale solid, positive to silver nitrate and permagnate spray reagents. The infrared spectrum indicated the presence of hydroxyl and water of hydration (3400 cm^{-1}) and a hydrogen bonded oxo function (1640 cm^{-1}). The nmr spectrum in deuterium oxide was devoid of proton signals. The compound analyzed for an empirical formula of $C_4H_6O_9$ which corresponds to a hemihydrate of oxalic acid $[(COOH)_2]_2\ H_2O$.

The infrared spectrum of the ether extract clearly indicated the presence of a carboxylic acid (1700 cm^{-1}). A GLC of the methyl esters of the ether extract suggested the presence of four compounds. Due to small amount of the material no further work could be done.

5. Kuhn Roth Oxidation of Palytoxin

Palytoxin was subjected to a modified Kuhn Roth oxidation (53). Analytical GLC of the esterified reaction mixture indicated the presence of 1,5-pentanedicarboxylic acid (pimelic acid), 1-decanoic acid, octane 1,8-dicarboxylic acid (sebacic acid) and 1-decanoic acid.

6. Oxidation of Palytoxin by Dimethylsulfoxide in Acetic Anhydride

Dimethyl sulfoxide in acetic anhydride oxidizes secondary alcohols to ketones. The reaction has recently been employed for making keto carbohydrates (76).

Palytoxin when subjected to dimethylsulfoxide-acetic anhydride oxidation furnished a chloroform soluble pale solid containing an oxo function as indicated by an infrared band (1720 cm^{-1}) and a positive DNP test. However, all attempts to prepare a crystalline dinitrophenylhydrazone failed.

7. Potassium Permagnate Oxidation of Palytoxin

Potassium permagnate oxidized palytoxin to a great number of products as indicated by paper chromatography.

No further attempts were made to pursue this mode of oxidation.

8. Sodium Metaperiodate Oxidation of Palytoxin

Sodium metaperiodate oxidation of palytoxin exhibited first order kinetics and a half life of about one minute (Fig. 11). Consumption of 13 μmoles of periodate was observed for one milligram of toxin. Therefore 2538 g (ca. 1 mole) of toxin would consume 33 moles of periodate. A minimum of 34 vicinal hydroxyls or their equivalent is suggested for palytoxin.

It was noted that no volatile aldehydes were generated during oxidation. Furthermore, simple titration experiments revealed the production of 8μ-mole equivalents acid for 1 mg or 20 moles of acid for 2500 g (ca. 1 mole) of toxin (44). This strongly indicates the presence of a minimum of 20 vicinal hydroxyls in one sequence or 22 vicinal hydroxyls in two sequences.

Infrared and mass spectra of the sodium salt of the acid(s) produced during oxidation proved the presence of sodium formate (44).

9. Large scale Sodium Metaperiodate Oxidation of Palytoxin

Large scale sodium metaperiodate oxidation of palytoxin at 0°, followed by extraction with chloroform furnished a chloroform extract and an aqueous portion. The chloroform extract after reduction with sodium borohydride was acetylated to furnish an acetate mixture. Fractionation of the acetate mixture over silica gel H provided five compounds which were designated C-107-A, C-107-C, C-104-d, C-109-A, and C-109-B. Homogeneity of these compounds was established by analytical thin layer chromatography.

The aqueous portion after freeing of excess periodate by ethylene glycol was extracted with 1-butanol. The butanol extract on reduction with sodium borohydride followed by acetylation furnished an acetate mixture, which could be fractionated into compounds C-116-C(A), C-116-D(A) and C-116-E(A) on thin layer chromatography. The homogeneity of the compounds was established as usual by thin layer chromatography.

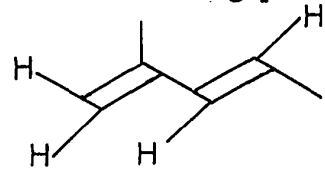
a. Compound C-107-A

Compound C-107-A is a mobile colorless oil which turns yellow on prolonged exposure to air and emits a strong sky blue fluorescence on silica gel HF. The homogeneity of the compound was indicated by chromatographic behavior. GLC of C-107-A over an OV 1, 3% on chromosorb W column (1/8" x 6') indicated the presence of essentially one compound (Rt 11' 24").

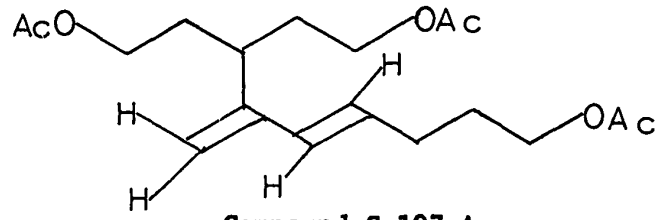
Compound C-107-A was negative to DNP spray reagent thereby suggesting the absence of an oxo function. The infrared spectrum of the compound depicted the presence of terminal methylene (900 cm^{-1} and 1640 cm^{-1}, b), methyl (1370 cm^{-1}) and acetate (1723 cm^{-1})

groups. The sharpness of the IR signal at 1723 cm^{-1} suggested the presence of only one kind of acetate. The nuclear magnetic resonance spectrum in deuterochloroform depicted the presence of methylenes and methines β to oxygen (1.64 and 1.75δ, 7H), acetate (2.03δ, 9H), allylic methylene and methine (2.51δ, cq, 2-3H), primary acetate methylene (4.01δ two or more superimposed triplets, 6H), terminal methylene (4.92 and 5.00δ, s, 1H each) and olefinic protons (5.50δ, C, 1H and 5.87δ, d, J=13 cps, 1H). The nuclear magnetic resonance spectrum depicted 28 protons in all.

Double resonance experiments indicated that the sextet at 4.10δ collapsed to a triplet (superimposed singlet) on irradiation of a signal at 1.75δ, thereby suggesting the presence of at least two kinds of primary acetate methylene. It was also indicated that the signal around 5.90δ are independent of the signals at 4.92δ and are only weakly coupled to the quartet at 2.51δ (J=1.1 Hz). These observations could be translated into the following partial structure.

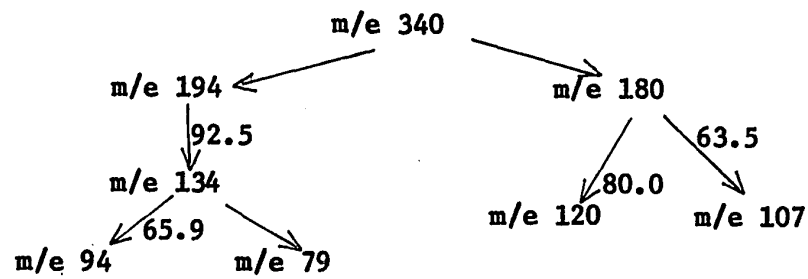


The ultraviolet maximum at 226.9 nm (calcd for a doubly substituted butadiene, 227 nm) confirmed the presence of a doubly substituted butadiene. The absence of secondary acetate methine suggested the presence of only primary acetates. The following structure could therefore be written on the basis of 28 protons and an interpretation of nmr signals.



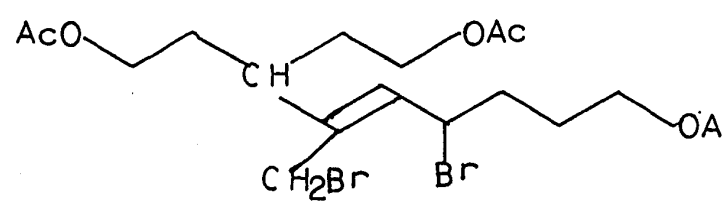
Compound C-107-A

The mass spectrum of compound C-107-A did not depict a parent ion. The largest ion at m/e 194 probably arose by a loss of $C_3H_5O_2$ and $C_2H_3O_2$ units. The next largest ion at m/e 180 arose by a loss of two $C_3H_5O_2$ units. The ion at m/e 194 and 180 both lost 60 ($C_2H_4O_2$) mass units to give m/e 134, and 120. The ion m/e 180 furnished m/e 107 by a loss of 73 ($C_3H_5O_2$) mass units



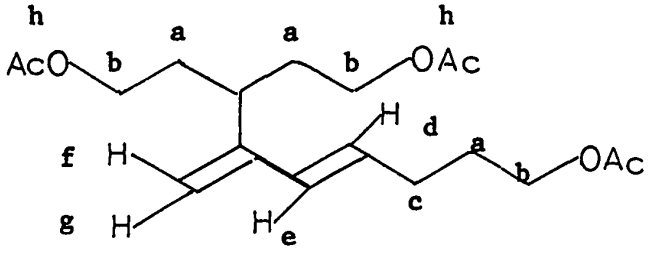
Bromination of C-107-A furnished two compounds, A and B.

Compound A did not furnish a parent ion in its mass spectrum. If it is assumed that compound A is a dibromo derivative of C-107-A, the highest mass peak at m/e 401-397 probably arose by a loss of 101 ($C_5H_9O_2$) mass units. The following structure consistent with the mass spectral fragmentation can be written



A single attempt to make Diels Alder adduct of C-107-A with maleic anhydride in toluene failed.

Complete assignments of nmr signals for compound C-107-A is as follows



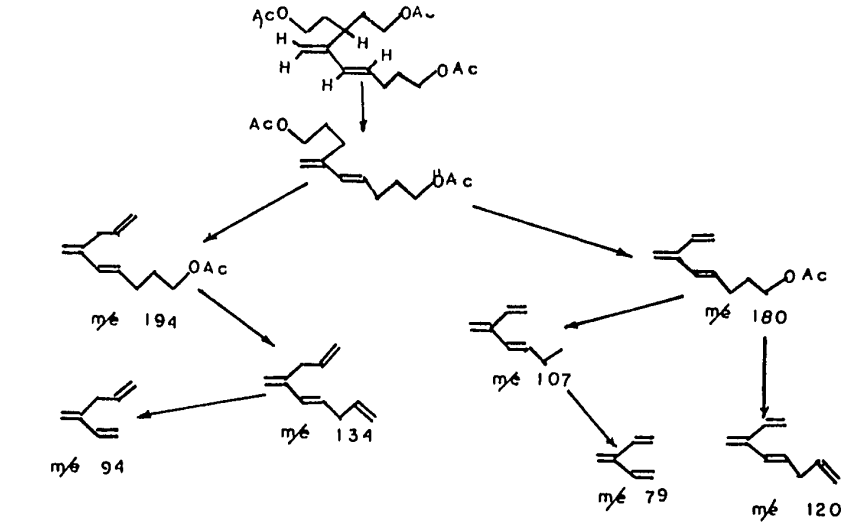
a	1.64δ and 1.74δ	f	4.98δ	
b	4.02δ	g	4.84δ	
c	2.52δ	h	2.00δ	
d	5.50δ		1.00δ	impurity
e	5.84δ			

The mass spectral fragmentation of compounds C-107-A and dibromo C-107-A are presented on page 216.

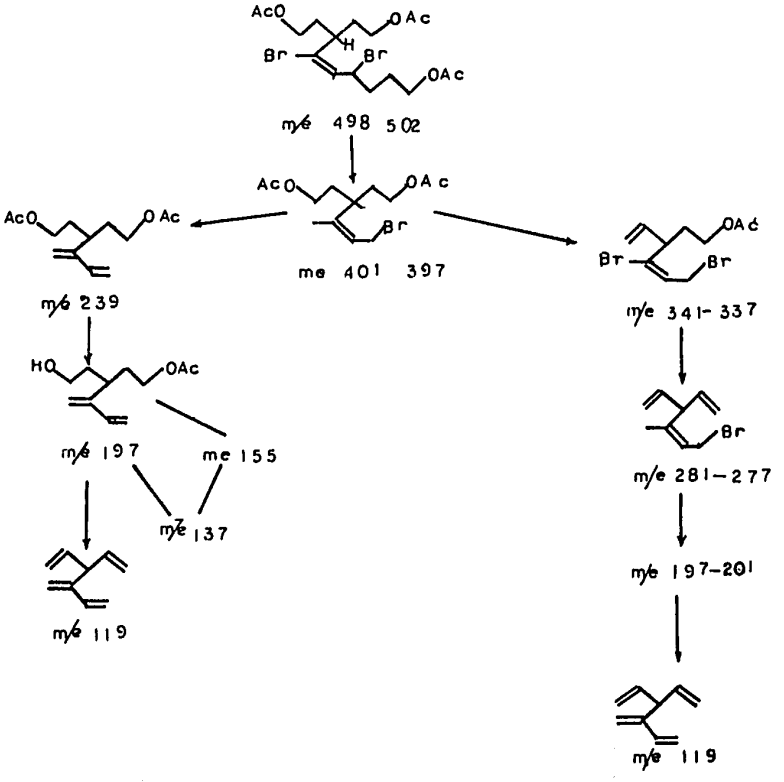
b. Compound C-107-C

Compound C-107-C was a non-fluorescent crystalline solid, mp 71-75°, transparent in the ultraviolet. The compound was negative to DNP spray and tetranitromethane and bromine tests indicating thereby the absence of keto and olefin functions. Absence of an oxo stretching

Mass spectral fragmentation for compounds C-107-A and dibromo C-1070A is outlined as follows.



DIBROMO C-107-A



band in saponified C-107-C confirmed the observation. Saponified C-107-C showed the presence of hydroxyl (3350, 1050 cm^{-1}), methyl and isopropyl (1350 and 1375 cm^{-1}) groups.

The infrared spectrum of C-107-C depicted the presence of hydroxyl (3250, 1070 cm^{-1}) and acetate (1730, 1240 cm^{-1}). Nuclear magnetic resonance spectra in deuterochloroform, pyridine-d_5 and benzene-d_6 (see Table XVIII) proved quite useful. The spectrum in deuterochloroform depicted a minimum of four methyl groups (0.87, 0.94, 1.19, and 125δ S, 3H each). The spectrum in deuteropyridine resolved the first two peaks to four sharp singlets (0.82, 0.88, 0.96 and 1.02δ). The nmr spectrum in deuterobenzene, wherein the signals moved to new positions (0.80, 0.86, and 1.00 and 1.06δ 6H) confirmed that the four singlets in the pyridine-d_5 spectrum constituted a pair of doublets. It also indicated that the signals either represent an isopropyl or two secondary methyls (54). The fragmentation m/e 298 ($C_{18}H_{34}O_3$) $-C_3H_7$ m/e 255 ($C_{15}H_{27}O_3$) clearly indicated the presence of an isopropyl group. The 1.190δ (3H) singlet underwent a solvent shift of +1.2 Hz in deuteropyridine (55) and -8.0 Hz in deuterobenzene (57) thereby suggesting its quaternary nature. The 1.250δ singlet coalesced with the broad singlet at 1.30δ in pyridine-d_5 and benzene-d_6. The nuclear magnetic resonance spectrum in pyridine-d_5 depicted the presence of aliphatic methylene and methyl (1.30δ, 16-18H) and a combination of alicyclic methylene and methylene β to hydroxyl oxygen (1.60δ, 14H). The same chemical shift for the latter signal in pyridine-d_5 and deuterochloroform (1.60δ) and relative higher integration ratio in deuteropyridine (57) suggested the presence of either a five or six

membered ring. The nmr spectrum in deuterochloroform depicted the presence of three acetates (2.02 and 2.07δ, 9H). The acetate signals underwent a relative solvent shift of 0.10 to 0.15δ in pyridine-d_5 and 0.30δ in benezene-d_6. Methylenes and methines attached to hydroxyl and acetate units appeared as complex signals (58) (4.0δ, 6-7H, 5.00δ, 1H) in deuterochloroform; however, the 4.0δ signal could easily be resolved to a triplet superimposed on a doublet (4.07δ, 4H J=6Hz) and a triplet (4.35δ, J=5.5 Hz 2H) corresponding to two primary acetate methylenes and two secondary alcohol methines. The broad methine acetate signal and hydroxy protons are buried under the water signal at 5.0δ.

Elemental analysis provided an empirical formula of $C_{34}H_{60}O_9$ which was confirmed by high resolution measurements (parent m/e 612 and m/e 539; $C_{31}H_{55}O_7$). Since compound C-107-C has three acetates and no double bond, the presence of two rings is suggested. If two of the remaining oxygens are hydroxyls as is suggested by the following mass spectral transformations, m/e $612 \xrightarrow{-73}$ m/e $539 \xrightarrow{-18}$ m/e $521 \xrightarrow{-18}$ m/e 503, the third is present as a cyclic ether (tetrahydrofuran and tetrahydropyran). The other ring could eitehr be a cyclohexane (1.2-cis disubstituted) (54) or cyclopentane. The intensity of the broad nmr signal (1.60δ, 14H) in pyridine-d_5 suggested the presence of three cyclic methylenes or a cyclopentane ring

$-(CH_2)_3-$	6 H
$-CH_2-CHOH-CH_2$	4 H
$-CH_2$ CHOH-	2 H
CH_2-C-O	2 H
Total	14 H

Compound C-107-C had a parent peak at m/e 612 and a base peak at m/e 298. The mass spectrum also depicted transformations arising by a loss of acetic acid, ketene and water molecules. All peaks above m/e 398 were less than 2% of the base peak. Peaks at m/e 582, 552, 539, 497, 479, 453, 357, 315, 298, and 255 increased in relative size at low electron voltage, thereby indicating their origin by primary processes. Compound C-107-C furnished fragment ions m/e 597, 594, 582, 552, and 539 by a respective loss of 15 (CH_3), 18 (H_2O), 30(CH_2O), 60($C_2H_4O_2$) and 73($C_3H_5O_2$) mass units. The ion m/e 539 further degraded to m/e 497, 479, 437, and 419 by a respective loss of 42 (C_2H_2O), 60($C_2H_4O_2$), 102($C_2H_4O_2$ + C_2H_2O)and 120 (2 x $C_2H_4O_2$) mass units. The parent ion directly fragmented to m/e 453 by a loss of 159($C_7H_{11}O_4$) mass units. The ion at m/e 298 arose from m/e 552 and 453 by a respective loss of 254 and 155 mass units.

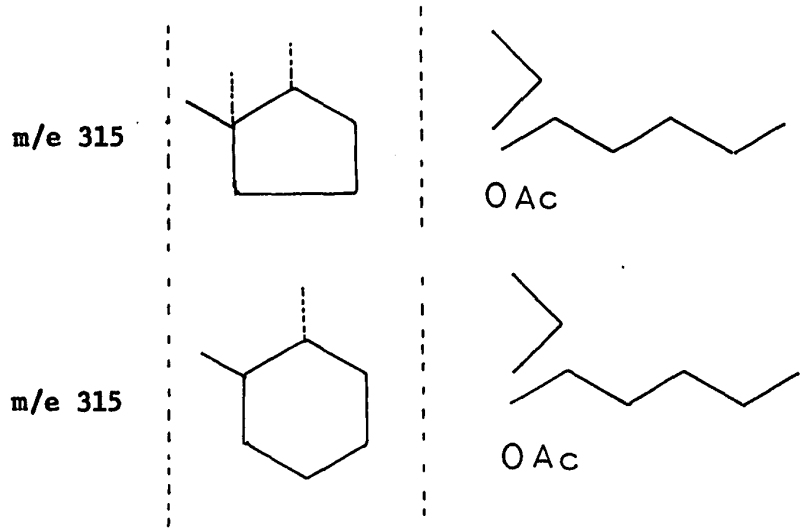
The presence of two ions at m/e 298($C_{18}H_{34}O_3$) and 315($C_{16}H_{27}O_6$) strongly suggested that the compound C-107-C is comprised of two portions. Furthermore the relative intensities of the two ions quite

expectedly indicated a greater charge retention on the less oxygenated fragment m/e 298.

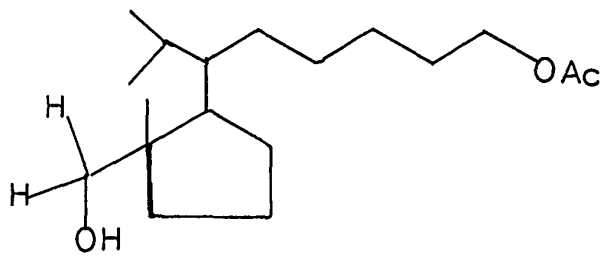
Fragment m/e 298. The ion m/e 298 further fragmented to m/e 280, 255 and 238 by a respective loss of 18(H_2O), 43(C_3H_7) and 60($C_2H_4O_2$) mass units. The ion m/e 255 furnished m/e 213, 195 by a loss of 42(C_2H_2O) and 60($C_2H_4O_2$) mass units. These observations clearly indicated that ion m/e 298 had one acetate, one hydroxyl and one isopropyl group situated in such a fashion that an isopropyl group was preferentially lost. The composition of the peak clearly indicated the presence of one element of unsaturation. The unsaturation could either represent a ring or a double bond generated during the fragmentation process. The ion m/e 298 by virtue of its intensity could hardly be a rearrangement peak, therefore, indicating the presence of a ring. Support for this conclusion was gathered by the absence of allylic cleavage and rearrangement ions, from m/e 298.

The ion m/e 582 ($C_{33}H_{58}O_8$) fragmented to furnish m/e 315 ($C_{16}H_{27}O_6$) and m/e 398 ($C_{22}H_{38}O_6$) respectively. Comparison of the two ions strongly suggested that the additional C_6H_{11} portion in m/e 398 is derived from m/e 298. It can therefore be concluded that fragment m/e 315 is attached to a cyclohexyl or methyl cyclopentyl moiety directly. (see top of page 221)

The facile removal of an isopropyl group from m/e 298 required that the isopropyl group is either attached to a carbinol or carbinol acetate (so that it may be removed by α cleavage) or be so situated that hydroxyl oxygen could eliminate it by a cyclic mechanism (60). The chemical shift of the isopropyl and lack of an appreciable

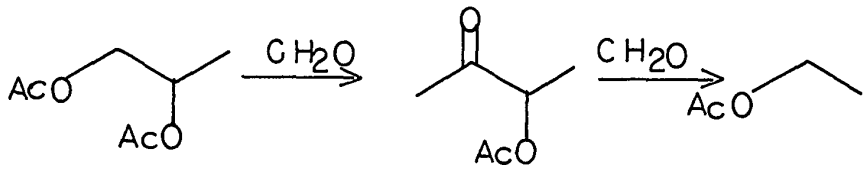


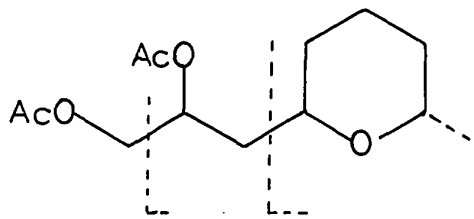
solvent shift in pyridine excluded the former possibility. We can therefore write the following structure for fragment m/e 298



It has already been mentioned that C-107-C furnished m/e 539 ($C_{31}H_{55}O_7$) and 453 ($C_{27}H_{49}O_5$) by elimination of $C_3H_5O_2$ and $C_6H_{11}O_4$ units. This information in conjunction with the generation of m/e 582 from the parent ion by loss of 30 mass units, indicated the presence of $C_6H_{11}O_4$ having a vicinal diol diacetate attached to tetrahydropyran. (see top of page 222)

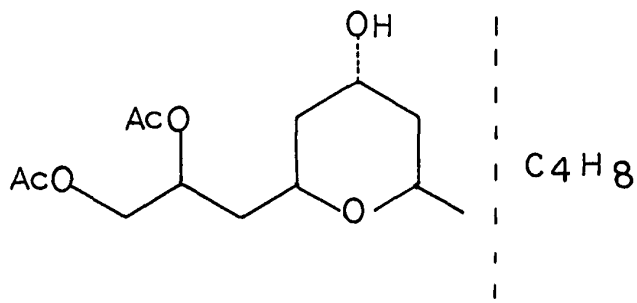
The presence of vicinal diol diacetate in compound C-107-C was confirmed by a positive benzidine-periodate test.





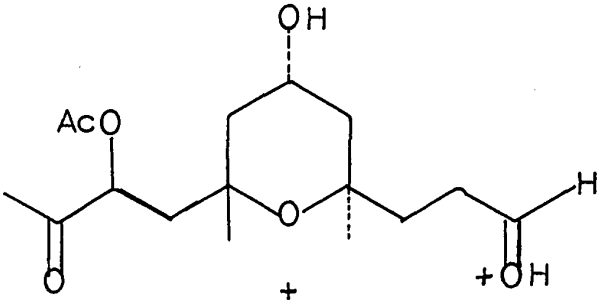
Since the singlet at 1.25δ underwent only a very slight solvent shift (5 Hz) in deuteropyridine it could be at C-1 or C-5 position of tetrahydropyran.

It was mentioned that compound C-107-C depicted a triplet at 4.35δ (J=5.5Hz, 2H). A large solvent shift of 0.20δ indicated that the signal corresponded to two secondary alcohol methines having adjacent methylenes. If it is assumed that one hydroxyl is at C-3 of tetrahydropyran, a partial structure can be written

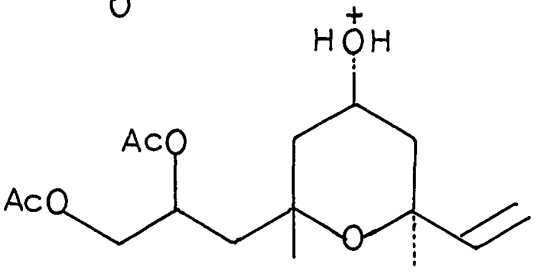


The reason for placing a hydroxyl group at C-3 of tetrahydropyran will become evident, while considering the related compound C-104-d from the periodate oxidation and also on biogenetic grounds.

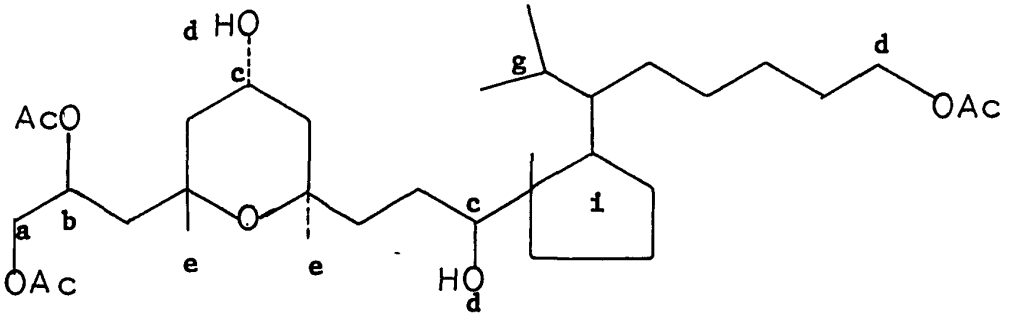
The ion m/e 315 may be represented by



or



Hence we write the following structure for compound C-107-C, along with an interpretation of its nmr signals.



a	4.1δ		f	1.30δ
b	5.0δ		g	0.98δ
c	4.1δ	4.35δ pyridine	i	1.60δ
d	2.50δ			
e	1.25δ			

The fragmentation pattern was consistent with the proposed structure and is presented on page 225.

c. Saponification of Compound C-107-C

Saponification of compound C-107-C with alcoholic sodium hydroxide furnished a crystalline white solid mp 79-82°. The compound was transparent in the ultraviolet. Its infrared spectrum indicated the presence of hydroxyl (3350, 1060 and 1130 cm^{-1}). The nuclear magnetic resonance spectrum in deuterochloroform exhibited the presence of an isopropyl (0.91δ d, J=6.0Hz, 6H), a quaternary methyl (1.190δ, s, 3H), aliphatic methylene (1.290δ, s,b) and methylene and methines attached to hydroxyl groups (3.56δ, c, and 3.84δ, c, 7H).

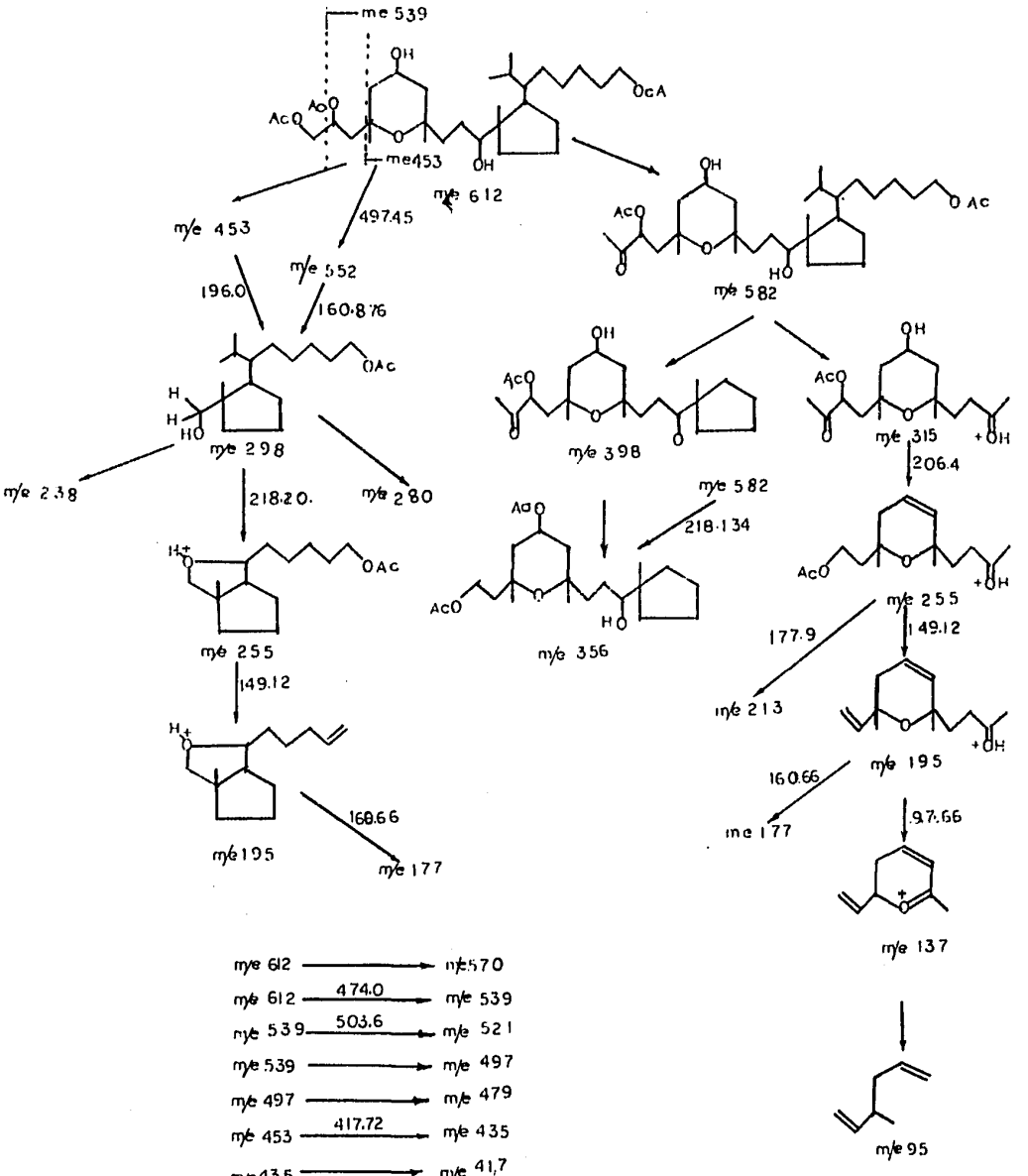
Bromobenzoylation of saponified C-107-C in pyridine furnished three bromobenzoates designated as band I, band II and band III. Infrared bands at 1580, 1450 and 845 cm^{-1} and UV maxima at 245 nm confirmed the incorporation of bromobenzoate groups. Mass spectra of band I exhibited strong peaks at m/e 440 and 438 corresponding to the m/e 298 fragment in the acetate. The ion m/e 440 and 438 fragmented to m/e 397 and 395 by loss of 43 mass units (C_3H_7), and m/e 250 by a loss of 4-bromobenzoyl cation. These observations clearly confirmed the presence of a hydrocarbon (corresponding to m/e 298) portion and a highly oxygenated portion.

Bands II and III depicted peaks at m/e 352, 324, 256, 240, 202, 200 and 185 and 183. No conclusion other than those already drawn could be arrived at.

d. Compound C-104-d

Compound C-104-d was a non-fluorescent colorless liquid, transparent in the ultraviolet. The compound was negative to DNP spray,

Mass spectral fragmentation pattern of the compound C-107-C is shown in the following schemes.



tetranitromethane and bromine tests, thereby indicating the absence of oxo and olefin functions. The infrared spectrum depicted the presence of acetate (1730, 1275 cm^{-1}) and hydroxyl (3450, 1050 and 1125 cm^{-1}) groups. Its nmr spectrum in deuterochloroform depicted the presence of an isopropyl (0.93δ, d, J=5.5Hz) a methyl (1.21δ, s, 3H) methylene and methyls (1.32δ, b, s), cyclic methylene (1.63δ, s, 6-10H), alicyclic methylenes (1.65δ, b), acetate methyls (2.07δ, s, c, 9H), hydroxyl (2.18δ 1-2H), methylene and methines attached to acetate or hydroxyl groups (4.0δ, c, 7-8H, 5.0δ, b, 1H). The isopropyl group could be resolved to four singlets in pyridine-d_5 but the solvent shift (4 Hz) clearly suggested that the isopropyl group could not possibly be attached to a carbinol or carbinol acetate function. The methyl singlet at 1.21δ did not exhibit any solvent shift. The broad signal at 1.32δ did not undergo a solvent shift, but an expanded spectrum portion depicted two spikes at 1.254δ and 1.300δ corresponding most probably to two methyl groups. The singlet at 1.63δ collapsed to a broad signal (1.63δ) and new peaks at 2.0δ. The region around 2.0δ in pyridine-d_5 depicted six peaks (1.94, 1.98, 1.99, 2.02, 2.04 and 2.07δ) and shoulders at 1.95, 1.99 and 2.005δ corresponding in all to ca 15H. Since the compound is a triacetate the additional 6H are a part of the 1.60δ singlet and have undergone a solvent shift of 0.30-0.4δ, characteristic of geminal or 1,3-diaxial deshielding (53).

First order analysis of the complex structure at ca 2.0δ in pyridine depicted the presence of two pairs of doublets at 1.960δ and 2.055δ (J=2.5Hz, 3H each), corresponding to three axial and three equatorial hydrogens, on the adjacent carbon (59) and three singlets

at 1.95, 1.99 and 2.04δ (9H), representing acetate methyls.

Methylenes and methines attached to acetate and hydroxyl groups could be resolved into a series of peaks around 4.0δ, consisting most probably of two pairs of triplets (3.88, 4.01δ, J=2.5Hz), one triplet (4.08δ, J=6.0Hz) and a doublet of doublet (4.16δ), and a broad signal at 5.0δ.

The mass spectrum of compound C-104-d depicted a parent peak at m/e 654 and a base peak m/e 298. High resolution measurements on m/e 654 revealed a composition of $C_{36}H_{62}O_{10}$. The ions at m/e 594, 581, 521, 440, 357, 315 and 255 increased in relative size at low electron voltage.

The parent ion m/e 654 fragmented to m/e 639, 639, 624, 612, 594, 581 by a respective loss of 15 (CH_3), 18(H_2O), 30(CH_2O), 42(C_2H_2O), 60($C_2H_4O_2$) and 73($C_3H_5O_2$) mass units. The presence of three acetates could be confirmed by the following sequential elimination

$$\text{m/e } 654 \xrightarrow{-60} \text{m/e } 594 \xrightarrow{-60} \text{m/e } 534 \xrightarrow{-60} \text{m/e } 474$$

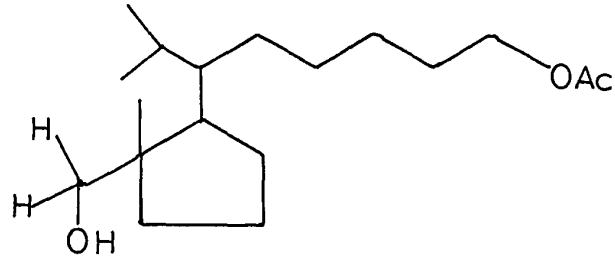
$$\text{m/e } 654 \xrightarrow{-73} \text{m/e } 581 \xrightarrow{-60} \text{m/e } 521 \xrightarrow{-60} \text{m/e } 461$$

The presence of ions m/e 624 and 581 arising by a loss of 30 and 73 mass units from the parent indicated the presence of a vicinal diol diacetate.

The ion m/e 612 further degraded to m/e 552, 492 and 474 by a respective loss of 60($C_2H_4O_2$), 120(2 x $C_2H_4O_2$) and 138 (2 x $C_2H_4O_2$ + H_2O) mass units. The ion m/e 624 ($C_{35}H_{60}O_9$) furnished m/e 440 ($C_{24}H_{40}O_7$) and m/e 357 ($C_{18}H_{24}O_7$), suggesting thereby that the

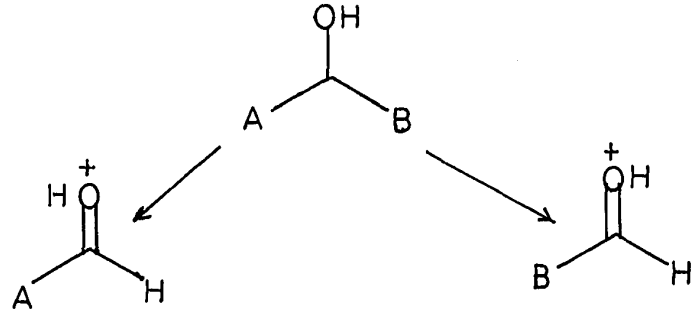
fragment m/e 357 is directly attached to a cyclohexyl or a methyl-cyclopentyl group.

The ion m/e 298 ($C_{18}H_{34}O_3$) arose from m/e 624 and m/e 594. The ion fragmented to m/e 280, 255, 238 by loss of 18(H_2O), 43(C_3H_7), and 60($C_2H_4O_2$) mass units. The ion behaved in all respects similar to the m/e 298 fragment in compound C-107-C. Therefore it could be assigned the following structure.



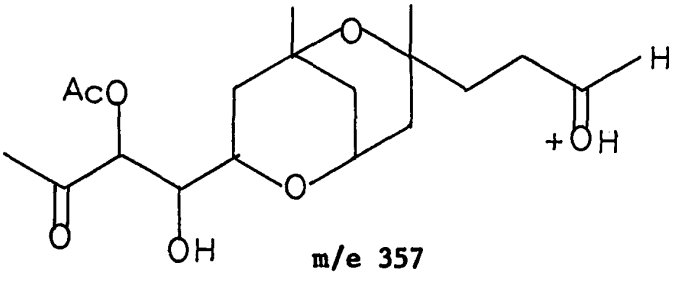
m/e 298

The ion m/e 357 ($C_{18}H_{29}O_7$) is most probably generated from m/e 624 ($C_{35}H_{60}O_9$). A comparison of the two fragments suggested that it is flanked by m/e 298 through a secondary carbinol which acts as a primary site of fragmentation

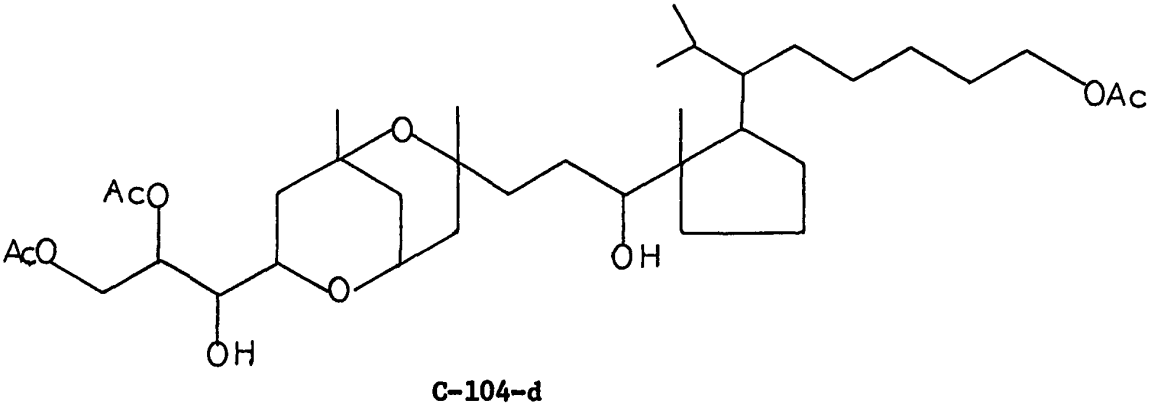


The ion m/e 357 furnished m/e 255 by loss of a mole each of acetic acid and ketene, and m/e 195 by loss of two moles of acetic acid and one mole of ketene, suggesting thereby the presence of a minimum of one acetate, one acetyl and one hydroxyl in the fragment. The ion m/e 195 ($C_{12}H_{19}O_2$) did not lose ketene or acetic acid indicating thereby the ethereal nature of the remaining two oxygens. Since the ions m/e 357, 297 and 255 depicted an unsaturation of two, the presence of two tetrahydropyran rings was not altogether speculative.

The mass spectral fragmentation of m/e 375 in conjunction with the nuclear magnetic resonance spectrum of C-104-d in pyridine-d_5 prompted us to write the following possible structures for the fragment m/e 357 and compound C-104-d

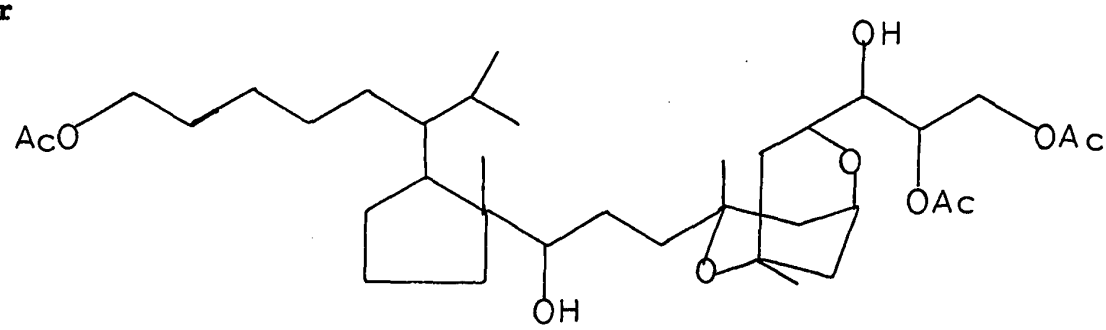


m/e 357

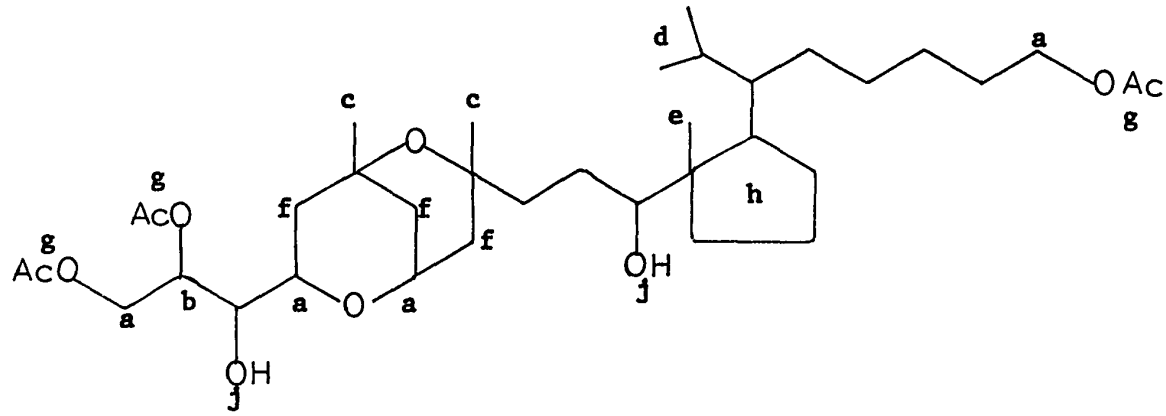


C-104-d

or



A complete nmr signal assignment is as follows,



a. 4.0δ (c, 7-8H)

b. 5.0δ (b, 1H)

c. 1.28-1.31δ (b, c)

d. 0.93δ (d, J 6 CPS)

e. 1.21δ (s)

f. 1.63δ (s) in pyridine 1.960, 2.05δ (d, J=2.5 Hz) 3H each

g. 2.07δ (s, 9H)

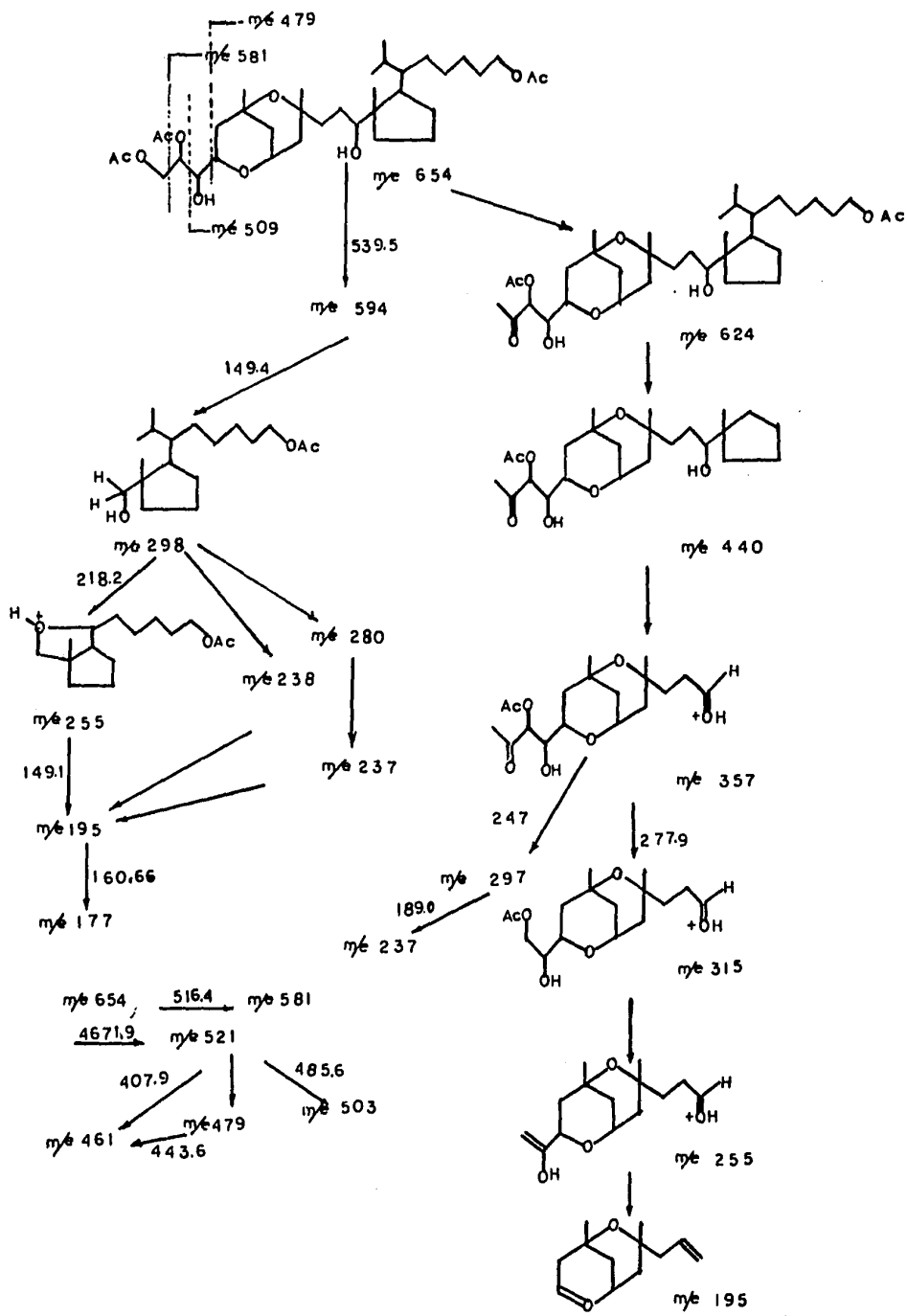
h. 1.65δ (b)

i. 1.32δ (b, s)

j. 2.07δ and 2.18 δ

and mass spectral fragmentation is presented on page 231.

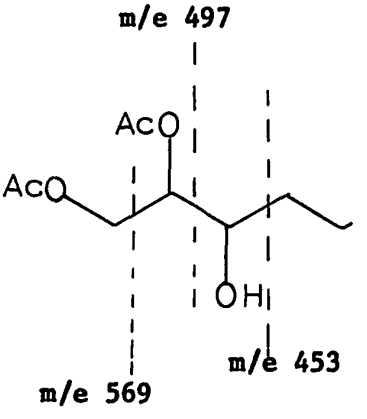
The following scheme details the mass spectral fragmentation pattern of compound C-104-d.



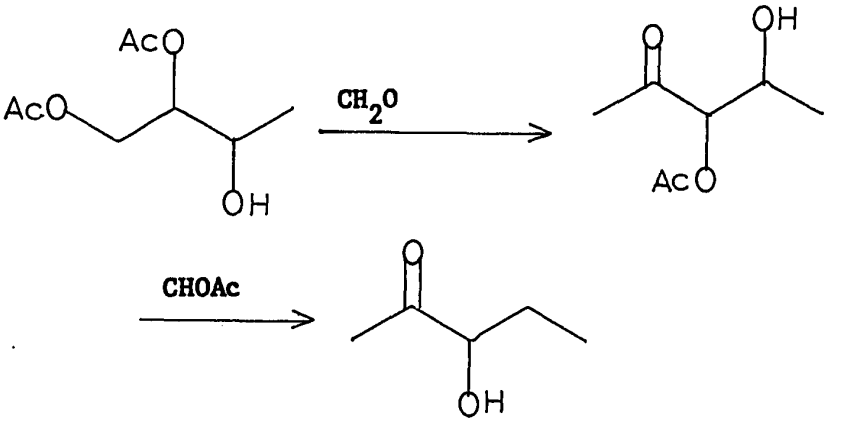
The symmetry of the two bridged tetrahydropyran rings is destroyed when the molecule makes a collision complex with pyridine-d_5 thereby resulting in a non-equivalence of axial and equatorial protons.

e. Compound C-109-A

Compound C-109-A was a colorless liquid, transparent in the ultraviolet. The compound was negative to DNP spray and tetranithromethane, but was positive to benzidine-periodate, indicating thereby the presence of vicinal diol and the absence of oxo and olefin functions. The infrared spectrum indicated the presence of hydroxyl (3400, 1050 cm^{-1}) and acetate (1730, 1240 cm^{-1}). The mass spectrum of the compound closely resembled the mass spectrum of C-107-C. The compound depicted a parent ion at m/e 642, 30 mass units higher than compound C-107-C. The chromatographic behavior of the compound indicated it to be more polar than C-107-C and less polar than compound C-109-B. The compound fragmented to ions m/e 569 and 612 by a respective loss of 73($C_3H_5O_2$) and 30(CH_2O) mass units, confirming thereby the presence of vicinal diol diacetate. The sequential loss of 73, 145 and 189 mass units resulting in ions m/e 569, 497 and 453 clearly suggested the presence of the following structural feature.

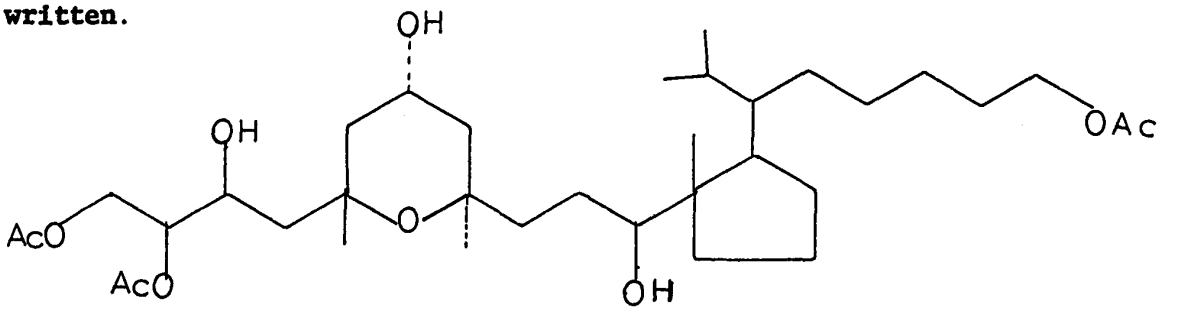


The fragment ion m/e 539 probably arose by a loss of 103 mass units from m/e 642 by the following process



The compound fragmented to major ions m/e 345, 327 and 298. The fragment m/e 345 arose from m/e 612 and further degraded to m/e 327 and 285 by a respective loss of 18 (H_2O) and 60 ($C_2H_4O_2$) mass units. The ion m/e 327 furnished m/e 267 by a loss of 60 ($C_2H_4O_2$) mass units, and m/e 225 by a loss of 102 ($C_2H_4O_2$ + C_2H_2O) mass units. The ion m/e 298 behaved identically to the ion m/e 298 in compound C-107-C.

A comparison of different ions (m/e 345 with m/e 315) with those in C-107-C clearly indicated that they differ by 30(CH_2O) mass units. Therefore the following structure for compound C-109-A may be written.



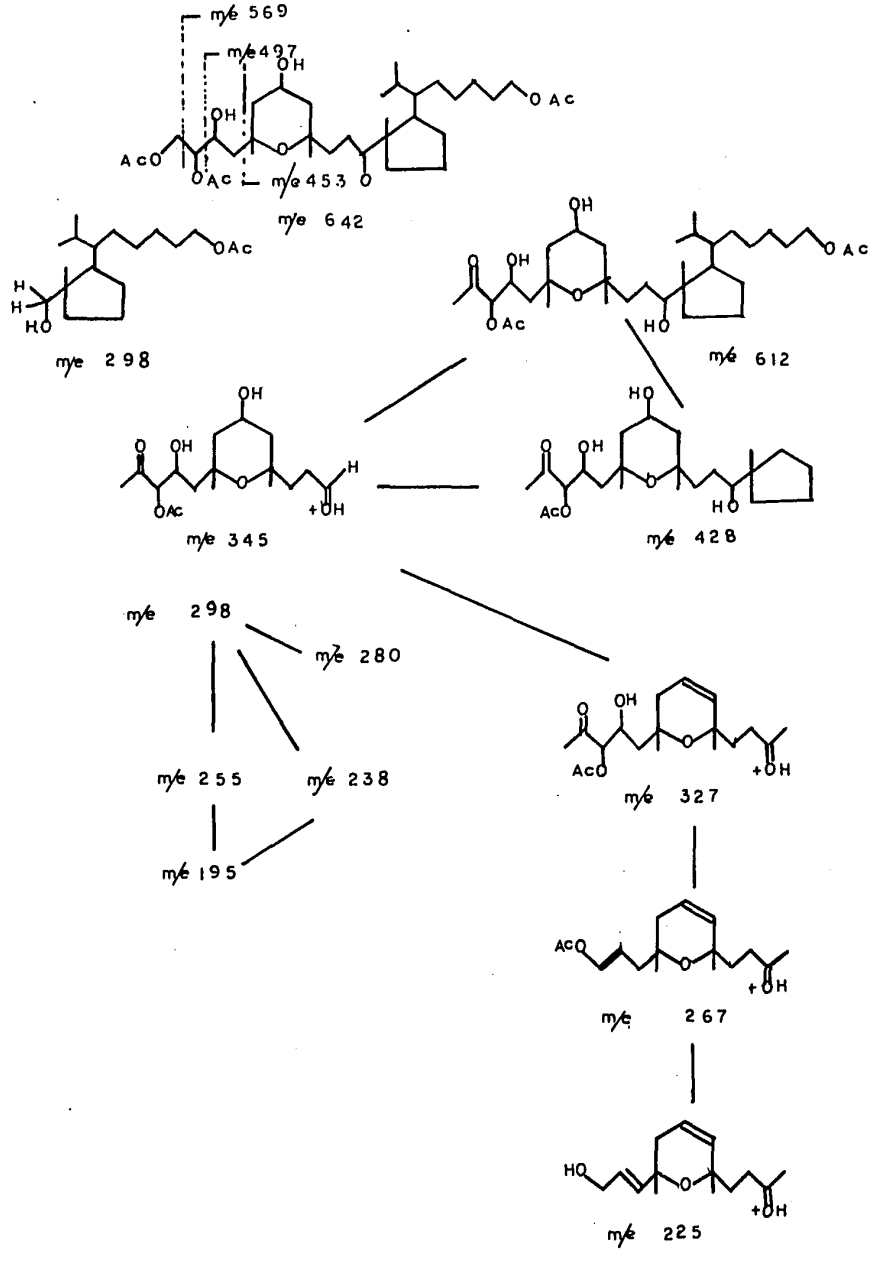
The mass spectral fragmentation of C-109-A is shown on page 235.

f. Compound C-109-B

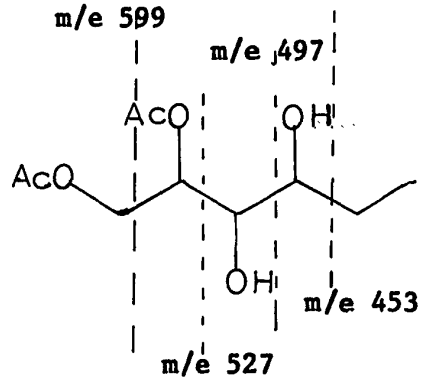
Compound C-109-B is a colorless non-fluorescent gum at room temperature. It freezes to a white granular solid at low temperature. The compound was negative to bromine and tetranitromethane tests, but was positive to benzidine-periodate spray reagent, indicating thereby the absence of olefin and presence of a vicinal diol functions. The compound was transparent in the ultraviolet. The infrared spectrum indicated the presence of hydrogen bonded hydroxyl (3350, 1040 cm^{-1}) and acetate (1725, 1250-1200 cm^{-1}) groups. The nuclear magnetic resonance spectrum in deuterochloroform depicted an isopropyl (0.88δ, J=6.0 Hz, 6H) which could be resolved into two doublets (0.825 and 0.935δ 3H each, J=7.0Hz) in pyridine-d_5. The nmr spectrum in deuterochloroform depicted a quaternary methyl (1.16δs, 3H) which underwent a solvent shift of +3 and -5 Hz in pyridine-d_5 and benzene-d_6. The signal at 1.280δ underwent a solvent shift of +1 Hz and +8 Hz in pyridine-d_5 and benzene-d_6 respectively. The spectrum indicated the presence of three acetates (2.10, 2.07 and 2.03δ). Methylenes and methines attached to hydroxyl and acetate groups appeared as a triplet (3.60δ, J=6Hz, 2H), complex broad signal (3.84δ, 2-3H) a complex structure (4.20δ, 2-3H) and a broad signal (5.0δ, 1H). The triplet at 3.60δ collapsed to a broad singlet on irradiation of the peak at 1.32δ, thereby suggesting it to be a primary alcohol methylene or ring alcohol methines.

70 And 21 EV mass spectra of the compound showed a parent ion at m/e 672 and base peaks at m/e 43 and 256. The parent ion gave rise to m/e 654, 642, 624, 599, 539, 479 by loss of 18(H_2O),

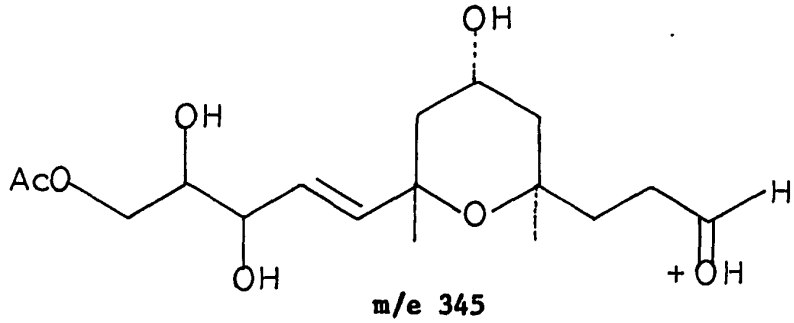
The following scheme details the observed mass spectral fragmentation pattern of compound C-109-A.



30(CH_2O), 48($CH_2O + H_2O$), 73 ($C_3H_5O_2$), 133 ($C_3H_5O_2 + C_2H_4O_2$), and 193 ($C_3H_5O_2 + 2\ (C_2H_4O_2)$ mass units. The parent ion directly fragmented to furnish m/e 599, 527, 497 and 453 by a respective loss of 73 ($C_3H_5O_2$), 145 ($C_3H_5O_2$ and $C_3H_4O_2$), 175 ($C_6H_9O_4$ and CH_2O) and 219 ($C_9H_{15}O_6$) mass units, thereby suggesting the presence of the following structural feature.

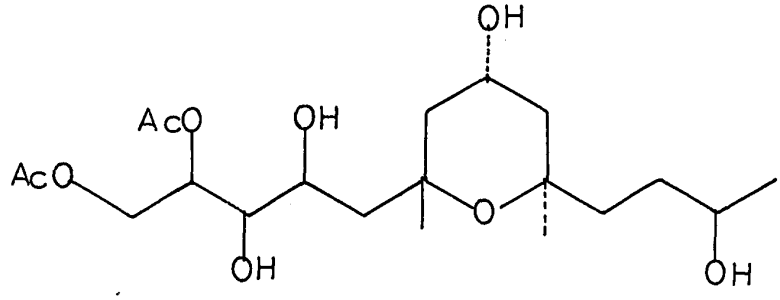


The ion m/e 453 degraded to m/e 411. The ion m/e 612 provided m/e 345 and also m/e 298, indicating thereby the presence in C-109-B of two portions similar to those in compounds C-107-C, C-104-d and C-109-A (m/e 298 and m/e 405 (345 + 60)). If m/e 298 is assumed to have the same composition as in other compounds, fragment, m/e 345 would have a composition of $C_{18}H_{29}O_7$. The ion m/e 345 further degraded to m/e 327, 285 and 225 with a respective loss of 18(H_2O), 60(CH_3COOH), and 120($C_4H_8O_4$) mass units respectively. The ion can thus be expressed

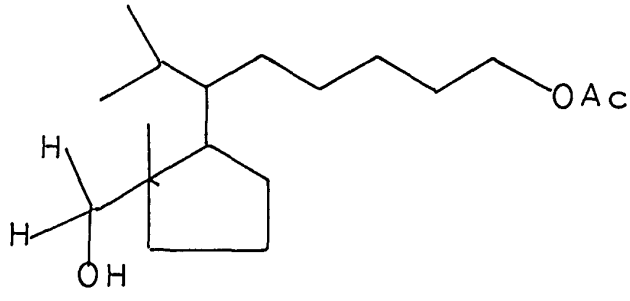


m/e 345

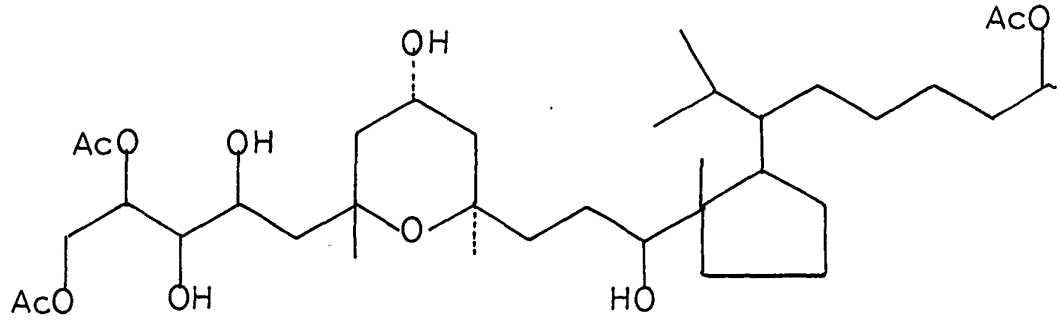
or the portion of the molecule as follows.



The ion m/e 298 has the following structure.

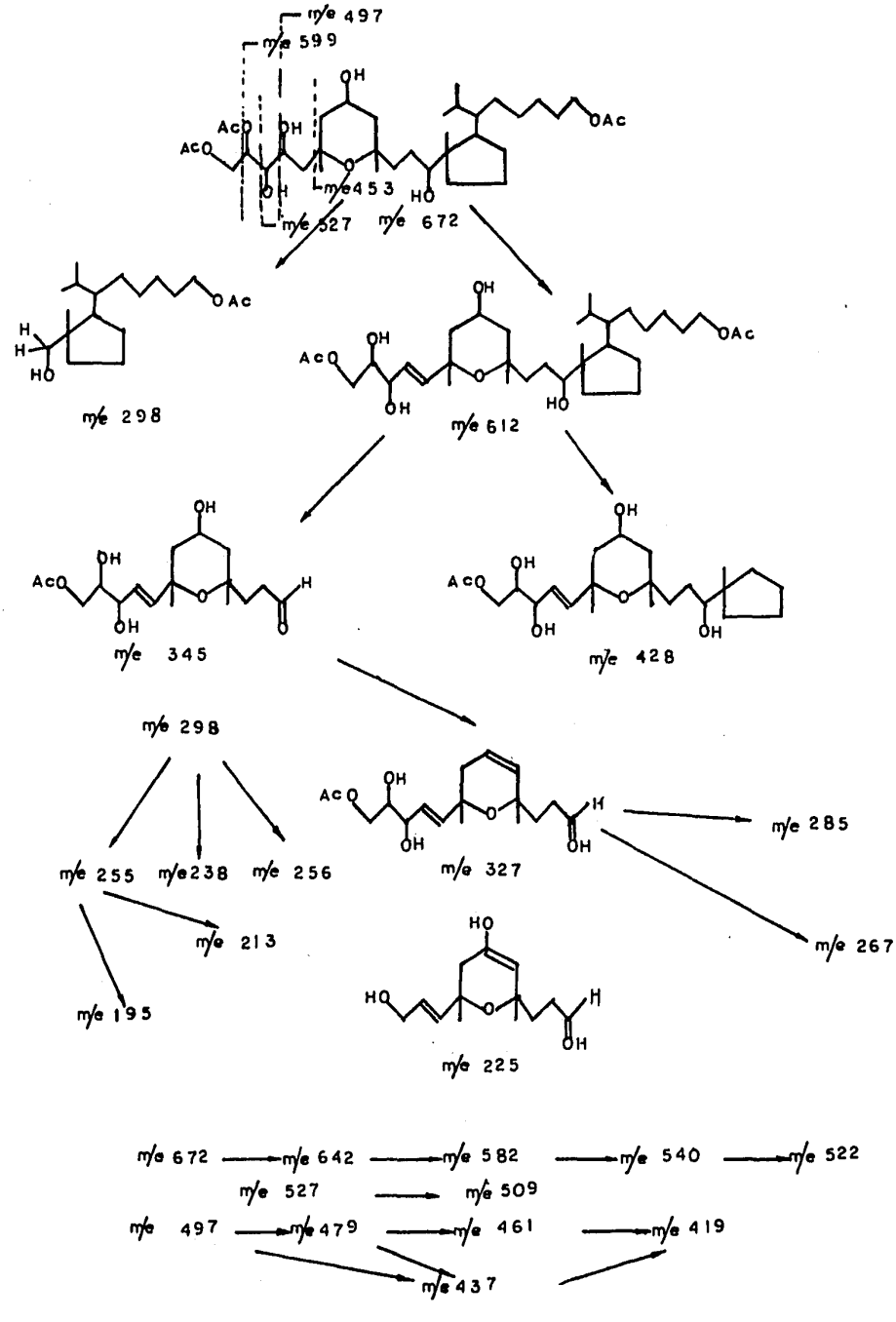


Therefore compound C-109-B may be represented by this structure.



The mass spectral fragmentation for compound C-109-B is shown on page 238.

The following scheme shows the observed mass spectral fragmentation pattern.



g. Interrelationship of Compounds C-107-C, C-104-d, C-109-A and C-109-B

It is evident from the structures of compounds C-109-B and C-109-A that they differ by 2 and 1 (CHOH) units from compound C-107-C. Inability of C-107-C to be further oxidized during periodate oxidation to give a compound of molecular weight by 30 units less sgrongly suggested that one of the vicinal hydroxyl is protected and is released for acetylation either during work-up or during borohydride reduction.

The nmr spectrum of the crude chloroform extract from the periodate oxidation of palytoxin depicted a singlet at 8.0δ which disappeared on prolonged standing or borohydride reduction of the extract. The chemical shift of the singlet strongly suggested it to be a formate ester.

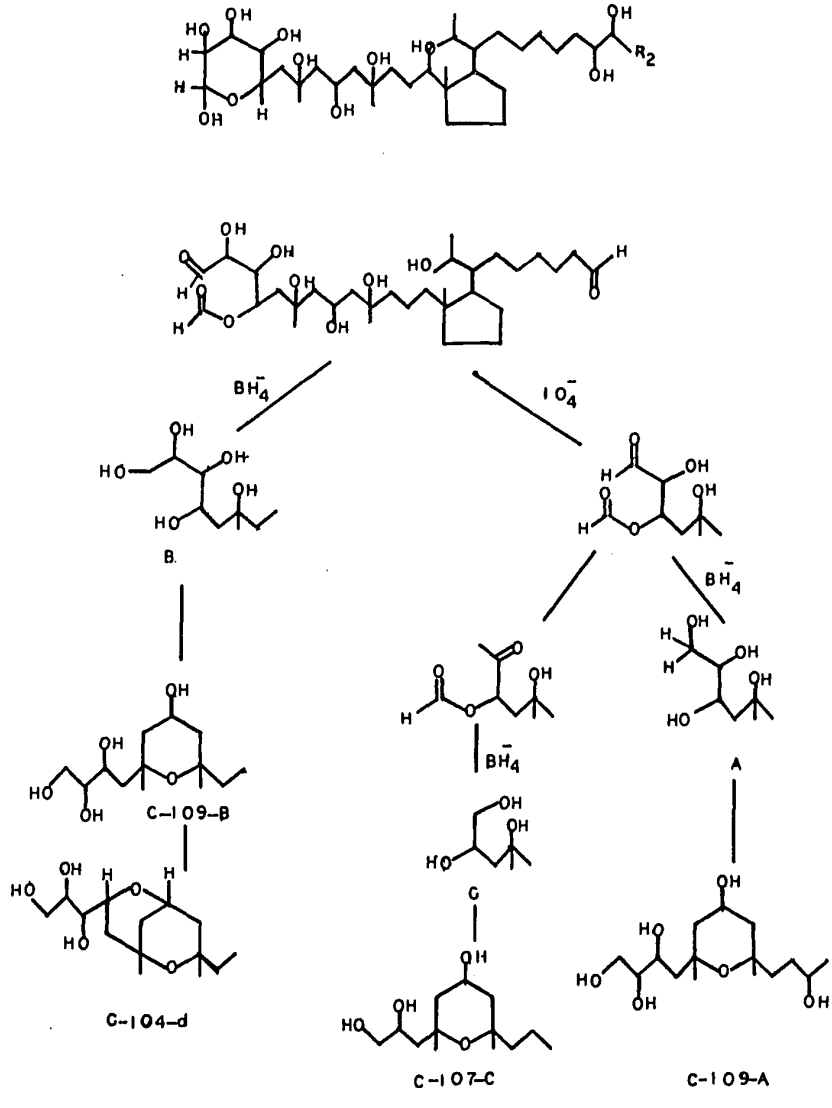
The compounds C-107-C, C-109-A, C-109-B, and C-104-d can easily be derived as shown on page 240.

h. Compound C-116-C(A)

Compound C-116-C(A) was a non-fluorescent colorless liquid. It decomposed on prolonged exposure to air and also on prolonged standing on silica gel thin layer plates. In the early stages of the work the compound had weak ultraviolet maxima at 267 and 257 nm, which were later shown to be due to a UV active impurity.

The infrared spectrum of purified C-116-C(A) (Fig. 33) depicted the presence of hydroxyl (3400, 1035 cm^{-1}) acetate (1730, 1240 cm^{-1}) and methyl (1360 cm^{-1}) groups. The nmr spectrum in deuterochloroform (Fig. 28) depicted a triplet (0.96δ, J=6Hz, 3H), corresponding

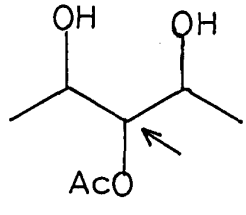
Generation of compounds C-107-C, C-104-d, C-109-A and C-109-B is presented thus.



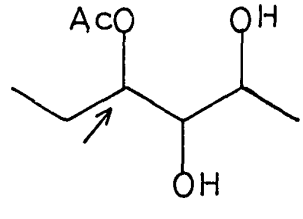
to a methyl attached to a methylene, a complex signal (1.25δ, b) corresponding to methylene attached to a methyl and probably β to oxygen, a series of singlets (2.00, 2.10, 2.45 and 2.50δ 21-23 H), corresponding to acetate methyl, a distorted triplet (3.90δ, J=6.0Hz, 4H) corresponding to primary acetate methylenes, a complex (4.60δ to 5.80δ, 3-5H), a complex (3.60δ 4H) corresponding to secondary alcohol and acetate methines respectively.

The methylene region (1.25δ, b in $CDCl_3$) underwent a solvent shift of +0.2 - 0.3δ to depict probably a quartet superimposed on a triplet (J=6.0 Hz, 5-6H) in benzene. The acetate methyl underwent a solvent shift of -0.2 to -0.25δ thereby exposing a broad singlet (2.05δ, 2H) corresponding to a hydroxyl group. The complex triplet at 3.90δ (4H) could be resolved to a triplet (3.86δ, 2H) and a doublet (4.02δ, 2H) in benzene, thereby suggesting the presence of the following functional groups, $-CH_2-CH_2-OCO-CH_3$ and $-CH-CH_2-OCO-CH_3$.

The complex (5.6δ, 4H) could also be resolved to a triplet (5.54δ J=5.5 Hz) and a doublet (5.67δ J=8.5 Hz) corresponding to the following functionalities.

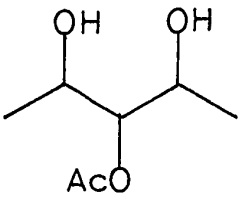


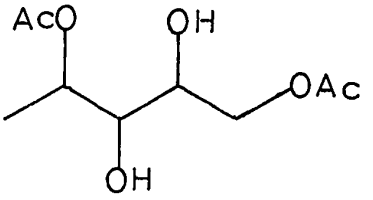
5.54δ



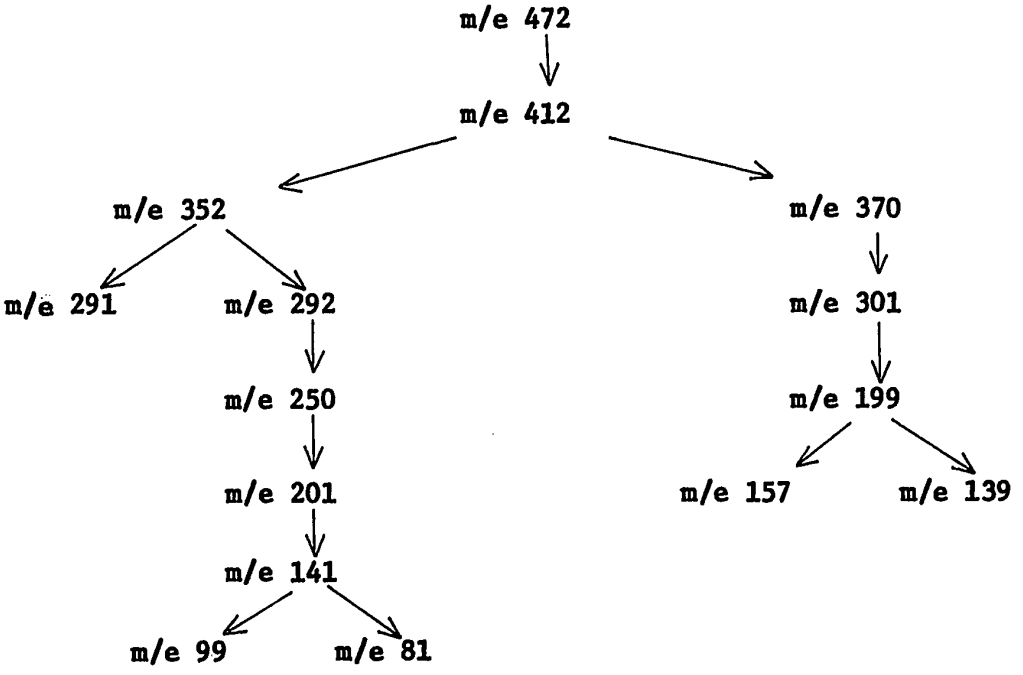
5.67δ

The triplet at 5.54δ collapsed to a singlet, while the doublet at 4.10δ was only tickled, when the signals at 4.52δ were irradiated, thereby indicating the presence of the following structural features.





Mass spectra of the compound C-116-C(A) did not reveal the parent ion. Highest mass peaks at m/e 472 and 412 could be seen in 70 and 20 EV mass spectra. Ion m/e 472 furnished m/e 412, 352, 292 and 250 by a respective loss of 60($C_2H_4O_2$), 120 (2 x $C_2H_4O_2$) and 162 (2 x $C_2H_4O_2$ + C_2H_2O) mass units. The fragmentation is outlined in the following scheme.



High resolution measurements for various peaks are presented in Table XXXI. Elemental analysis on a relatively small sample of C-116-C(A) (0.25 mg) furnished only an approximate analysis. Since the parent ion could not be observed, the composition of the compound was arrived at by working back from ions m/e 250 and 199 and corroborating the results with nmr and elemental analysis, in the following manner.

m/e 250	$C_{14}H_{18}O_4$	m/e 199	$C_{10}H_{15}O_4$
m/e 292	$C_{16}H_{20}O_5$	m/e 301	$C_{14}H_{21}O_7$
m/e 352	$C_{18}H_{24}O_7$	m/e 370	$C_{18}H_{26}O_8$
m/e 412	$C_{20}H_{28}O_9$	m/e 412	$C_{20}H_{28}O_9$
m/e 472	$C_{22}H_{32}O_{11}$	m/e 472	$C_{22}H_{32}O_{11}$
m/e 532	$C_{24}H_{36}O_{13}$	m/e 532	$C_{24}H_{36}O_{13}$
m/e 592	$C_{26}H_{40}O_{15}$	m/e 592	$C_{26}H_{40}O_{15}$
m/e 652	$C_{28}H_{44}O_{17}$	m/e 652	$C_{28}H_{44}O_{17}$

Calcd for $C_{28}H_{44}O_{17}$: C, 51.58; H, 6.80 %.

Found: C, 51.30; H, 10.30 %.

(Total number of protons as indicated by nmr 42-44.)

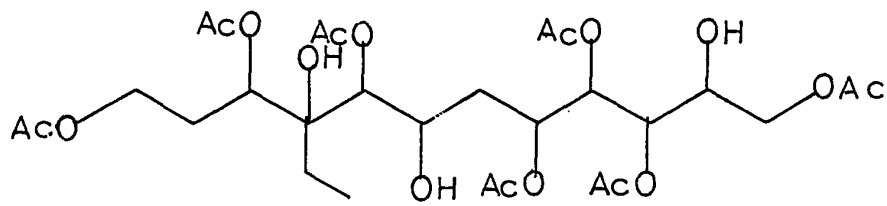
Since the nmr spectrum indicated the presence of seven acetate units, the compound corresponds to a parent $C_{14}H_{30}O_{10}$ and has no unsaturation.

The absence of unsaturation is further substantiated by the absence of a C=C stretching signal in the ir and a negative bromine test.

Periodate oxidation of hydrolyzed C-116-C(A) (by hydrochloric acid in methanol) and subsequent conversion of the reaction products to dinitrophenylhydrazones failed to depict any aldehyde and

ketone bigger than three carbons, based on an examination of the mass spectrum of the mixture.

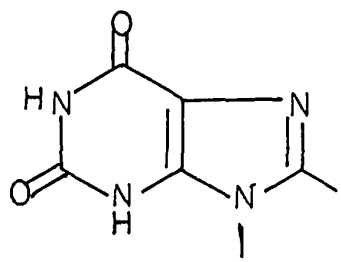
On the basis of the above information we may write the following structure.

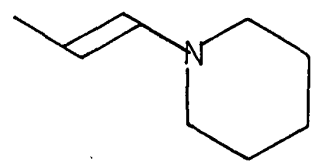


The mass spectral fragmentation of C-116-C(A) is presented on page 245.

i. Compound C-116-E(A)

Compound C-116 -E(A) is a colorless liquid and depicts maxima at 286 and 230 nm in its ultraviolet spectrum corresponding probably to the following chromophores





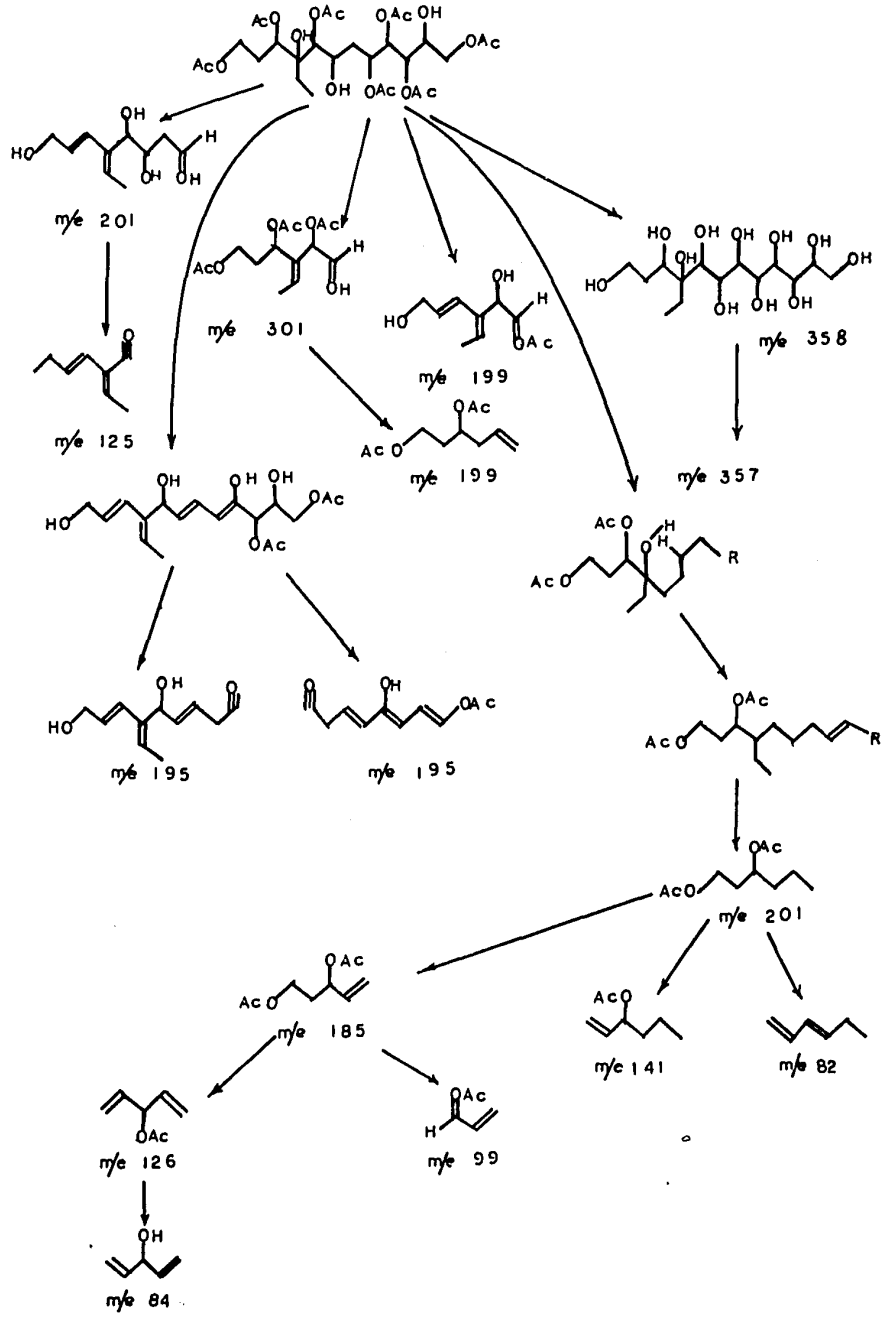
λ_{max} 267 (7,900) pH 5.0

λ_{max} 240 (8,000), 277 (8,300) pH 9.99

λ_{max} 283 (8,700) pH 13.00

λ_{max} 228 (10,000)

A scheme for mass spectral fragmentation of compound C-116-C(A) is presented thus.



The compound was negative to ninhydrin, Dragendorff and DNP sprays indicating thereby the absence of primary or tertiary amines, quaternary ammonium and oxo functions.

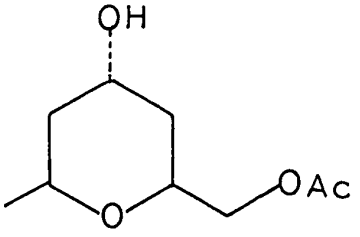
The infrared spectrum of the compound indicated the presence of hydroxyl (3450, and 1050 cm^{-1}) and acetate (1735 and 1235 cm^{-1}). A broad band at 1650-1600 could possibly represent olefin and amide functions.

The nuclear magnetic resonance spectrum depicted a broad signal at 1.66δ corresponding to cyclic methylenes and methylenes adjacent to carbinol, a sharp singlet at 2.04δ (both signals constitute 26-28H), corresponding to the acetate methyls, a broad signal at 4.06δ (7-8H) corresponding to primary acetate methylene and carbinol methines, a very broad signal at 5.10δ (1H) methine attached to an acetate, a sharp singlet at 6.22δ representing chloroform and olefinic protons.

The mass spectrum of the compound depicted the highest mass peak at m/e 558 and a base peak at m/e 173 in the 20 EV spectrum and at m/e 43 in the 70 EV spectrum. The ion m/e 558 fragmented to give m/e 498, 456 and 396 by a respective loss of 60 ($C_2H_4O_2$), 102 ($C_2H_4O_2$ + C_2H_2O) and 162 (2 x $C_2H_4O_2$ + C_2H_2O) mass units. The ion m/e 558 further fragmented to give m/e 485 by a loss of 73 ($C_3H_5O_2$) mass units. The ion m/e 498 furnished m/e 438 and 425 by a respective loss of 60 ($C_2H_4O_2$) and 73 ($C_3H_5O_2$) mass units. High resolution measurements at m/e 396 furnished its composition $C_{21}H_{24}N_4O_4$. Working back to peak m/e 558 is shown as follows and arrives at a composition of $C_{27}H_{38}N_4O_{11}$ for C-116-E(A).

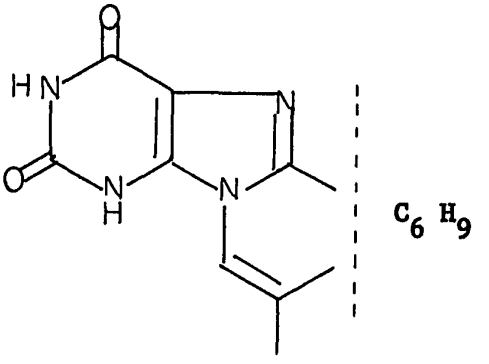
$C_{21}H_{24}N_4O_4$	m/e 395
$C_{23}H_{28}N_4O_6$	m/e 456
$C_{25}H_{30}N_4O_7$	m/e 498
$C_{27}H_{34}N_4O_9$	m/e 558
$C_{27}H_{36}N_4O_{10}$	m/e 576
$C_{27}H_{38}N_4O_{11}$	m/e 594

The assumption was made that the ion m/e 558 had been generated by dehydration of the parent ion at m/e 594. The need for this assumption became quite evident by the lack of any significant olefinic protons in the nmr spectrum. The presence of more than three rings was precluded on the basis of fragmentation (m/e 347 and 303). The formation of a base peak at m/e 173 at 22 EV clearly suggested that it arises by a primary process. Unfortunately, high resolution measurements on this peak could not be affected because of the small size of the sample. If, however, it is assumed that it has no nitrogen, then it can be written as follows.

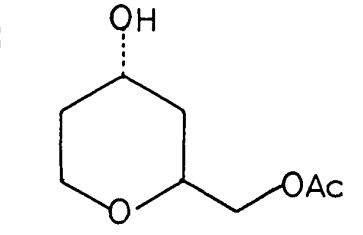


Evidently this portion of the molecule is expected to lose, CH_3COOCH_2 (73) and m/e 173 units readily. The formation of m/e 396, 347, 345 and 303 fragments containing 4 nitrogen atoms suggested

that the nitrogens are close together in the molecule. This in conjunction with the UV spectrum prompted us to write the following purine fragments. We think that the purine portion itself is generated by dehydration or by some other process during oxidation.



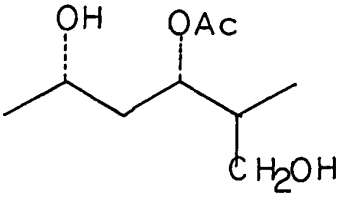
C_6H_9



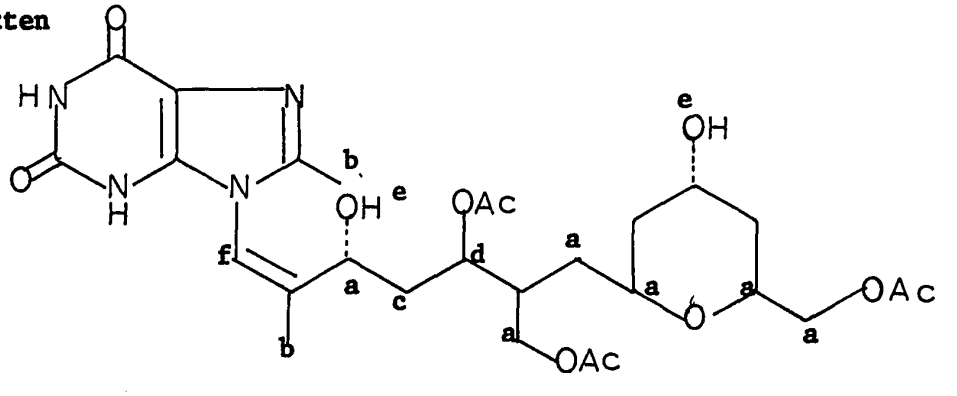
2 - OAc

1 - OH

Since the compound is negative to benzidine-periodate spray and has no more than one secondary acetate and a minimum of 6 protons corresponding to primary acetate and methines attached to hydroxyl, the C_6H_9 fragment may be expressed thus



This was further favored on biogenetic grounds. The following structure along with an interpretation of its nmr can be written



a = 4.06δ (b, 7-8H)

b = 2.04δ (s)

c = 1.66δ (b, c)

d = 5.10δ

e = 5.60 or 2.0 or 4.02δ

f = 7.22 or 5.66δ

It should be emphasized that lack of the availability of high resolution measurements of peak m/e 173 and others and direct evidence for the presence of purine or tetrahydropyran rings make this structure highly tentative. The mass spectral fragmentation of C-116-E(A) is presented on page 250.

10. Periodate Oxidation of Palytoxin (Jamaican) Followed by 4-Bromobenzoylation

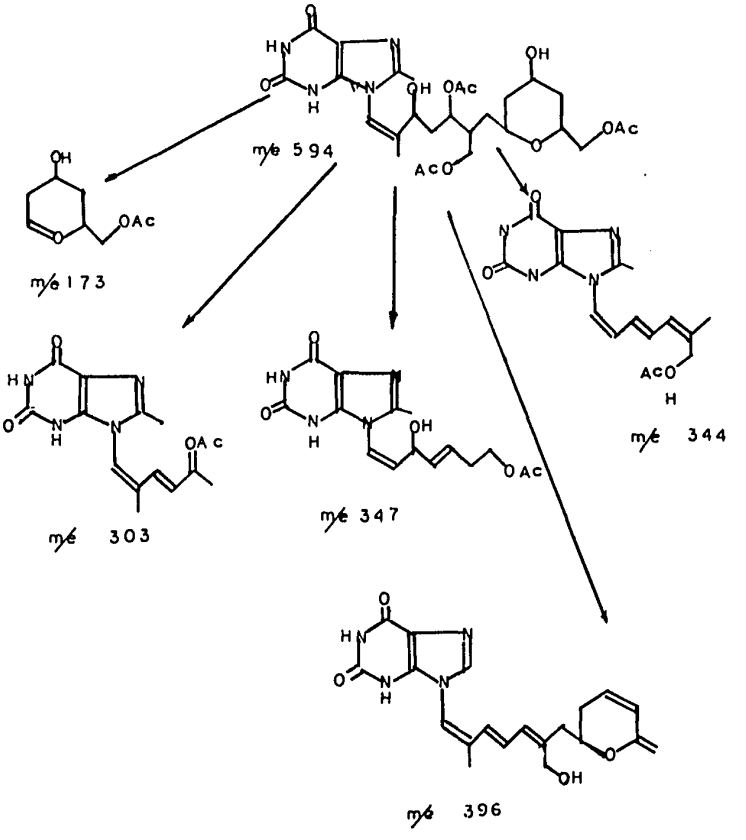
In another periodate oxidation (Jamaican palytoxin), the barohydride reduced chloroform extract was reacted with 4-bromobenzoyl chloride in pyridine. The reaction mixture was fractionated into three compounds A(Rf 0.565), B(0.32) and C(0.20) by thin layer chromatography.

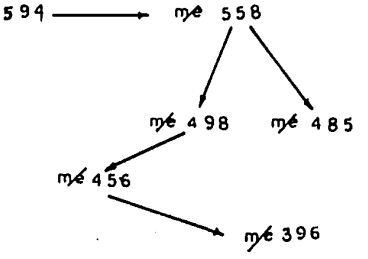
Band A could be crystallized as white needles from aqueous methanol, mp 84.5°. The ultraviolet maximum at 245.5 nm and infrared bands (1710, 1580, 1450 and 845 cm^{-1}) confirmed the presence of 4-bromobenzoate. The nmr spectrum exhibited the presence of an isopropyl (0.86δ, d, J=6Hz) methyl (1.11δ, s), methylene (1.260δ, b, s), alicyclic methylene (1.60δ, b), primary and secondary alcohol methylene and methines (3.84 and 4.26δ, c) and 4-bromobenzoate group (7.54δ, A_2B_2, q).

The mass spectrum did not reveal the parent ion. The highest significant ions m/e 440 and 438 (49%) lost 43 mass units to

C-116-E(A)

The mass spectral fragmentation can be expressed as shown.





furnish m/e 397 and 395 (30%). Both ions corresponded to m/e 298 and 255 in the acetates.

Band B was also crystalline.

Both bands B and C contained 4-bromobenzoate (1710, 1580, 1450, and 848 cm^{-1}) groups. These bromobenzoates may be suitable for x-ray diffraction studies.

11. Lead Tetraacetate Oxidation of Palytoxin

Lead tetraacetate like sodium metaperiodate oxidizes vicinal diols to aldehydes and ketones (61). Palytoxin was oxidized with lead tetraacetate in aqueous acetic acid and pyridine, in the hope to obtain a less complex reaction mixture and/or smaller fragments of known structure.

In a quantitative lead tetraacetate oxidation of palytoxin 13.2 moles of oxidizing agent was consumed per mg of toxin (33-34 moles for 2538 g toxin); however, no volatile aldehydes could be detected during oxidation.

In one experiment palytoxin was oxidized by lead tetraacetate in aqueous acetic acid. The evaporated reaction mixture was extracted with chloroform and both chloroform and aqueous portions on examination as dinotrophenylhydrazones by thin layer and paper chromatography indicated the presence of six and three compounds respectively.

In another experiment, the chloroform extract from lead tetraacetate oxidation was directly reducted with lithium aluminum hydride. Surprisingly enough the reaction product had infrared bands assigned to oxo (1725 cm^{-1}) and hydroxyl (3450, 1050 cm^{-1}) functions. The 70 EV mass spectrum of the above crude product mixture

exhibited an ascending pattern characteristic of a long hydrocarbon chain. Some significant fragments could be seen at m/e 420, 414, 400, 386, 372, 340, 299, 284, 270, 256, 242, 228, 213, 199, 185, 171, 157, 143 and 139.

On the basis of available information from the periodate oxidation, it is speculated that peaks at m/e 420 and 414 arise from m/e 546 (triacetate m/e 672, C-109-B) and m/e 486 (triacetate m/e 612, C-107-C) by loss of 126 ($7H_2O$) and 72 ($4H_2O$) mass units. The remaining ions are produced by loss of methyl and ethylene and differ from each other by 14 mass units. The ion m/e 256 (probably corresponds to m/e 298 in acetate) fragmented to m/e 213 by loss of 43 (C_3H_7) mass units.

In still another experiment, the chloroform extract from a lead tetraacetate oxidation (in pyridine), after reduction with sodium borohydride was silylated with hexamethyldisilazane and examined by GLC. Gas chromatography indicated the presence of four major and two minor components, (Rt 3'40", 5'30", 16'52", 16'38", 1'12" and 2'00"). Thin layer chromatography of the acetylated-reduced chloroform extract sub stantiated this observation and indicated the presence of compounds corresponding to C-107-A, C-107-C, C-109-B and C-104-d from the periodate oxidation.

GLC of the acetates of the reduced aqueous portion indicated the presence of eight compounds.

12. Comparison of Periodate and Lead Tetraacetate Oxidation of Palytoxin.

While sodium metaperiodate oxidation of palytoxin involved an easier work-up and relatively mild reaction conditions, it suffered

from the disadvantage of furnishing a greater number of products. On the other hand, while lead tetraacetate furnished a cleaner reaction mixture, the yields of chloroform extract were relatively low (LTA. 10-20%, periodate 30-35%) and the aqueous portion was composed of small molecules.

It was observed that lead tetraacetate oxidation of palytoxin was completed in 6 hr at 7° while consumption of periodate could be observed even over a week, at room temperature.

13. Summary of Structural Data

In concluding our study on palytoxin, we must point out some salient features of the present work.

Our best estimate of the molecular formula of palytoxin $C_{120}H_{200}N_4O_{52}$ (M Wt 2528) is based upon elemental analysis of palytoxin, palytoxin acetate, acetate-content of palytoxin acetate and molecular weight of palytoxin acetate.

We have shown that palytoxin has three UV chromophores, two of which are triply substituted butadienes while the third may be a butadiene attached to an amide.

Our hydrolytic studies have indicated the absence of a glycosidic linkage.

We have shown that palytoxin consumes 33 moles of oxidant and generates 20 mole-equivalents of acid. No volatile aldehydes are generated. Periodate oxidation of palytoxin generated a relatively large number of compounds, of which only seven (C-107-A, C-107-C, C-104-d, C-109-A, C-109-B, C-116-C(A) and C-116-E(A)) could be obtained in quantities sufficient (2 to 6 mg) for spectral and chemical

studies. These compounds account for only $C_{75}H_{138}N_4O_{30}$ of the proposed molecular formula. The remaining portion $C_{45}H_{60-70}O_{22}$ containing eight to twelve elements of unsaturation and ten to fifteen vicinal hydroxyls, is probably lost as formic acid, other acids and other small molecules. The characterized compounds are summed up as follows:

Compound	Acetate	Parent
C-107-A	$C_{18}H_{28}O_9$	$C_{12}H_{22}O_6$
C-107-C	$C_{34}H_{60}O_9$	$C_{28}H_{54}O_6$
C-116-C(A)	$C_{28}H_{44}O_7$	$C_{14}H_{30}O_{10}$
C-116-E(A)	$C_{27}H_{38}N_4O_{11}$	$C_{21}H_{32}O_8N_4$
		$C_{75}H_{138}N_4O_{30}$

Palytoxin has 21 elements of unsaturation while the above C-75 fragment accounts for only 10. Therefore the lost fragment carries ca 11 elements of unsaturation.

It is evident that we do not have enough information even to speculate about the total structure of palytoxin.

Regarding the future of the problem, the author maintains that oxidative reactions in particular periodate and lead tetraacetate oxidations using oxidative workup potassium permagnate (62) or silver oxide (63) and reductive (LAH) workup will be extremely useful.

High pressure hydrogenation using a method described by Cedar (64) may indeed prove to be very informative.

H. Lipids of Toxin Palythoa

1. Fatty Acids

Fatty acid analysis of toxic Palythoa indicated the presence of caproic acid (C-6), caprylic acid (C-8), capric acid (C-10), myristic acid (C-14), isopentadecanoic acid (C-15), pentadecanoic acid (C-15), palmitoleic acid (C-16, 2H), palmitic acid (C-16), isoheptadecanoic acid (C-17), heptadecanoic acid (C-17), linolenic acid (C-18, 4H), linoleic acid (C-18, 2H), stearic acid (C-18), eicosadienoic acid (C-20-4H), arachidic acid (C-20), 19-methyl eicosanoic acid (C-21), and iso C-22 acid.

The fatty acid content resembles closely that of other marine invertebrates (65).

2. Pigments

Pigment PG_2 had an ultraviolet spectrum very similar to that of chlorin, thereby suggesting the presence of dihydroporphyrins (66).

No further work was done.

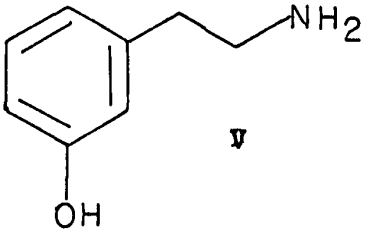
Pigment PO_1 had an ultraviolet spectrum and Rf values identical to that of β-carotene (authentic sample) thereby indicating it to be β-carotene.

I. UV-Active Constituents of Toxic Palythoa

1. Isolation Procedure I

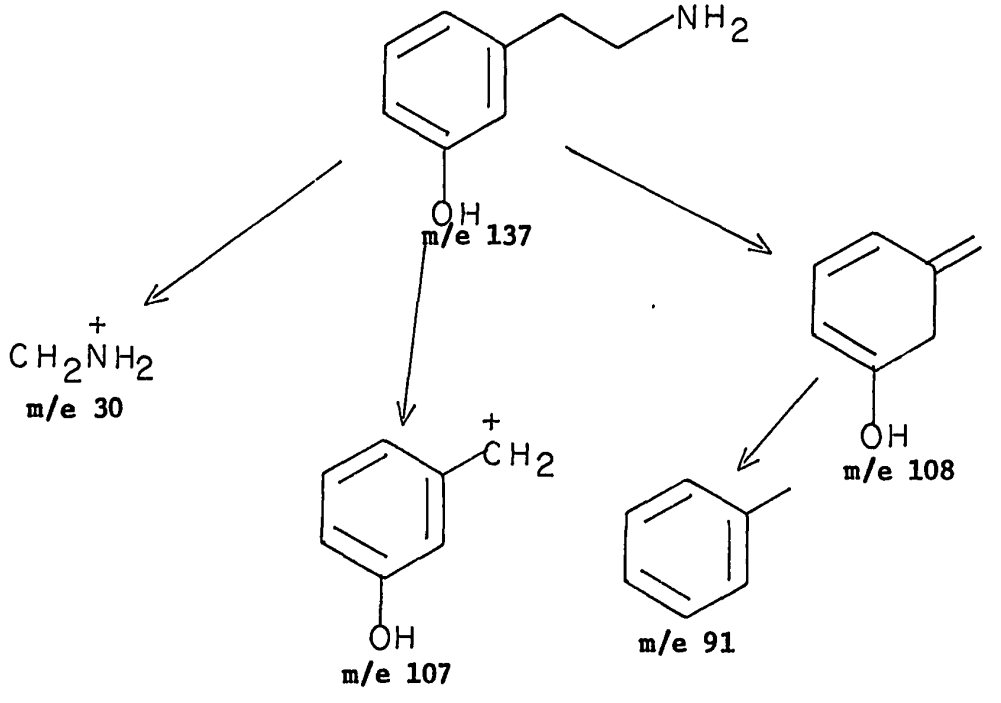
Compound Z-1 was isolated from the aqueous effluents of Tahitian toxic Palythoa by a process involving column chromatography over Amberlite IRc 50 (Hydrogen form), Dowex 50 W-X4 (Hg^{+2} form), Dowex 50W-X4 (H^+ form) and finally preparative paper chromatography.

The elution volumes from the Dowex 50-W-X4 column clearly indicated that the compound is strongly basic. The compound was found to be negative to the Ehrlich and Molisch tests, but positive to both ninhydrin (purple spot) and phenolic hydroxyl and amine moieties. The ultraviolet absorption spectrum had a maximum at 274 nm with a shoulder at 278.5 nm. The UV maxima underwent a strong base shift to 291.75 nm thereby providing support for the presence of a phenolic hydroxyl group. The infrared spectrum indicated the presence of a primary amine hydrochloride (3000 cm^{-1}, 1495 cm^{-1}) a phenolic hydroxyl group (3333, 1156 cm^{-1}) and a metadisubstituted benzene ring (778.2 and 752 cm^{-1}, 2959 and 1587 cm^{-1}). The nuclear magnetic resonance spectrum in deuterium oxide indicated two sets of signals around 3.0δ (4H) and 6.9δ (4H). The former corresponded to an A_2B_2 system while the latter to an ABCD. The nuclear magnetic resonance spectrum in trifluoroacetic acid resolved the A_2B_2 portion into a distinct triplet (2.7δ J=6.0 Hz) and a complex broad signal (3.15δ) thereby indicating that the B_2 part of the A_2B_2 system is attached to an amine function. The mass spectrum (70 EV) exhibited a base peak at m/e 30 and a parent peak at m/e 137 (58%). The compound could be acetylated to a diacetate having an ultraviolet maximum at 272 nm uneffected by acid or base. The nmr spectrum showed singlets at 2.10 and 2.30δ, corresponding to three protons each, and the mass spectrum had a parent at m/e 221 (18.5%) and a base peak at m/e 120. The compound Z-1 on reaction with diazomethane furnished a monomethyl ether having a singlet at 4.70δ in its nmr spectrum. On the basis of above information, the compound could be expressed as 2-[m-hydroxyphenyl] ethyl amine (V).

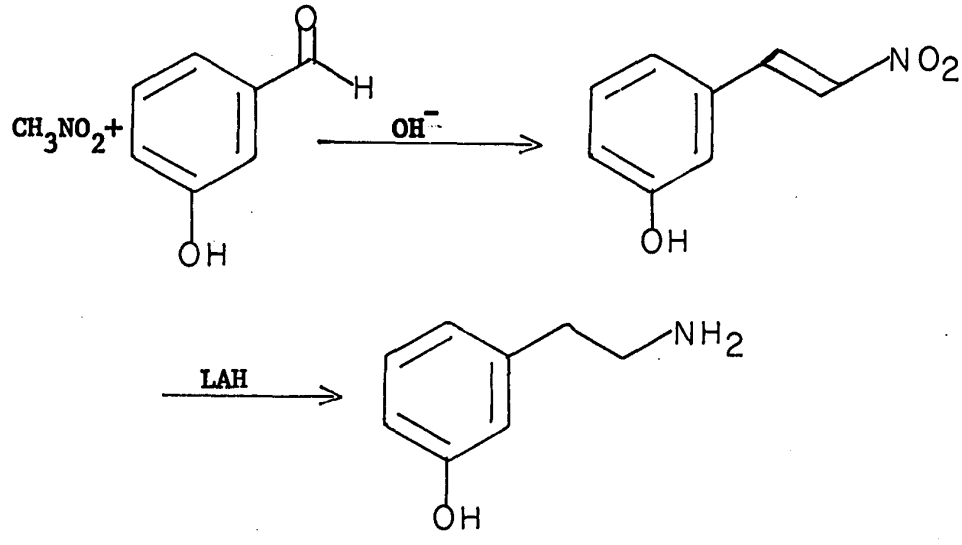


V

The major mass spectral fragmentation are shown in the following scheme.



Proof for the structure was obtained by direct comparison of its spectral properties with that of a synthetic sample prepared by the sequence shown on the following page.



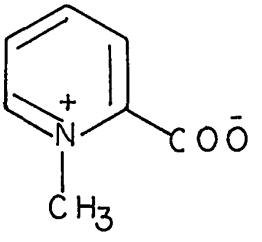
2. Isolation Scheme II

The aqueous effluents from the polyethylene chromatography of toxic Palythoa on purification over Dowex 50W-X4 (H^+ form) column, furnished three compounds Z-2, Z-3 and Z-4.

a. Compound Z-2

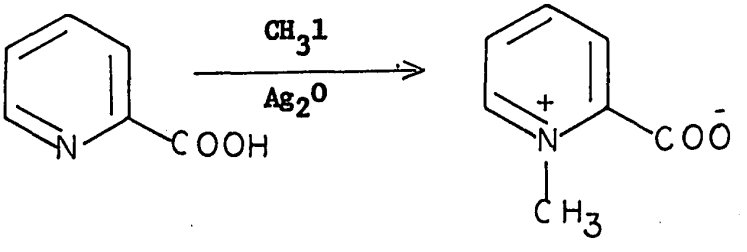
Compound Z-2 was a pale hygroscopic solid negative to both ninhydrin and Pauly's sprays but positive to Dragendorff reagent, thereby indicating the presence of tertiary or quaternary nitrogen. The compound had a UV absorption maximum at 272 nm which was not affected by base and underwent only a small hypsochromic shift of 2 nm in 1N-hydrochloric acid, thereby indicating the absence of phenolic hydroxyl and aromatic amine moieties. The infrared spectrum indicated the presence of a carboxylic acid (broad band at 3000 cm^{-1}), methyl groups (1370 cm^{-1}), and another disubstituted aromatic system (787.4 and 840.3 cm^{-1}). The nuclear magnetic resonance spectrum indicated the presence of two sets of signals, a singlet (4.425δ) corresponding

to a quaternary N-methyl and two sets of complexes (8.05 and 8.80δ) corresponding to an ABCD system. The mass spectrum had its highest mass peak at m/e 121 (6.45%) and a base peak at m/e 36. Compound Z-2 could be hydrogenated over catalytic platinum to a UV inactive white solid (did not melt up to 230°). The infrared spectrum of the hydrogenated Z-2 indicated the presence of NH (3300-3000 cm^{-1} broad band), and carboxylic acid anion (1630 cm^{-1}). The nuclear magnetic resonance spectrum depicted three complex patterns at 1.35, 1.65 and 3.15δ. The complexes at 1.35δ and 1.55δ (6H) represented the ring methylene groups while a complex at 3.15δ (3H) represented the methylene and methine adjacent to nitrogen. The spectrum resembled in all respects the spectrum of pipecolic acid. On the basis of the above observations and the comparison of the ultraviolet spectrum with that of homarine (28) compound Z-2 was assigned the structure (IX)



IX

The structure of the compound Z-2 was proved by synthesis of homarine from picolinic acid.

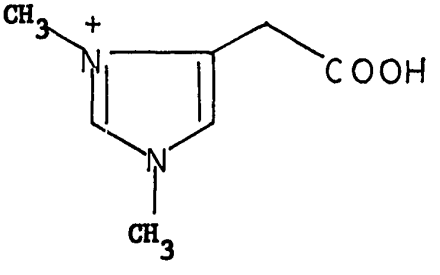


b. Compound Z-3

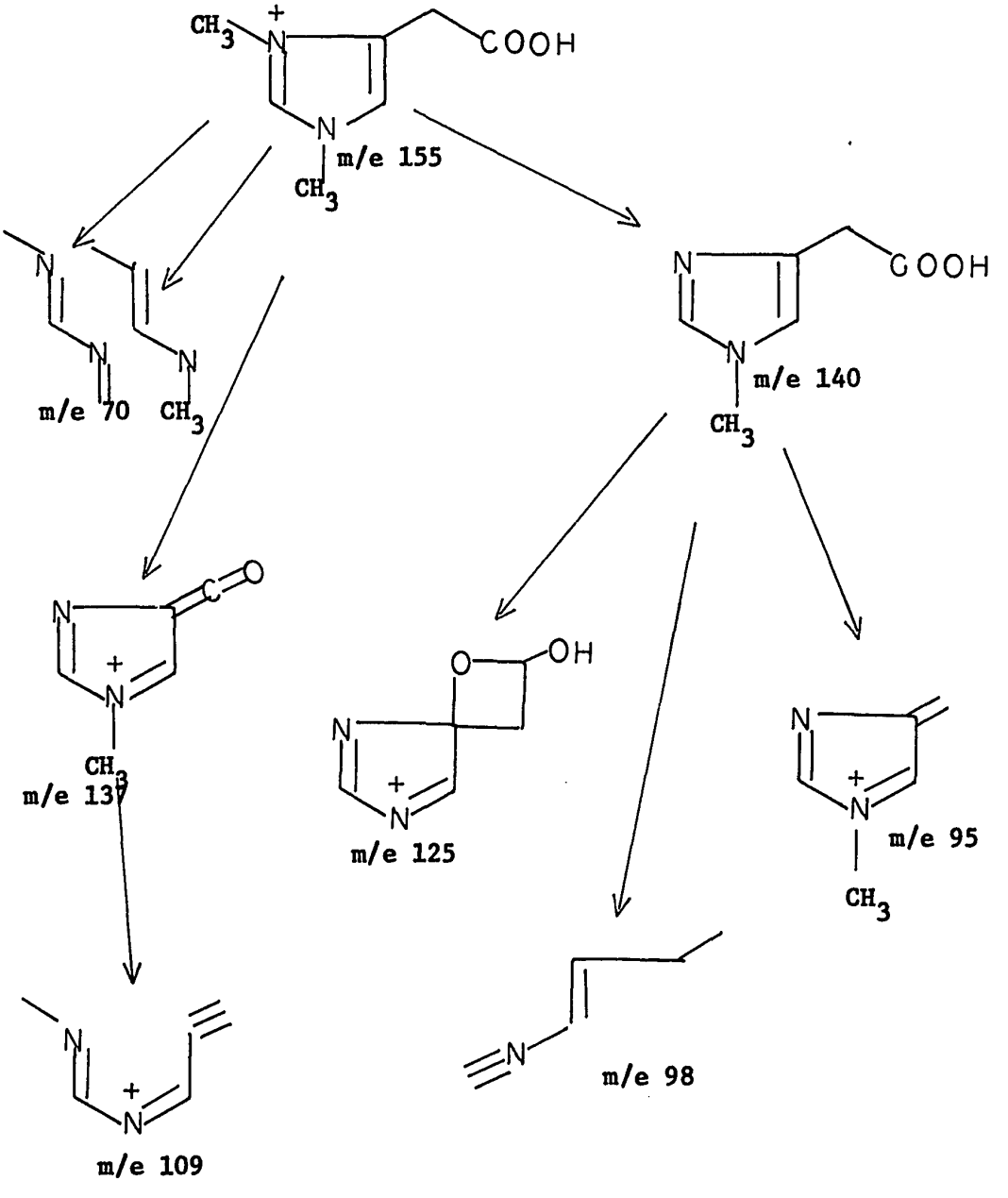
Compound Z-3 was isolated as a colorless hygroscopic semi-solid by preparative thin layer chromatography of the fractions 80-100. The compound was negative to ninhydrin, Pauly's and Dragendorff reagents thereby suggesting the absence of primary amino phenolic and tertiary amino functional groups. The strong ultraviolet absorption at 214 nm indicated the presence of an imidazole ring which was further substantiated by infrared bands at 1325, 1200 and 1100 cm^{-1} corresponding to the ring stretching and ring breathing modes. The intense broad infrared band at 3400 cm^{-1} represents a combination of water of hydration and carbon hydrogen stretching signals.

Whereas the band at 1630 cm^{-1} most probably represents a carboxylate anion (zwitterionic structure), the nuclear magnetic resonance spectrum depicted three singlets at 3.40, 3.80 and 3.90δ representing three protons each, and two singlets at 4.90 and 7.50δ corresponding to one olefinic proton each. The singlets around 3.80δ most probably represent three alkoxy methyls or methyls on aromatic nitrogens. The mass spectrum of compound Z-3 exhibited a base and a parent peak at m/e 140 and major peaks at m/e 125 (30%), 109 (42.5%), 98 (5%), 95 (9%), 83 (9%), 70 (21%), 68 (13%), 44 (69%) and 43 (43%).

The above data can be translated in a structure commonly known as anemonins (29) (II)



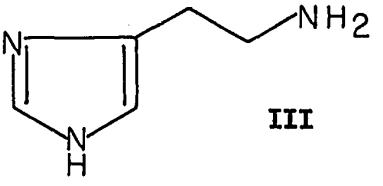
The mass spectral fragmentation could be expressed as follows



d. Compound Z-4

Compound Z-4 was isolated as a white crystalline solid, mp. 238°. The compound gave pink color with Pauly's reagent indicating thereby the presence of an imidazole ring. This observation was confirmed by ultraviolet maxima at 214 mm and infrared bands at 1324 and 1227 cm^{-1}. Infrared spectrum further indicated the presence of primary amine hydrochloride (3030 cm^{-1}).

Nuclear magnetic resonance spectrum showed the presence of an A_2B_2 pattern (3.03δ, 4H) corresponding to two methylene groups and two singlets at 7.03δ (1H) and 7.73δ (1H) corresponding to two aromatic protons. Mass spectrum showed a parent ion at m/e 112 and a base peak at m/e 30. On the basis of spectral properties compound could be expressed as histamine (III)



III

This was confirmed by comparison with an authentic sample.

e. Compound Z-5

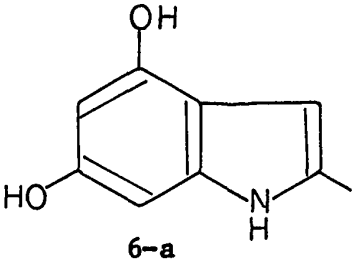
Compound Z-5 was isolated as a hygroscopic pale solid by column chromatography over Sephadex cation and anion exchangers. The compound was negative to ninhydrin, isatin and Prochazkas' reagents but gave a yellow spot for Ehrlich and a brownish green spot for purine tests. The ultraviolet absorption spectrum showed strong absorption at

330 nm which was irreversibly destroyed with sodium carbonate. The infrared spectrum showed a number of bands between 1600 and 950 cm^{-1} most probably representing the N-H and C-N stretching modes respectively. The nuclear magnetic resonance spectrum depicted a series of sharp singlets at 2.650δ, 2.850δ, 2.960δ, 3.25δ, 3.27δ, 3.725δ and 4.10δ probably corresponding to $NH-CH_3$, $N(CH_3)_2$, $-\overset{O}{\overset{\|}{C}}-NH-CH_3$, $-\overset{+}{N}-(CH_3)_3$, $N-CH_3$ and $-OCH_3$ respectively. The mass spectrum of the compound Z-5 could not be run. No combustion data of compound Z-5 were obtained.

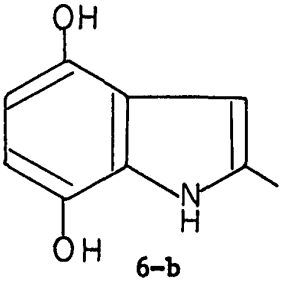
f. Compound Z-6

Compound Z-6 was isolated by repeated thin layer chromatography of concentrated aqueous effluents over silica gel HF. Since the amount of the material was small, only ultraviolet and mass spectra were recorded. Compound Z-6 had ultraviolet maxima at 276 nm and 219.5 nm with a shoulder at 283 nm (219.5/270 nm=3.14). The compound exhibited a strong bathochromic shift of 15 nm in base. The ultraviolet spectrum suggested the presence of an indole moiety. The mass spectrum of compound Z-6 exhibited a base peak at m/e 162 and a parent peak at m/e 163 (73%). The presence of a strong M-1 peak (100%) suggested the presence of a methyl group at 2 position of indole (67). The overall pattern of the mass spectrum clearly proved the suspicion that the compound is a simple indole. The spectrum had, M-15, M-31, M-42, M-72 peaks at m/e 147, 132, 121, and 91 arising most probably from loss of a methyl, a combination of methylene and hydroxide, and loss of combination of methyl cyanide and hydrogen. The presence of 2- and 3-oxo indoles was ruled out on the basis of the ultraviolet spectrum and by the absence of a predominant m/e 125 peak arising by a loss of 28 mass units (C=O) from the parent ion.

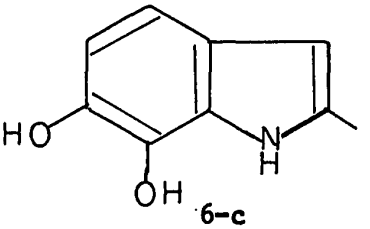
Position 5 of the indole could not possibly carry a hydroxyl group because of a weak M-15 peak at m/e 147. Therefore the two hydroxyls (the number of hydroxyls determined by difference) could be placed at 4, 6 and 7 positions of indole.



6-a

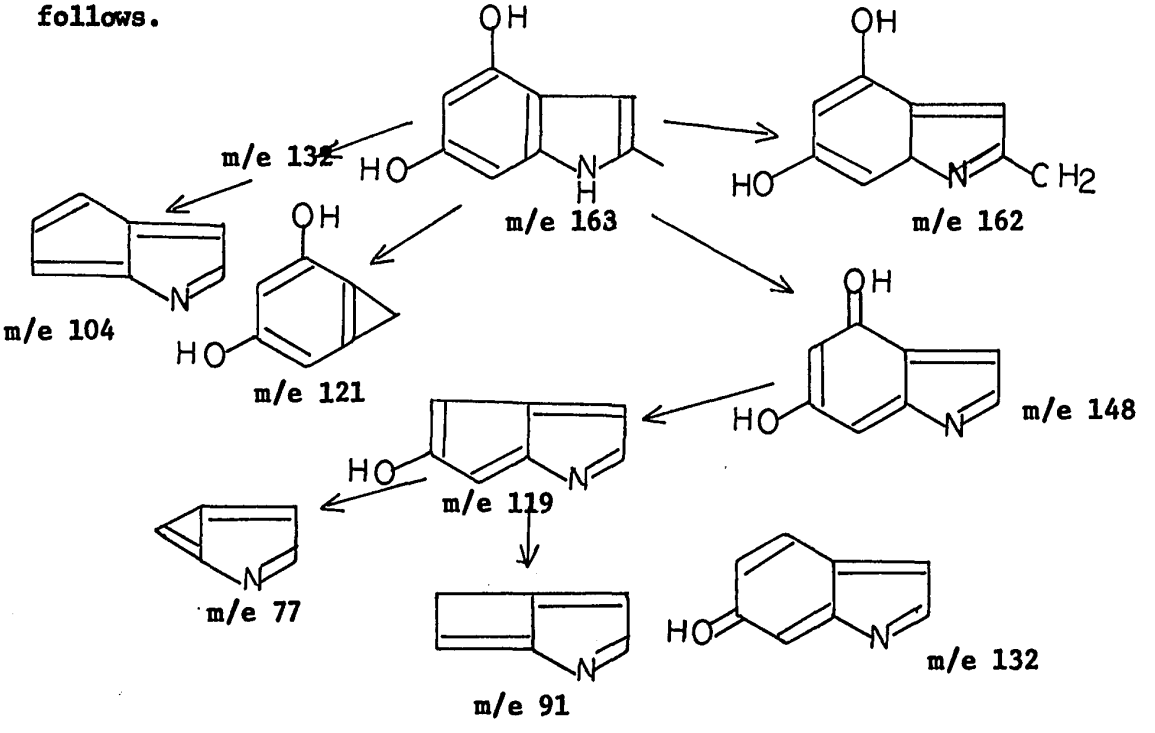


6-b



6-c

A mass spectral fragmentation for structure 6-a may be written as follows.

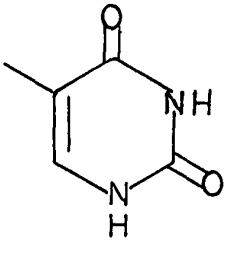


J. UV-Active Constituents of *Ophiacoma insularia* and *Ophiacoma erinaceous*

The aqueous concentrate from *Ophiacoma insularia* and *O. erinaceous* were extracted with 1-butanol. The butanol extract, on chromatography over a Sephadex-Cm column followed by thin layer chromatography over silica gel HF, furnished a crystalline solid (mp 225.5%) Ins-A, and an amorphous white solid, Ins-B.

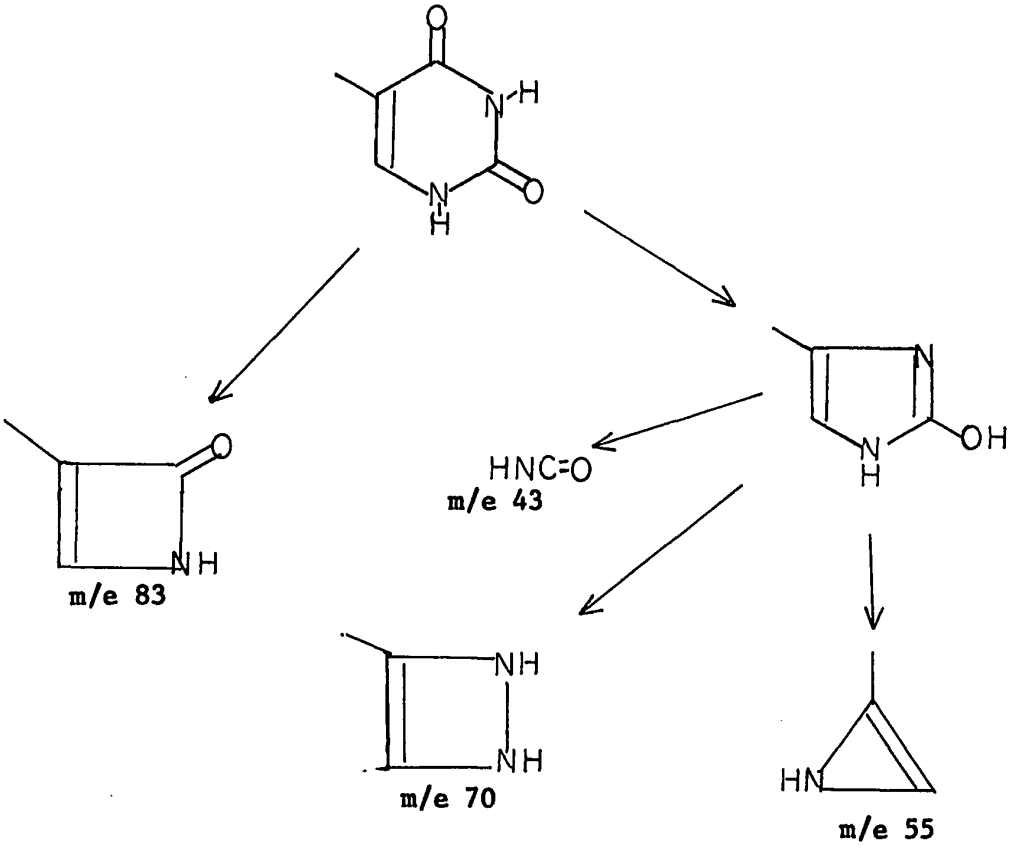
1. Ins-A

Ins-A was crystallized from ethanol as white needles, mp 225.5°. Ins-A had an ultraviolet maximum at 256 nm. An infrared absorption at 1660 cm^{-1} suggested the presence of a lactam moiety while the band at 1305 cm^{-1} suggested the presence of a methyl. The nuclear magnetic resonance spectrum exhibited a doublet at 1.90 δ (J=1.5 Hz) corresponding to three protons and a quartet at 7.40 δ (J=1.5 Hz) corresponding to one proton. The mass spectrum of Ins-A showed a parent peak at m/e 126 (78%), and a base peak at m/e 55. The other major peaks were at m/e 97, 83, 70, 53 and 39 respectively. The above information could readily be translated into structure.



THYMINE

Mass spectral fragmentation pattern of thymine is presented as follows.



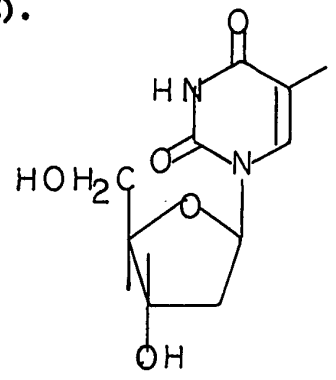
Comparison with an authentic sample of thymine proved the structure of Ins-A.

2. Ins-B

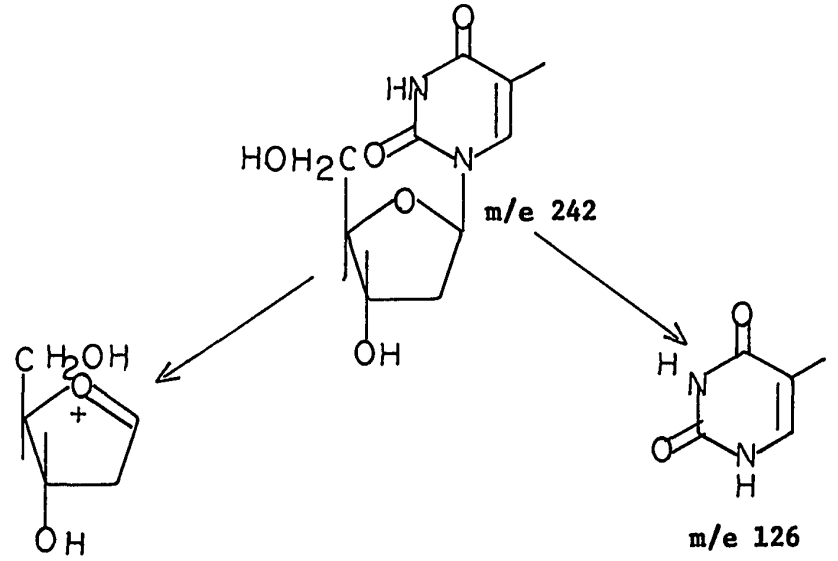
Ins-B was isolated as an amorphous white solid, mp 178°, with an ultraviolet absorption maximum at 265 nm. The infrared spectrum indicated the presence of a lactam, or amide (1650 and 1710 cm^{-1}), methyl (1380 cm^{-1}), a triply substituted double bond (850 cm^{-1}) and hydroxyl

(3250 and 1000 cm^{-1}) groups. The nuclear magnetic resonance spectrum exhibited a doublet at 1.95δ (J=1.5 Hz), complex structures at 3.79δ, 3.85δ, two triplets at 4.50δ (J=4 Hz) and 6.28δ (J=7 Hz), a quartet at 4.03δ (J=3.5-4 Hz) and a quartet (J=1.5 Hz) at 7.65δ. The spectrum was identical with that of thymidine.

The mass spectrum further proved the identity of Ins-B as thymidine by the presence of a parent peak at m/e 242 (1%), a base peak at m/e 43 and major fragments at m/e 126 (45%), 117 (27%), 113 (43%) and 83 (18%).



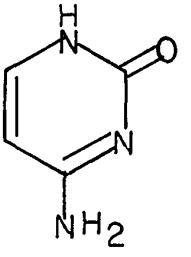
Scheme of the mass spectral fragmentation pattern of thymidine.



K. UV-Active Constituents of Echinothrix diadema and Holothuria atra

Compounds Ech-A, H_{A-A} and H_{A-B} were isolated. Ultraviolet maximum at 265 nm and the mass spectrum of Ech-A indicated it to be thymine.

Ultraviolet maxima at 267 nm and 265 nm and mass spectra of compounds H_{A-A} and H_{A-B} indicated them to be cytosine and thymine respectively.



CYTOSINE

L. Conclusion

Since some of the UV active compounds of toxic Palythoa accompanied palytoxin in the isolation scheme, it was thought that they might be structurally related to palytoxin. As of now, we have failed to establish any relationship.

We have, though, succeeded in establishing the identity of homarine (IX), m-tyramine (V) histamine (III) and anemonine (II) and have indicated the presence of alanine, sarcosine (by two dimensional thin layer chromatography), serotonine (XII) (mass spectrum of the mixture), and absence of choline esters other than acetyl choline in toxic Palythoa. In contrast, a study of non-toxic Palythoa (Palythoa mamilosa) indicated the presence of p-tyramine and tyrosine (paper chromatography). Similar differences have already been noted by Gupta (68) in his study of the sterols of coelenterates. He established the presence of 24-methylenecholesterol in toxic Palythoa and

cholesterol, brassicasterol, 22,23-dihydrobrassicasterol, gorgosterol and 24-β-ethylcholesterol in non-toxic Palythoa (Palythoa spp).

Homarine is a betaine which is widely distributed in marine organisms. Distribution and function of homarine has recently been reviewed by Gasteiger et al. (69). They have indicated the distribution of homarine in a rather systematic manner along their particular phylogenetic tree, increasing in concentration with evolution from simpler to more complex forms. Their study also revealed that the highest concentrations are reached in the large marine invertebrates such as Loligo Busycon and Limulus and are directly related to cellular activity. Quite in accordance with some previous observations on Echinodermata (69), we did not find homarine in Echinothrix diadema, Ophiacoma insularia and Holothuria atra.

m-Tyramine is not commonly found in marine animals. Hartman (70) referred to an enzymic preparation from O. bimaculatus capable of decarboxylating m-tyrosine but failed to establish the presence of m-tyramine or m-tyrosine. Bacq (11) has, however, invoked m-tyramine. A related interesting compound leptodactyline (m-hydroxyphenylethyltrimethyl ammonium) has been reported from skin extracts of a South American amphibian of the genus Leptodactylus by Erspamer (75).

Ackerman (8) isolated anemonine from Anemonia sulcata and established its structure by synthesis. Distribution of anemonine in marine animals has recently been reported by Welsh et al. (6).

In addition to the observation of Alender (21) on two diadematid, Echinothrix diadema and Echinothrix calamaris, we have found thymine in Echinothrix diadema, and thymine and thymidine in Ophiacoma insularia

and Ophiacoma erinaceous. Our studies on Holothuria atra demonstrated the presence of cytosine and thymine.

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