Organic constituents of the marine sponge Dysidea etheria, the nudibranch Hypselodoris zebra, and the gorgonian soft coral Briareum polyanthes
by Stephen Howard Grode

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Chemistry
Montana State University

Abstract:
This thesis comprises a report of research into the chemical constituents of the marine sponge Dysidea etheria, the gorgonian soft coral Briareum polyanthes, and the nudibranch Hypselodoris zebra. A total of eleven compounds were characterized, six of which were new molecules.

The compounds isolated and characterized from D. etheria were the sesquiterpenes furodysinin, 27, and the heretofore unreported furodysinin lactone, 56, and the ceramides α-hydroxy N-acylsphingo-sines, 60, and N-acylsphingosines, 61. The extraction and subsequent fractionation of H. zebra yielded the furanosesquiterpenes furodysinin, euryfuran, 34, 5-acetoxy nakafuran—8, 24, and 5-hydroxynakafuran8, 59. Briareum polyanthes was the source of a series of novel, highly functionalized diterpenes which possessed the briaran carbon skeleton. These molecules were given the trivial names, brianthein X, 69, Y, 68, and Z, 67.

Characterization of all isolates was accomplished, predominantly, by analysis of NMR, MS, IR, and UV data on the purified compounds and their chemical derivatives. The structure of brianthein Y was confirmed by an X—ray diffraction analysis.

ORGANIC CONSTITUENTS OF THE MARINE SPONGE *Dysidea etheria*, THE NUDIBRANCH *Hypselodoris zebra*, AND THE GORGONIAN SOFT CORAL *Briareum polyanthes*

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APPROVAL

of a thesis submitted by

Stephen Howard Grode

This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

8 Mar 1983
Date

John M Carsellman III
Chairperson, Graduate Committee

Approved for the Major Department

8/March/1983
Date

Edwin H. Abbott
Head, Major Department

Approved for the College of Graduate Studies

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Signature Stephen Grode

Date 11 March 1983

To my grandparents, whose foresight and fortitude brought them to the shores of this great nation.

To my parents, who allowed me the freedom to find my own way.

To my wife.

ACKNOWLEDGMENT

Acknowledging all those responsible for my professional development at Montana State University would not be possible. I would, however, like to thank Thomas R. James, Jr., for the isolation of the brianthein series. Thanks are also due to Dr. Robert T. Orth for helpful discussions on mass spectrometry and Richard Taylor and Dr. Mark Waddington for helpful instruction on the NMR spectrometer. The assistance of William Grey in setting up the plant pathogenic antimicrobial assays is also gratefully acknowledged. Dr. Bradford P. Mundy, Dr. P.W. Jennings, and their respective research groups also deserve to be thanked for allowing me the occasional use of their chemicals and glassware.

The most important acknowledgement is due my advisor, Dr. John H. Cardellina II, whose wit and good cheer were a source of constant encouragement during the trials and tribulations encountered during the course of this research project.

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ABSTRACT

This thesis comprises a report of research into the chemical constituents of the marine sponge Dysidea etheria, the gorgonian soft coral Briareum polyanthes, and the nudibranch Hypselodoris zebra. A total of eleven compounds were characterized, six of which were new molecules.

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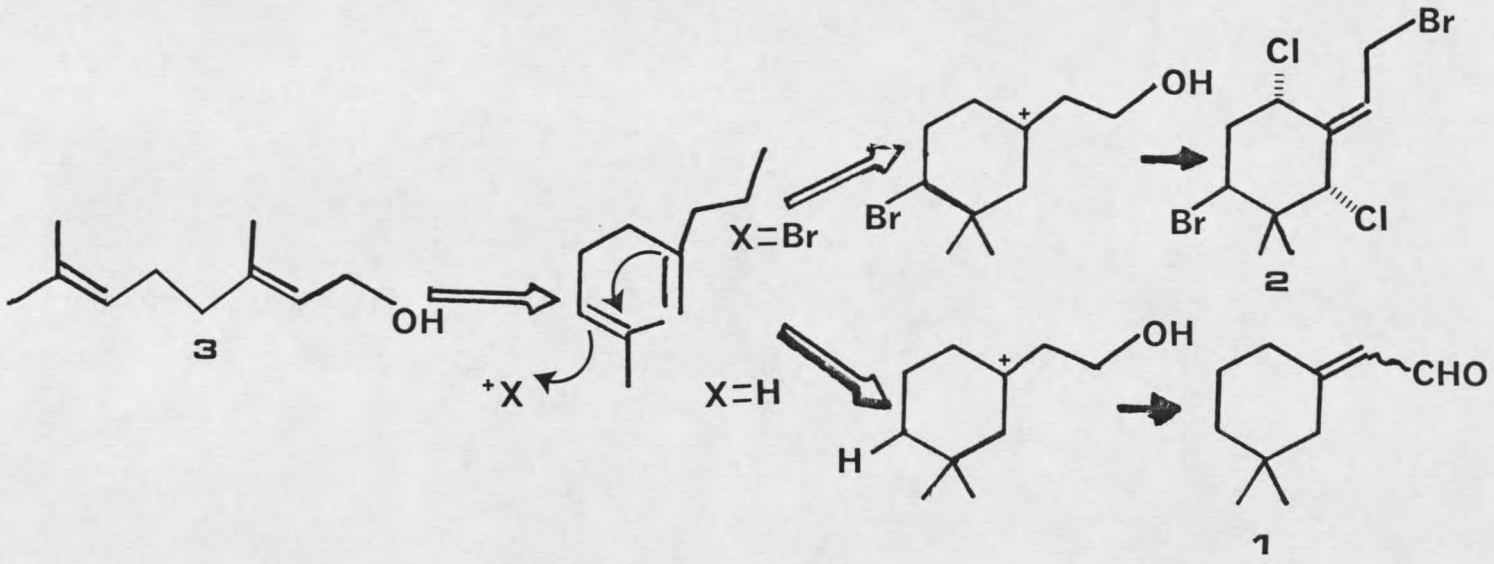
INTRODUCTION

The natural products chemist is primarily motivated by the search for new and unique compounds. From a biological perspective, the uniqueness might be inherent in biological activity (e.g., antibiotic, antimicrobial, antiviral, or antineoplastic), a role in chemical communication, or use as a taxonomic marker. From a chemical perspective, the uniqueness would be inherent in novel structure.

It is quite reasonable to suspect that the high ionic content of the ocean would be conducive to formation of unique compounds; that is, it would be possible to form biogenetic intermediates in the marine environment that would be unstable or highly unlikely in the terrestrial environment. An example of the differing intermediates formed in the marine and terrestrial environments, as presented in Scheme I, is the biosynthesis of cis and trans-3,3 dimethyl-$\Delta^{1,\alpha}$-cyclohexaneacetaldehyde, **1**, a sex pheromone from the male boll weevil Anthonomis grandis (1), and ochtodene, **2**, isolated from tropical red seaweeds of the family Rhizophyllidaceae (2). Starting from the same precursor, geraniol, **3**, the first step in the terrestrial environment is proton induced cyclization, whereas the first step in the marine environment is presumed to be bromonium ion induced cyclization. The next step in both is proton loss to form the alkene. Ochtodene is then further halogenated.

The intense interest in marine natural products began about fifteen years ago and was spurred by the aforementioned search for



Scheme I. Proposed Biosynthetic Pathways from Geraniol in the Marine and Terrestrial Environments.

novel structures. Systematic studies have been directed toward a particular phylum, such as sponges (3), toward a particular class of compounds (4-8), or toward a class of compounds in a phylum (9-13). Since many of these novel structures demonstrated biological activity, three major pharmaceutical firms, Hoffman La Roche in Australia, Roussel in Brazil, and Suntory in Japan, as well as independent researchers (14,15), have undertaken systematic screening of marine organisms for biological activity.

One of the most intensely studied invertebrate phyla is Porifera, with a steady stream of publications dating back to the late eighteen hundreds (16). Sponges are among the lowest and simplest forms of animal life. They are primitive multicellular animals that are more complicated than the protozoa and less complicated than the typical coelenterate. Some of the more interesting species, from a chemical standpoint, belong to the genus Dysidea. There are three classes in the phylum Porifera; Dysidea belongs to the class Demospongiae, order Dictyoceratida and family Disideidae. About one hundred different species of the class Demospongiae have been examined, yielding about two hundred new molecules, many of them novel and not observed in the terrestrial environment (12).

Most natural products chemists concentrate their search on secondary metabolites (e.g., terpenes, sterols, carotenoids, tyrosine derived bromo compounds, and bromopyrrole derivatives) leaving the primary metabolites (amino acids, carbohydrates, and

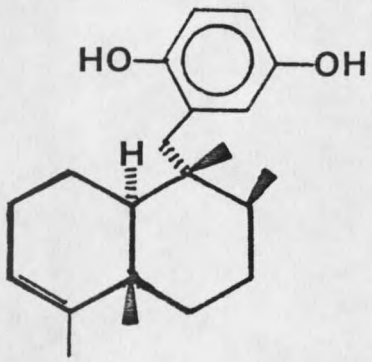
proteins) to the biochemists. Consequently, the following review of natural products will concentrate solely on secondary metabolites.

The secondary metabolites reported from species of Dysidea can be separated into three main classes: phenolic compounds, polychlorinated amino acid derived metabolites, and the terpenes. Avarol, **4**, is a sesquiterpenoid hydroquinone isolated from Dysidea avara and has been shown to inhibit cell division in sea urchin eggs (17). The (+) enantiomer of chromazonarol, **5**, was isolated from D. pallescens (18). Chromazonarol was originally isolated from Dictyopteris undulata, a brown seaweed (19).

Several polychlorinated amino acid derived metabolites were all isolated from Dysidea herbacea; however, they may actually be products of symbiotic blue-green algae or bacteria (20). The structure of dysidin (21), **6**, was determined by single crystal X-ray analysis, while the diketopiperazine (22), **7**, and dysidenin (23), **8**, were identified by NMR, IR, UV, and MS studies.

Terpenes are the most abundant secondary metabolites to be isolated from sponges. Most of these have been isolated from the order Dictyoceratida, to which Dysidea belongs. Sesquiterpenes are the most abundant terpenoids to be isolated from Dysidea species and all but one have a furan or oxidized furan ring.

A series of sesquiterpenes have been isolated from D. pallescens. Identification was made by chemical degradations, spectral data, biogenetic considerations, and establishment of the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OH
H

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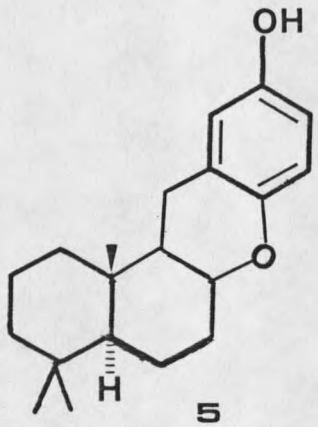
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O
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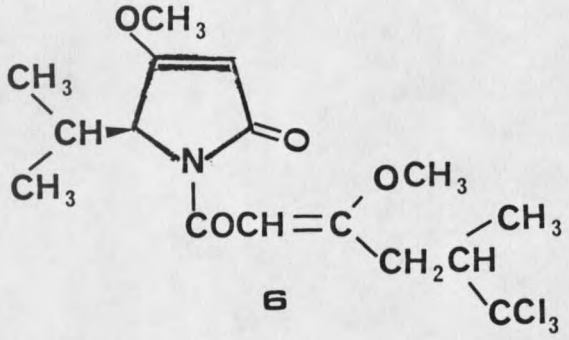
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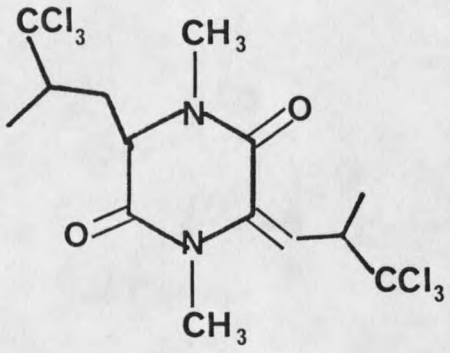
CH3
CH
CH3
N
O
COCH=C
OCH3
CH3
CH2CH
CCl3

6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O
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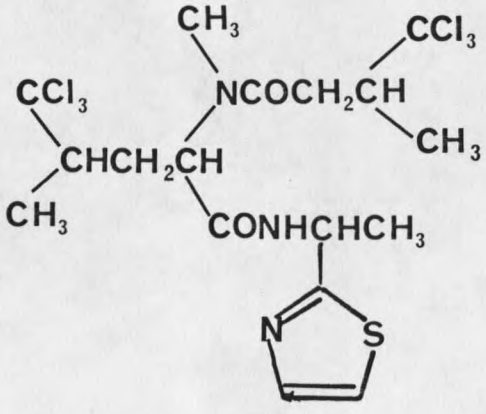
CCl3
CH3

7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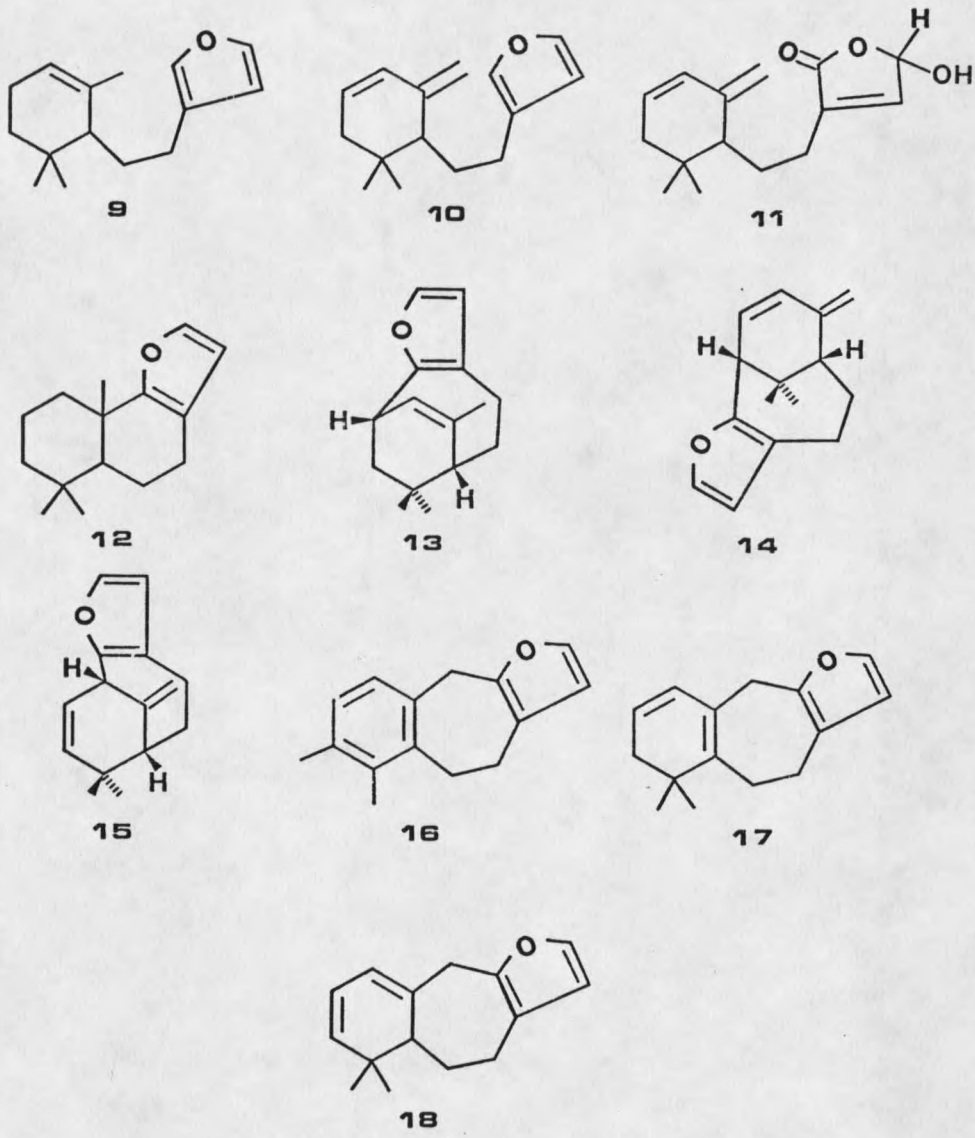
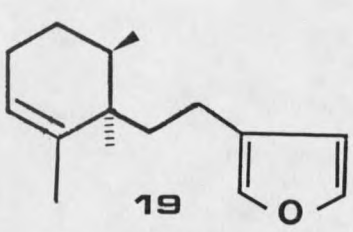
CH3
CONHCHCH3
N
S

8



9 10 11 12 13 14 15 16 17 18

interrelationship amongst them. Pallescensin-1, **9**, -2, **10**, and -3, **11**, are of the known monocyclofarnesane type (24) while pallescensin A, **12**, B, **13**, C, **14**, D, **15**, E, **16**, F, **17**, and G, **18**, are of previously unknown skeletons (25,26). Isolates from D. fragilis include, microcionin-2, **19**, and -4, **20**, (27) (originally isolated from Microciona toxystila (28)), nakafuran-8, **21**, and -9, **22**, (29)



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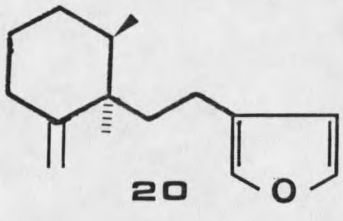

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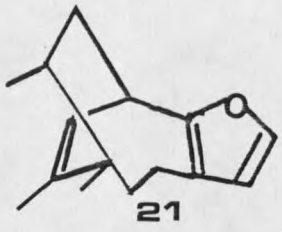

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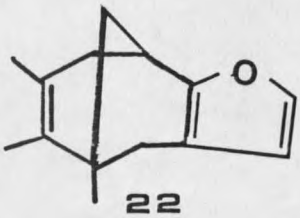

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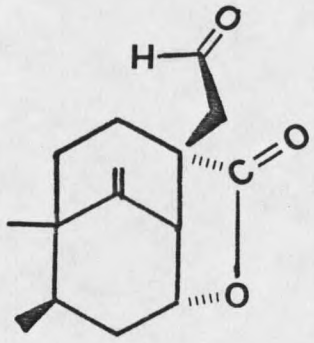

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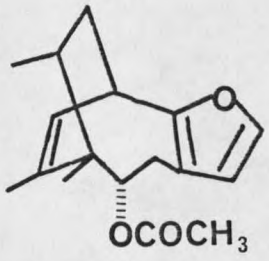


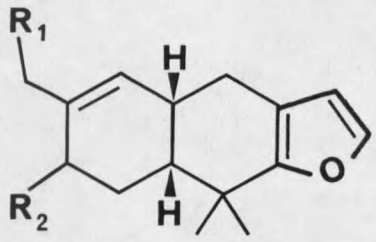
24

and upial, **23**, (30). All were identified by analysis of spectral data and chemical degradations. The 5-acetoxy nakafuran-8, **24**, was isolated from D. etheria and was also identified by spectral data and chemical degradations (31-33). Another series of sesquiterpenes has been characterized from D. herbacea (20). They are furodysin, **25**, thiolfurodysin, **26**, furodysinin, **27**, and thiolfurodysinin, **28**, (previously reported from an unidentified Dysidea species (34)), (4aS*,7R*,8aR*)-6,9,9, trimethyl-4,4a,7,8,8a,9-hexahydronaptho[2,3-b]furan-7-ol, **29**, (4aR*,6R*,8aS*)-4,4,7 trimethyl-4,4a,5,6,8a,9-hexahydronaphtho[2,3-b]furan-6-ol, **30**, (4aR*,7R*,8aS*)-4,4,7-trimethyl-4,4a,5,6,8a,9-hexahydronaphtho[2,3-b]furan-6-ol, **31**, 4,4-dimethyl-7-methylene-cis-4,4a,5,6,7,8,8a,9-octahydronaptho[2,3-b]furan, **32**, (2Z,4E,6E)-3-(4,8-dimethylnona-2,4,6-trienyl)furan, **33**, and euryfuran, **34**. All were identified using spectral data only. The probable precursor of furodysin and furodysinin is spirodysin,

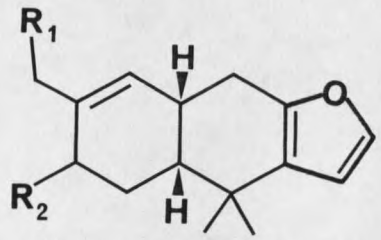
35, also isolated from D. herbacea (35). Furodysin and furodysinin were obtained by exposure of spirodysin to boron trifluoride etherate or heat (35).

Until a few years ago, diterpenes isolated from the marine environment were quite rare. Recently, however, compounds have been identified from seaweeds (36–38), coelenterates of the orders Alcyonacea (39–43) and Gorgonacea (44–50) and from a few sponges (51). A series of diterpenes have been isolated from Dysidea amblia (52). Ambliol A, **36**, dehydroambliol A, **37**, ambliolide, **38**, ambliofuran, **39**, and ambliol B, **40**, were identified by spectral data and chemical degradation. The sponge was steeped in methanol for two weeks at 0°C, homogenized, and then Soxhlet extracted with fresh methanol for two days. Ambliolide, therefore, was probably formed by air oxidation of ambliol A in the methanolic solution.

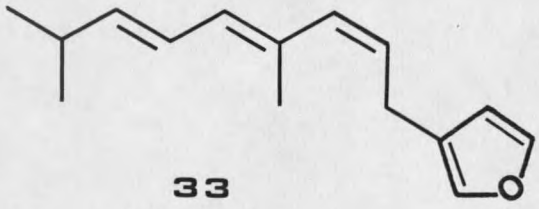
Sesterterpenes are very rare in terrestrial fauna and flora but are more commom in the marine environment, especially in sponges. Disidein, **41**, isolated from D. pallescens is a pentacyclic sesterterpene, whose identification was accomplished primarily by the mass spectral framentation pattern of the diester degradation product (53). Its biogenesis, as presented in Scheme II, is probably the proton catalyzed cyclization of the linear phenolic sesterterpene, **42**. Furospongolide, **43**, is a twenty one carbon terpene isolated from D. herbacea collected in the Red Sea (54). Tetranorsesterterpenes are among the most unusual terpeniod



	R_1	R_2
25	H	H
26	SAc	H
29	H	βOH



	R_1	R_2
27	H	H
28	SAc	H
30	H	βOH
31	H	αOH



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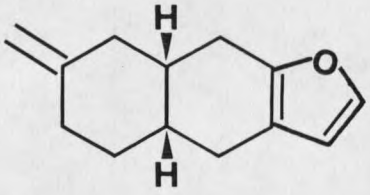

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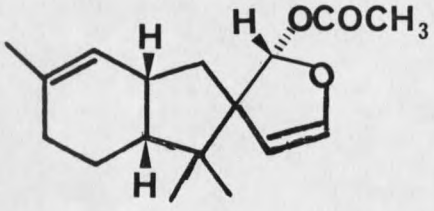

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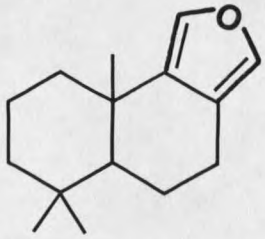

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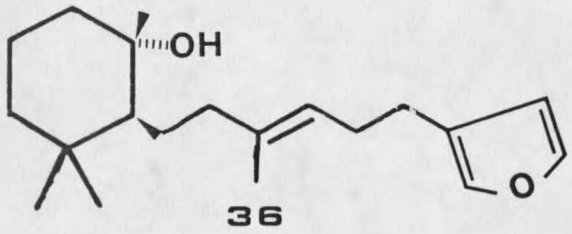

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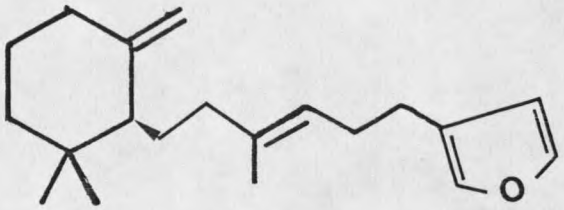

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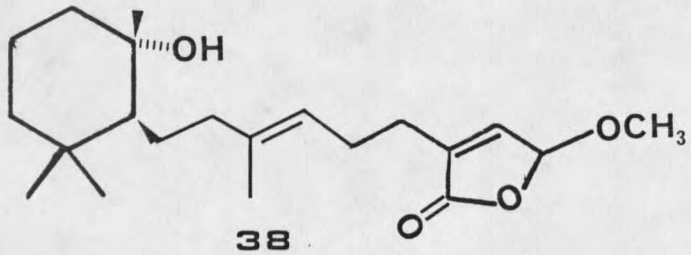

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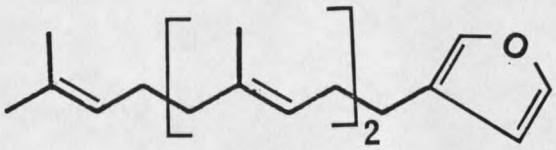

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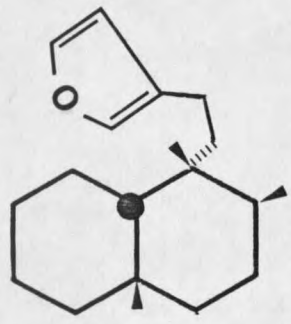

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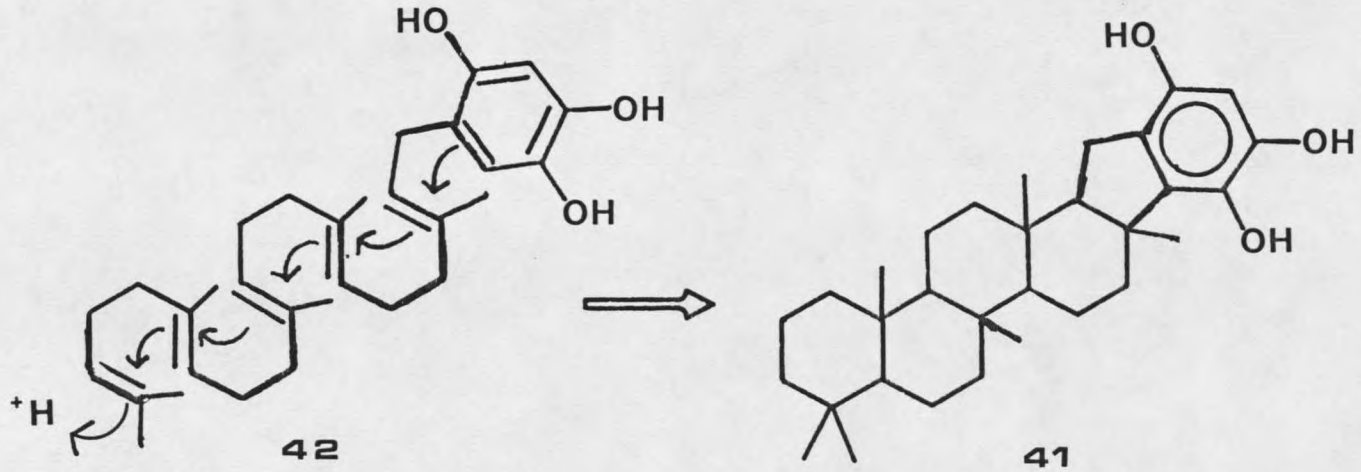


Scheme II. Proposed Biosynthetic Pathway of Disidein.

compounds isolated from marine sponges and are thought to arise from oxidative degradation of sesterterpenes.

Nudibranchs, gastropod mollusks of the order Nudibranchia (subclass Opisthobranchia) are often observed feeding upon marine sponges. Their often vibrant coloring and lack of external shell would, seemingly, make them easy targets for predation, yet they are not sought after by fish, other mollusks, and crab predators; carnivorous fish have been observed to reject nudibranchs. The suborder Doridaceae has been the most intensely studied; two chemical defense mechanisms have been observed, secretion of a strong acid (55,56) or secretion of a neutral toxic or noxious substance (57,58). The compounds responsible for the latter mechanism are presumed to be of dietary origin.

Recently, dorid nudibranchs of the genus Hypselodoris have been the subject of interest. The secondary metabolites isolated are predominantly furanosesquiterpenes. Faulkner's group at Scripps Institution of Oceanography examined four nudibranchs of the genus Hypselodoris and isolated a total of nine furanoterpenes (59). Agassizin, **44**, was found in H. agassizi collected at Cruz de Juanacaxtle, Nayarit, Mexico, and nakafuran-9, **22**, the butenolide of nakafuran-9, **45**, dendrolasin, **46**, and the furanoditerpene ghiselinin, **47**, were isolated from H. ghiselini collected at Isla Danzante, Gulf of California. Specimens of H. californiensis collected at Isla San Jose, Gulf of California yielded dendrolasin and nakafuran-8, **21**, while those collected at Point Loma, California contained furodysinin, **27**, euryfuran, **34**, and pallescensin A, **12**.

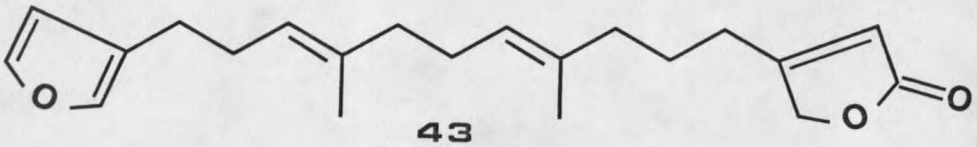
Six specimens of H. porterae found feeding on Dysidea amblia, also collected at Point Loma, California, contained furodysinin and euryfuran. The marine sponge Euryspongia sp., collected at Casa Cove, La Jolla, California, was found to contain euryfuran and is thought to be the source of that isolated from Hypselodoris species.

H. godeffroyana, observed feeding on Dysidea fragilis in Kaneohe Bay, Oahu, contained nakafuran-8 and nakafuran-9. D. fragilis was subsequently shown to be the source of the metabolites (29). H. daniellae, collected at the Ala Wai Canal, Honolulu, posessed spiniferin-2, 48, (60) first isolated from the sponge Pleraplysilla spinifera (61).

It is presumed all furanoterpenes isolated from Hypselodoris species have antifeedant properties (59), however, it has been demonstrated only in nakafuran-8, nakafuran-9, furodysinin, and pallescensin A (29,59,60).

Soft corals are sessile, soft bodied invertebrates usually found in tropical waters. They belong to the phylum Cnidaria, class Anthozoa, and subclass Octocorallia. There are three orders, Stolonifera, Alcyonacea, and Gorgonacea. Recently much interest has been devoted to the gorgonian soft corals due to the isolation of prostaglandins (62-64) and unique terpenoid compounds therefrom (9). One of the most examined genera from Gorgonacea is Briareum.

Briareum asbestinum, heretofore the only species investigated, has yielded, a series of highly functionalized diterpenes. Specimens collected at St. Thomas, Virgin Islands, and at Grand



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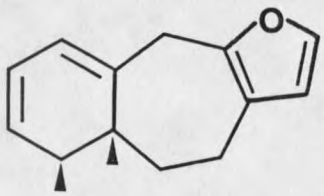

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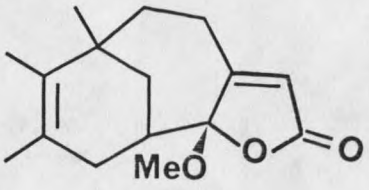

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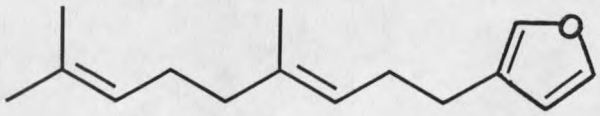

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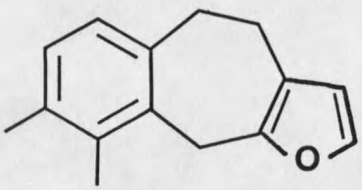

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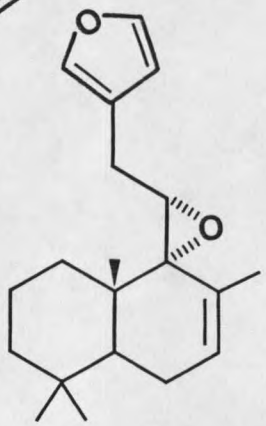

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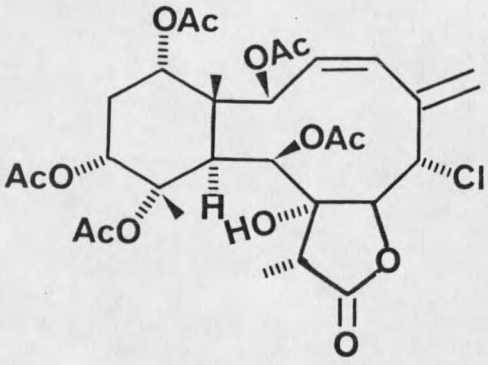
Bahama yielded the not fully characterized briareins C and D (47), and from a Jamaican collection, briarein A, **49**,(48). From a collection off the coast of Belize, briarein B and asbestinins -1, **50**, -2, **51**, -3, **52**, -4, **53**, and -5, **54**, have been isolated (49). A collection from Roatan Island, Honduras produced asbestinin epoxide, **55**, and asbestinin-5 acetate, **56**, (50). The structure of briarein A and the diol obtained by hydrolysis of asbestinin-1 were elucidated by single crystal X-ray. The structure of briarein B, briarein A with a butyrate ester replacing one of the acetate groups, is not fully solved. Characterization of the remaining asbestinins was accomplished by analysis of spectral data and chemical interconversion. Asbestinin-1 and its hydrolysis product, and asbestinin-5 were shown to antagonize the effects of acetylcholine on guinea pig ileum preparations (50).



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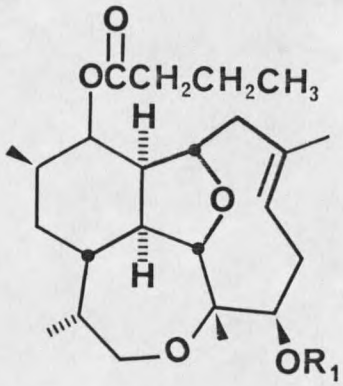
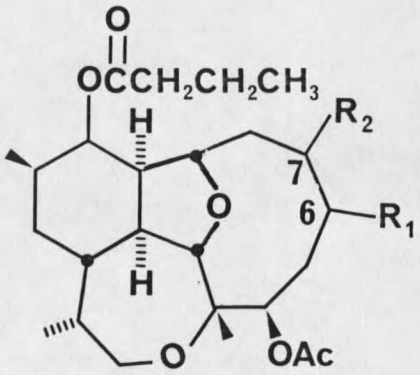
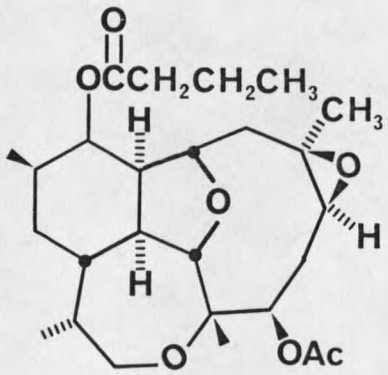


	R_1
50	Ac
52	H



	R_1	R_2
51	H	CH_3, $\Delta^{6,7}$
53	=O	$=CH_2$
54	····OH	$=CH_2$
56	····OAc	$=CH_2$



55

STATEMENT OF THE PROBLEM

The central problem confronting the natural products chemist is structure elucidation, thus the primary objective of the research described herein is the characterization of novel compounds. The criterion in choosing which marine organism to examine was the uniqueness of compounds isolated from related organisms and the lack of literature pertaining to the particular organism. The marine sponge Dysidea etheria and the soft coral Briareum polyanthes met both criteria. In addition, the weakly antibiotic activity of Dysidea etheria, the taxonomic identification of Briareum polyanthes, and the role of chemical communication in the isolates of Hypselodoris zebra, prompted this investigation. Antimicrobial activity was to be assessed for all compounds isolated.

RESULTS AND DISCUSSION

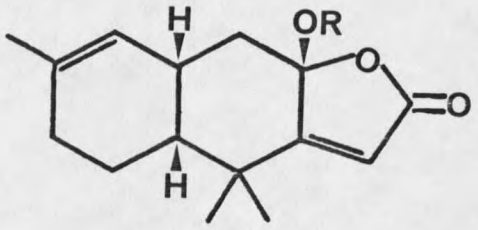
Sesquiterpenes from Dysidea etheria

Dysidea etheria was first described as a new species by M. W. deLaubenfels in 1936 (65). The description of the beauty of a tropical reef and his encounter with D. etheria is as follows:

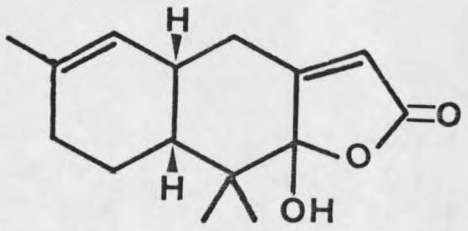
> As one walks about on the bottom of the sea near the Dry Tortugas, using a diving apparatus, at depths of less than 10 meters... Gaudy fish swim through the forests of purple and yellow bush-like coelenterates (corals and gorgonians). Not the least striking nor least beautiful are the sponges in this habitat. Big dark Spheciospongia vesparias stand up as big as barrels, and shaped somewhat like them. Graceful vase-shaped Hircinia campanas abound. There are rich red bushes of Haliclona rubens, chrome-yellow bushes of Verongia fulva, two or three Haliclonas of different shades of green, and dark purple bushes of Iotrochota birotulata. There are small flaming orange sponges such as Myriastra kallitetilla and such azure blue ones as Dysidea etheria... Comparisons to a 'fairyland' are appropriate.

Literature accounts of D. etheria are sparse. Jakowska and Nigrelli (66) reported extracts of D. etheria demonstrated weak antimicrobial activity against Escherichia coli and poikilotherm and bovine forms of tubercle bacilli and, after this research was completed, a brief reference to the appearance of furodysinin in D. etheria was reported (67).

D. etheria was collected from a variety of shallow water habitats in Bermuda, primarily in Harrington Sound, and stored in acetone at -5°C prior to extraction. The sponge was homogenized and then extracted with acetone, followed by dichloromethane.



56 R=H
58 R=CH_3



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Antimicrobial assays on freshly prepared extracts of D. etheria were weakly positive for Bacillus subtilis and negative for Staphylcoccus aureus, Escherichia coli, Pseudomonas aeruginosa, and Candida albicans. The crude dichloromethane soluble extracts exhibited weak activity against S. aureus, C. albicans, and Mucor rhaminianus and no activity against B. subtilis, E. coli and P. aeruginosa. The water soluble extracts exhibited trace activity against S. aureus and C. albicans and no activity against B. subtilis, E. coli and P. aeruginosa.

Chromatography of the dichloromethane soluble extracts concentrated the marginal antibiotic activity in relatively polar fractions eluted with ethyl acetate and small quantities of methanol in ethyl acetate. Gel permeation chromatography and subsequent reversed phase HPLC of those fractions yielded small quantities of a colorless solid which was subsequently shown to be the sesquiterpene lactone **56**.

Inspection of the ^{13}C-NMR spectral data (see Figure 1) for **56** revealed the presence of one carbonyl, two olefinic linkages, a quaternary sp^3 carbon substituted by two heteroatoms (δ104.91), one other quaternary carbon, two methines, three methylenes, and three methyl groups. The total of fifteen carbons, including three methyl groups, indicated a probable sesquiterpene. Accurate mass spectral measurements (see Figure 2) delineated a molecular formula of $C_{15}H_{20}O_3$. The carbonyl and two carbon-carbon double bonds left three sites of unsaturation, which were attributed to a tricyclic skeleton.

From the ^1H-NMR data (see Figure 3), the two olefinic protons, a singlet at δ5.67 and a broad doublet at δ5.36, had to be on separate trisubstituted double bonds. A broad D_2O exchangeable singlet at δ3.5 was assigned to a hydroxyl group (3580, 3330 cm^{-1}). An unsaturated γ-lactone, 1745 cm^{-1} and λ_{max} 221 nm (ε=8700), accounted for one ring and the other oxygens, meaning that the quaternary carbon at δ104.91 bore both the hydroxyl group and the lactone oxygen, as illustrated in **56a.**

Careful integration in the ^1H-NMR between δ1.50 and 1.75 indicated six protons instead of the apparent five protons. The LIS experiments revealed the hidden proton and extrapolation to zero equivalents indicated its chemical shift to be δ1.63. Spin-spin decoupling of the normal spectrum and that recorded after addition of 0.552 equivalents $Eu(fod)_3$ suggested part structure **56b.** Examination of coupling constants (see Table 1) indicated a <u>cis</u> ring juncture ($J \approx 2.5$ Hz between H_6 and H_{11}) of the two carbocyclic rings.

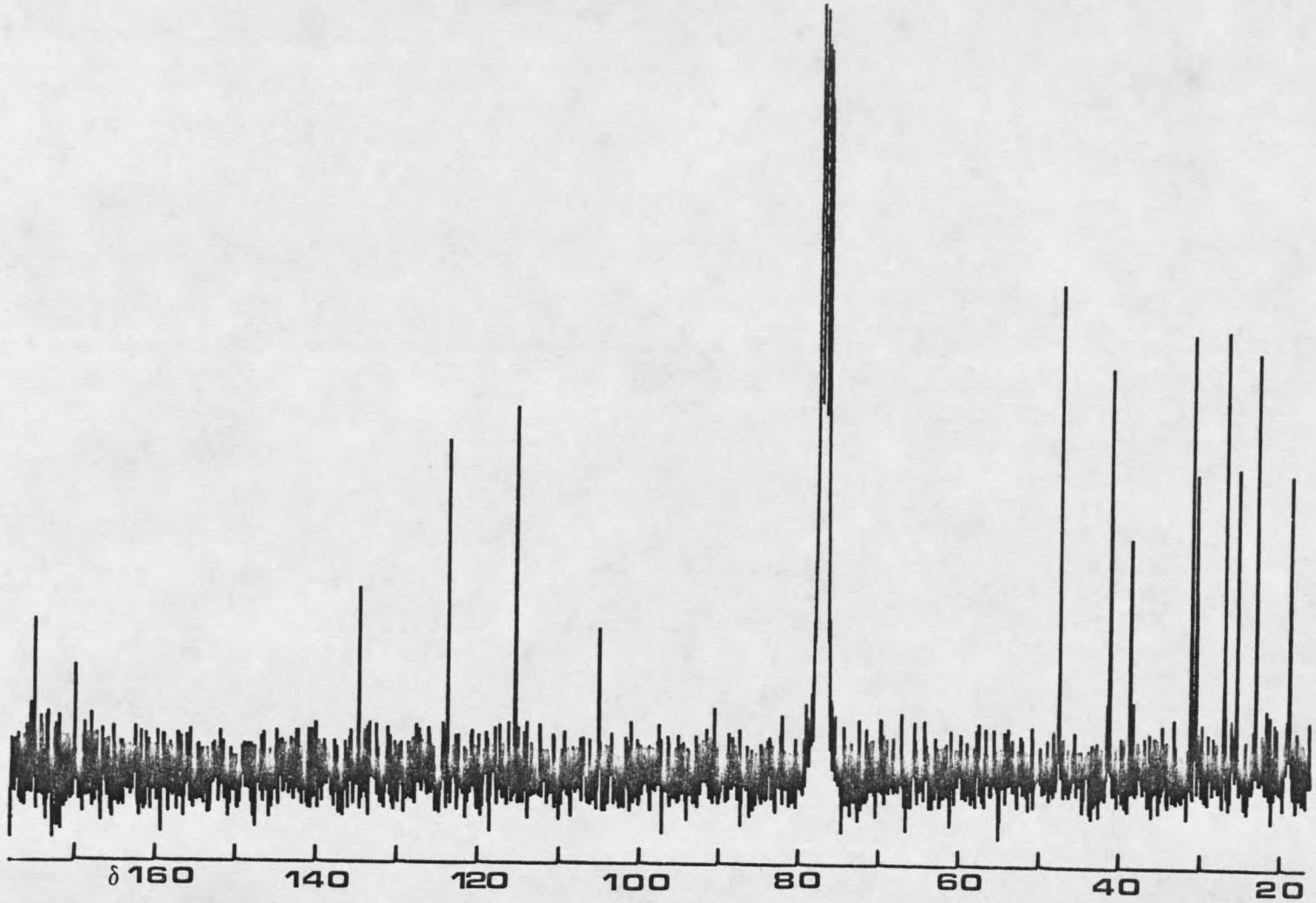


Figure 1. ^{13}C-NMR Spectrum of Furodysinin Lactone.

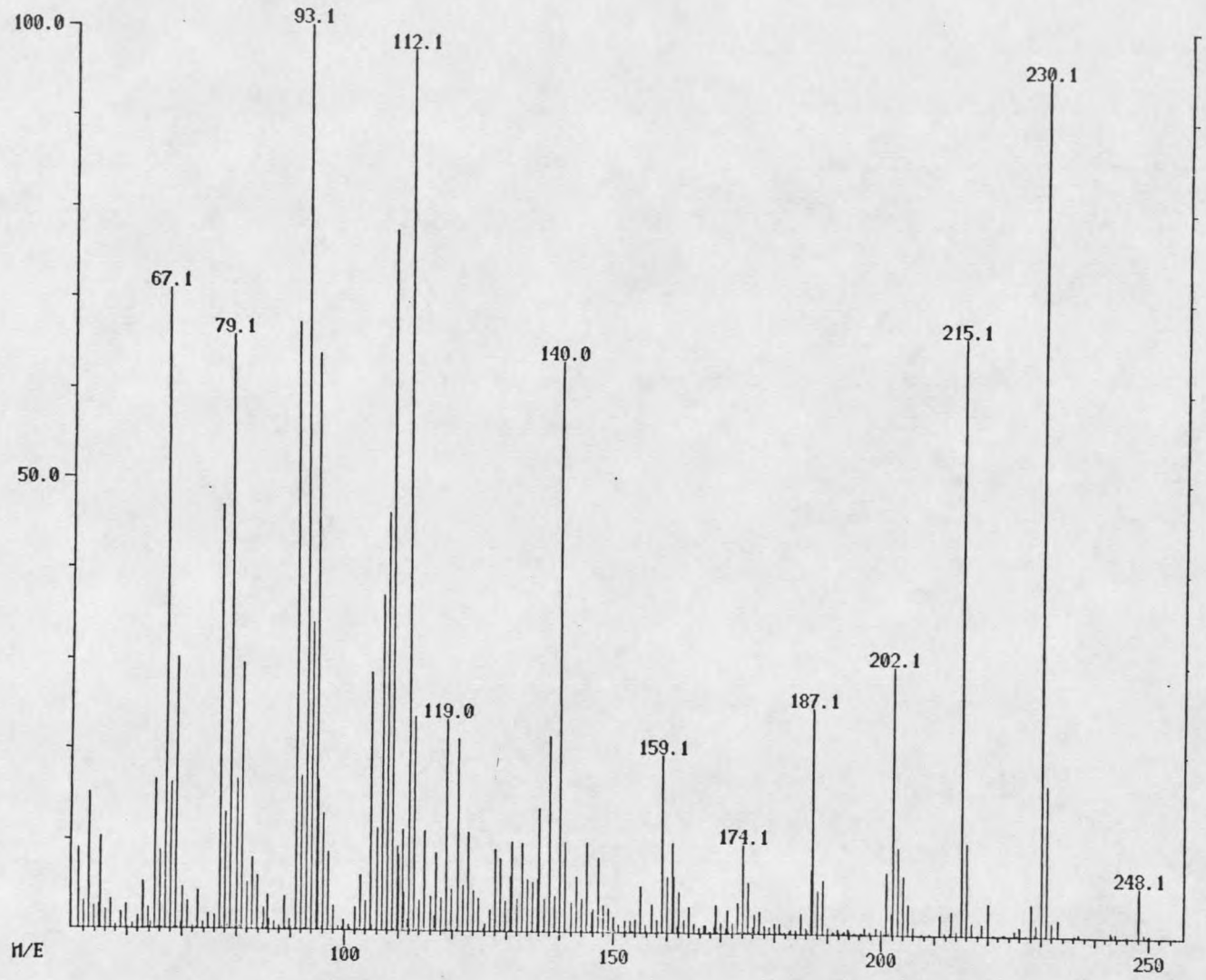


Figure 2. Mass Spectrum of Furodysinin Lactone.

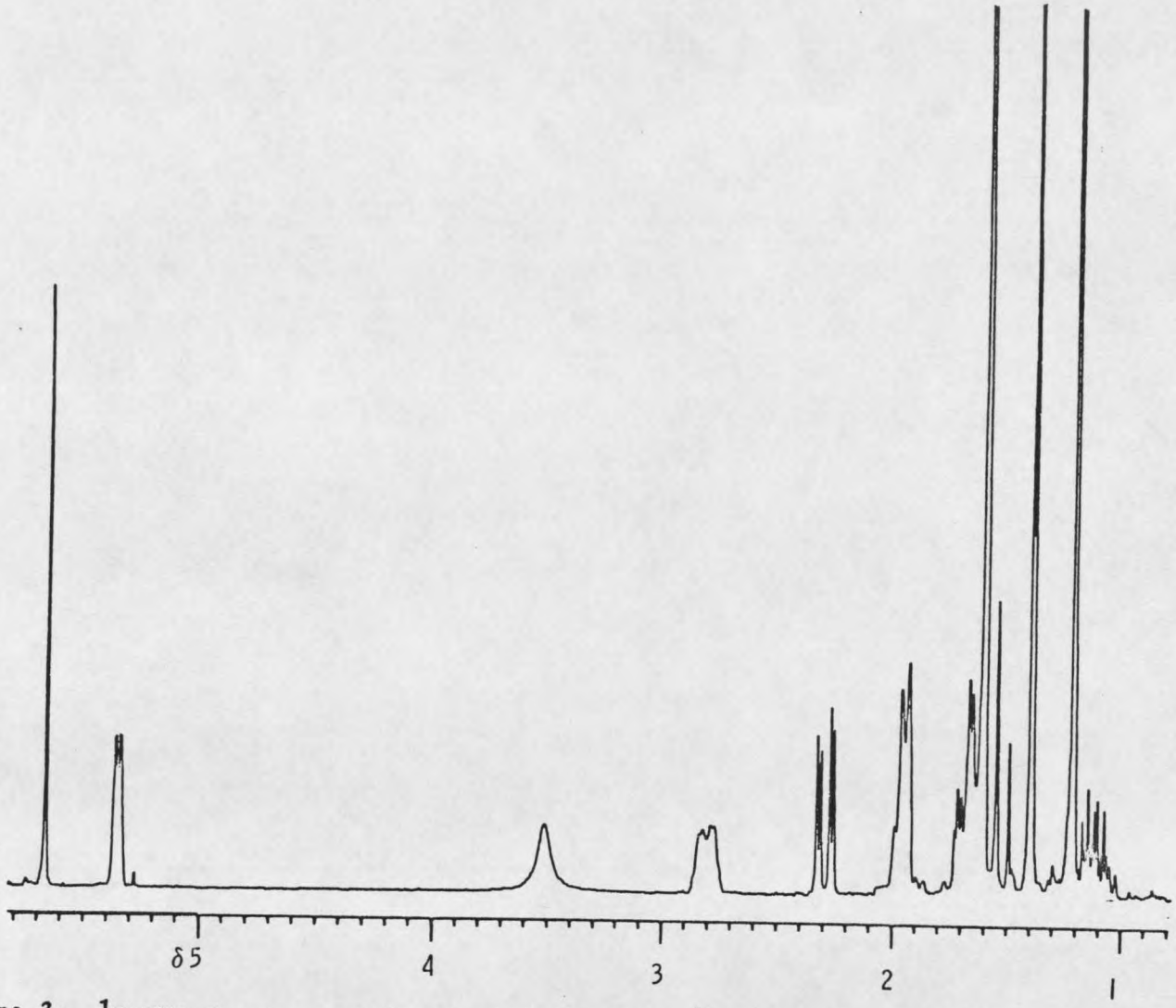


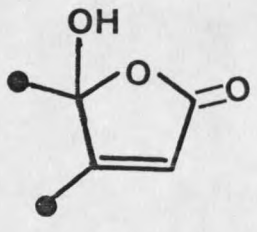
Figure 3. ^{1}H-NMR Spectrum of Furodysinin Lactone.

Table 1. ^{1}H-NMR Assignments for Furodysinin Lactone

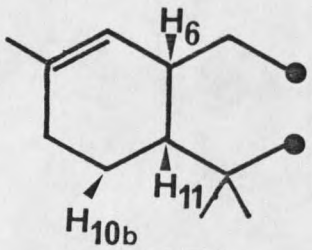
Hydrogen	Chemical Shift(δ)	Multiplicity	Coupled to (J, Hz)
H_2	5.67	s	
OH	3.5	br s	
H_{5a}	1.57	dd	H_{5b} (14), H_6 (13)
H_{5b}	2.28	dd	H_{5a} (14), H_6 (3.8)
H_6	2.80	m	H_{5a} (13), H_7 (5.7) H_{5b} (3.8), H_{10b} (3.7)* H_{11} (2.5)**
H_7	5.36	br dd	H_6 (5.7), $H_{9a,b}$ (1.4)
$H_{9a,b}$	1.96	overlapping m	H_{10a} (9.5), H_7 (1.4)
H_{10a}	1.12	br ddd	H_{10b} (12.6) H_{11} (12.6) $H_{9a,b}$ (9.5, unresolved, small)
H_{10b}	1.70	br ddd	H_{10a} (12.6), H_6 (3.7)* H_{11} (3.1) $H_{9a,b}$ (unresolved, small)
H_{11}	1.63**	m	H_{10a} (12.6), H_{10b} (3.1) H_6 (2.5)**
H_{13}	1.38	s	
H_{14}	1.21	s	
H_{15}	1.61	br s	

*W coupling

**Not resolved in normal spectrum; chemical shift obtained by extrapolation to zero equivalents $Eu(fod)_3$; after addition of 0.554 eq., an apparent dt (J ≅ 13.3, 2.5, 2.5).



56a

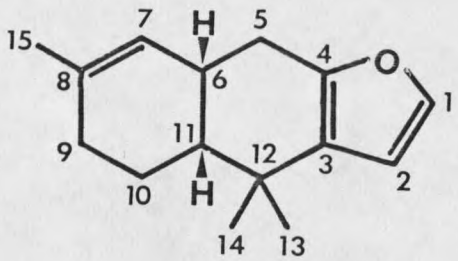


56b

Somewhat perplexing at first glance was the coupling between H_6 and H_{10b}, but construction of a Dreiding model illustrated that when the dihedral angle between H_6 and H_{11} was minimized, H_6 and H_{10b} were aligned quite properly for W coupling. The two methyl singlets (δ1.38 and 1.21), IR absorptions at 1371 and 1348 cm^{-1}, and a fully substituted sp^3 carbon (δ38.47) were ample evidence for a germinal dimethyl group and accounted for the remaining elements in the molecule.

Assembling the substructures gave **56** or **57**. Originally, **57** was favored because it followed the isoprene rule. Two subsequent experiments; however, rigorously established **56** as the proper structure.

The ^{1}H-NMR of the lactone bore a resemblance to the ^{1}H-NMR of a major non polar component isolated from the first fraction of the aforementioned Florisil chromatography. Purification could not be accomplished by gel permeation (Sephadex LH-20 and Bio-Beads S-X8), silica gel chromatography (flash and gravity), argentation chromatography (15% silver nitrate impregnated SiO_2) and HPLC



27

(Ultrasphere-Cyano, ODS, and Si). Thus, all spectral work was done on nearly pure material. The analysis of the ^{1}H-NMR (see Figure 4), which indicated a 2,3-disubstituted furan (δ7.21 and 6.22) and a resemblance between the signals at δ1.96, 1.70, and 1.61 of **56** and δ2.03, 1.71, and 1.66 of the furanoid compound suggested molecules with a similar skeleton. Mass spectral analysis (see Figure 5) indicated a molecular formula of $C_{15}H_{20}O$ and the fragmentation pattern along with the inverse gated ^{13}C-NMR (see Figure 6) unequivocally established the compound as the non-isoprenoid furodysinin **27** (20).

In order to confirm the apparent relationship of furodysinin and the newly discovered lactone, the oxidation of **27** was undertaken. Photoinduced oxygenation (68) of **27** in methanol gave, exclusively, a ketal-lactone shown to be **58** by ^{1}H-NMR (δ3.12, 3H, s). The ketal could not be hydrolyzed to the hydroxy lactone under a variety of acidic conditions: 4% HCl at room temperature for 8 hours, 4% HCl at 50 C for 2.5 hours, and 4% HCl at reflux for 2.25 hours.

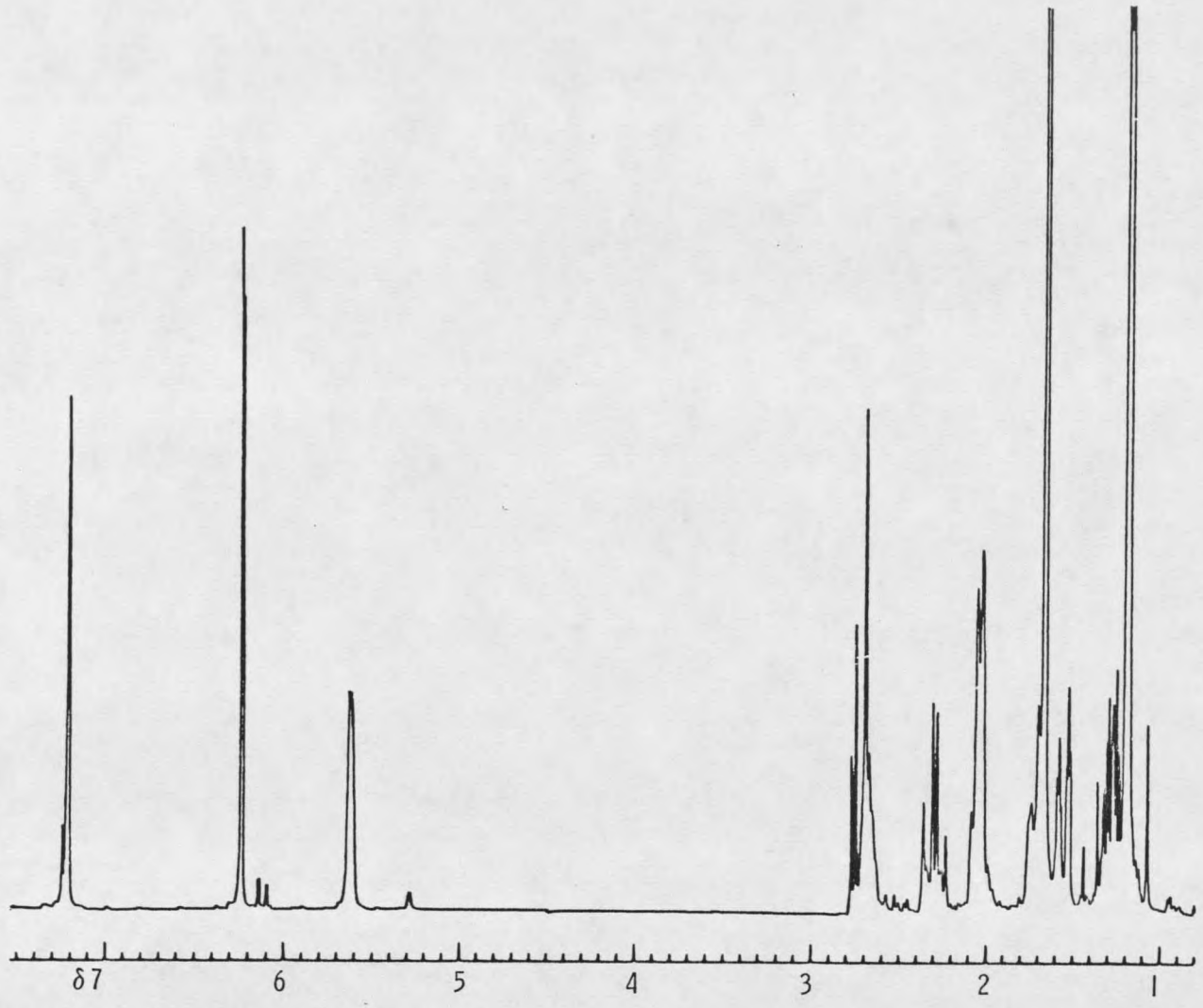


Figure 4. ^{1}H-NMR Spectrum of Furodysinin.

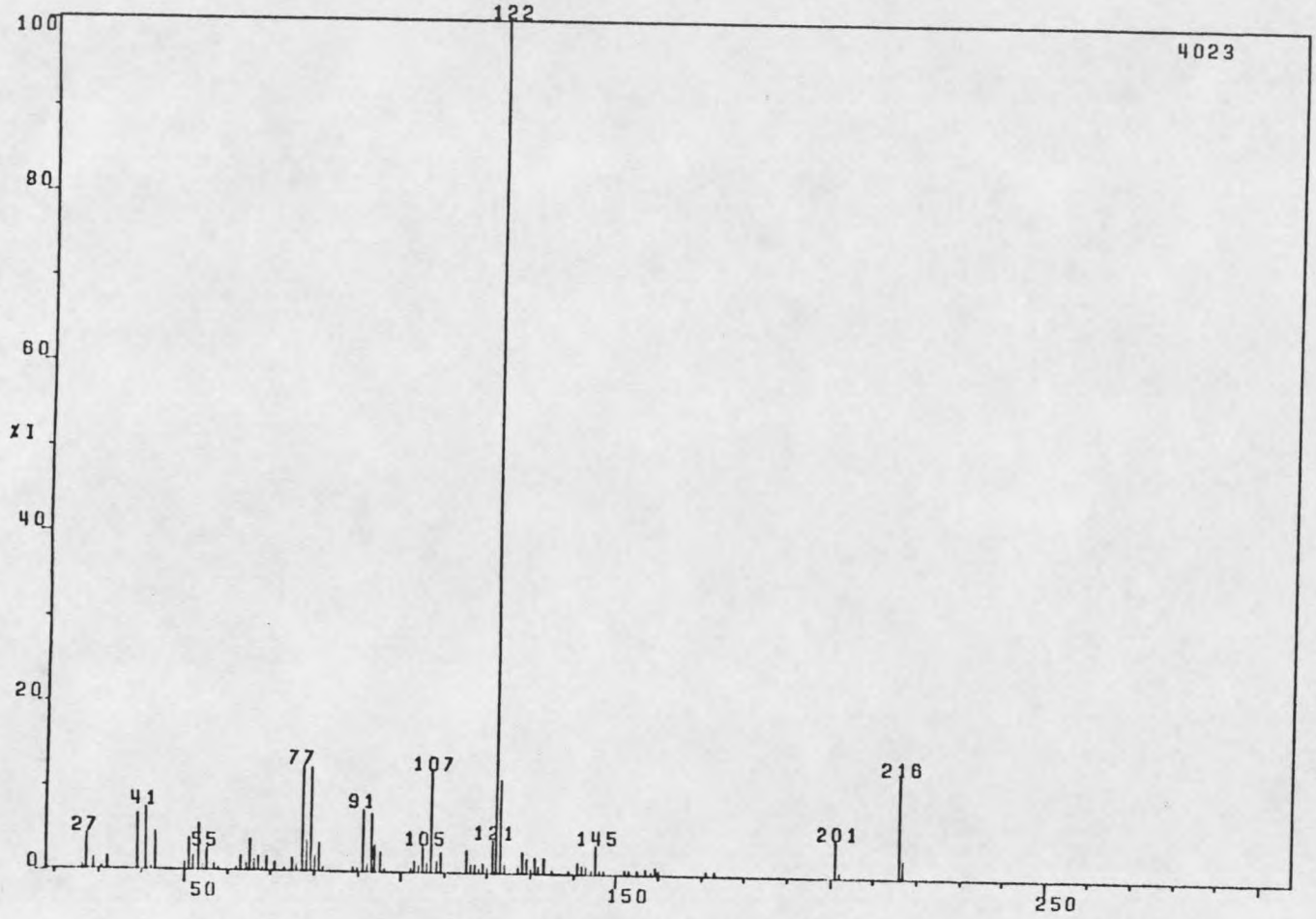


Figure 5. Mass Spectrum of Furodysinin.

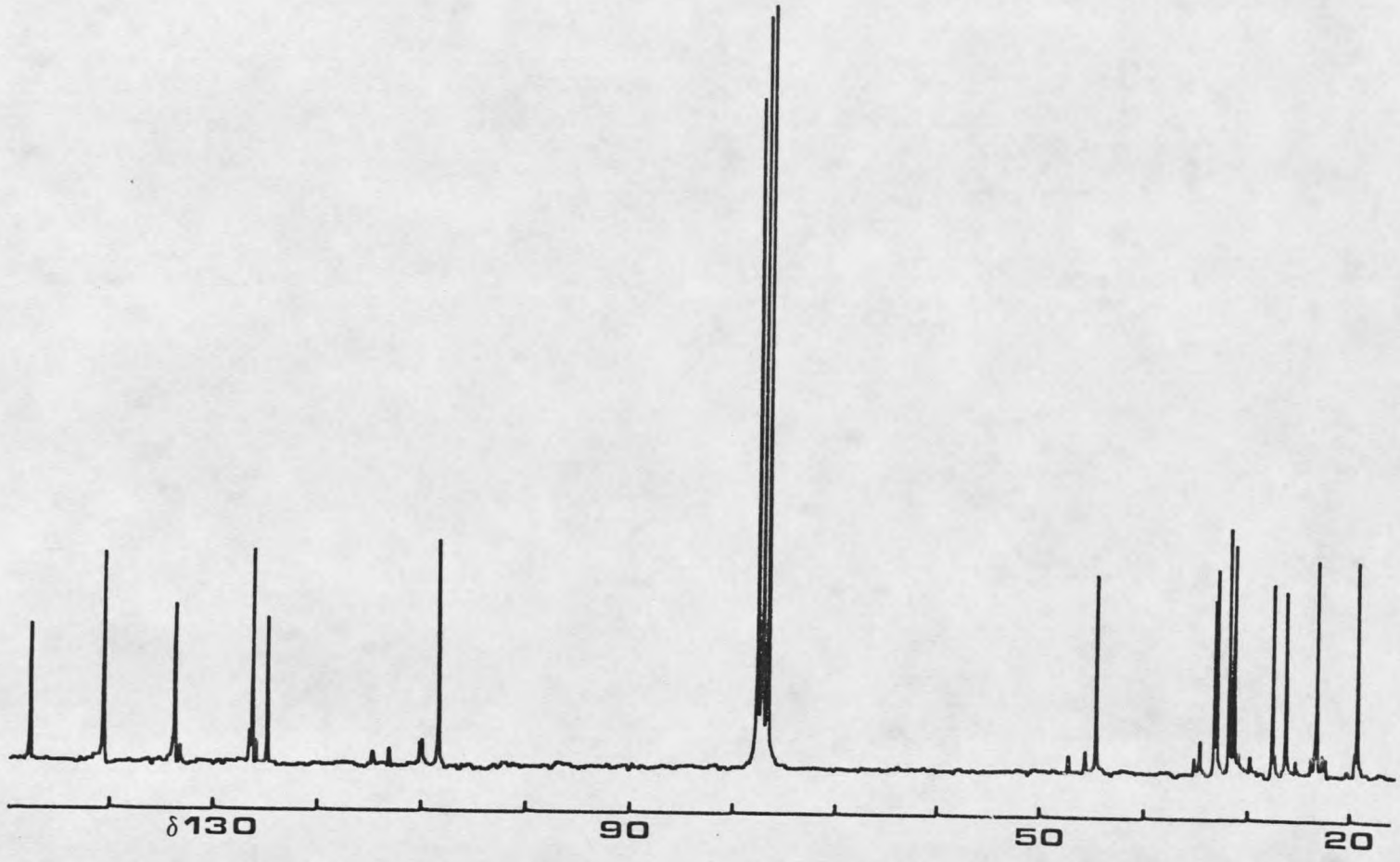


Figure 6. Inverse Gated ^{13}C-NMR Spectrum of Furodysinin.

Repetition of this oxidation, using tetrahydrofuran-water in place of the methanol, gave mostly starting material. The conversion was finally achieved by a two step oxidation with meta-chloroperbenzoic acid, followed by Jones reagent (29). The sole product of this sequence gave a ^{1}H-NMR spectrum superimposable with that of the natural lactone, indicating that one of the diastereomers of **56** (epimers at C-4) was the structure of both the natural product and the semisynthetic lactone.

The ^{13}C-NMR assignments for **56** and **27** are presented in Table 2. The assignments for furodysinin lactone followed from an off-resonance decoupled and three single frequency off-resonance decoupled experiments (SFORD) irradiating at δ2.80, 2.28, and 1.12. The assignments for furodysinin were not as simple to ascertain. The six sp^2 carbons were assigned with the aid of an off-resonance decoupled experiment, but the upfield region, due to the congestion of the four signals between δ33.2 and 31.2, required additional experiments for full assignment. The off-resonance decoupled spectrum provided the multiplicity of five sp^3 carbons (Figure 7, spectrum b), a spin-echo experiment (0.5/J) identified the quaternary carbon (δ33.13) (Figure 7, spectrum d), and a second spin-echo experiment (1/J) established the remaining CH_2 (δ31.69) and indicated that the resonances at δ31.22 and 32.85 were due either to methine or methyl groups (Figure 7, spectrum c). The information desired from a J-modulated spin-echo experiment is dependent upon the time between the 90° pulse and the 180° pulse (the echo delay). An echo delay of 0.5/J (J is the 13C-H coupling

Table 2. ^{13}C-NMR Assignments, Furodysinin Lactone and Furodysinin

	Furodysinin Lactone		Furodysinin			
					J-Modulated Spin Echo	
Carbon #	δ	ORD	δ	ORD	0.5/J	1/J
1	174.76	s	140.51	d		
2	115.27	d	108.20	d		
3	169.82	s	124.71	s		
4	104.91	s	147.48	s		
5	41.17	t	27.56	t		pos
6	30.37	d	31.22			neg[c]
7	123.54	d	126.18	d		
8	134.49	s	133.62	s		
9	30.96	t	31.69			pos
10	18.65	t	19.26	t		pos
11	47.33	d	44.60	d		neg
12	38.47	s	33.13		s	pos
13	25.28[a]	q	32.85[b]			neg[d]
14	26.84[a]	q	26.21[b]	q		neg
15	22.98	q	23.12	q		neg

[a] assignments interchangeable

[b] assignments interchangeable

[c] a methine (see text)

[d] a methyl (see text)

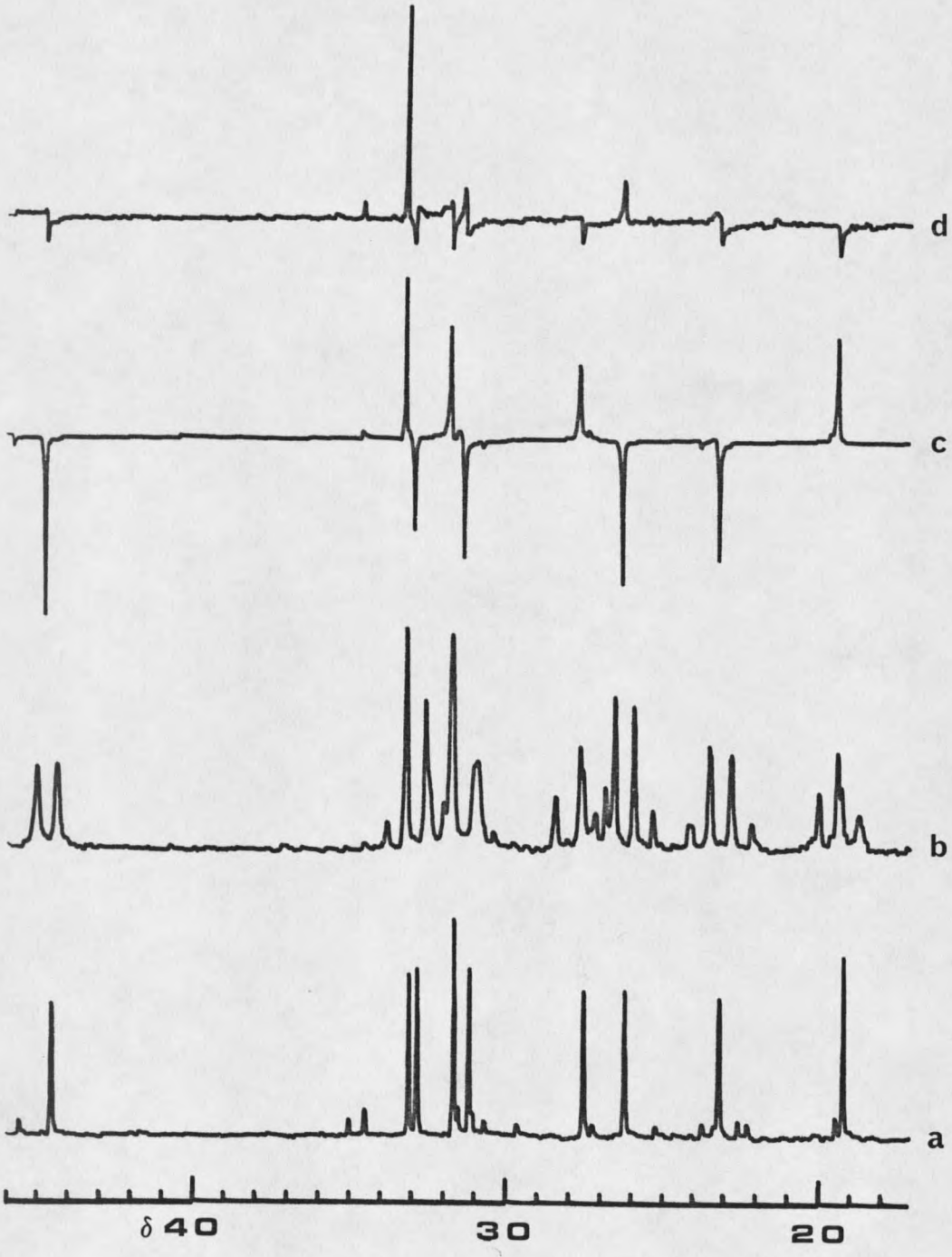


Figure 7. 13C-NMR Spectra of the Highfield Region of Furodysinin

constant) will give a spectrum exhibiting only quatenary carbons (defined as the positive phase), while a 1/J echo delay will show quatenary and methylene carbons as the positive and methine and methyl carbons as the negative phase (69). Correlation of the off-resonance decoupled and spin-echo data indicated that the signal at δ31.22 was the methine and that at δ32.85 the methyl. An SFORD (irradiating δ1.61) identified the allylic methyl (δ23.12) and another SFORD (irradiating at δ2.65) confirmed the resonance at δ31.22 as the methine.

A lanthanide induced chemical shift study (LIS) was undertaken to establish the full relative configuration of **56**. A full equivalent of $Eu(fod)_3$ was added in increments to a $CDCl_3$ solution of the lactone and a ^1H-NMR spectrum recorded after each addition. Table 3 contains the slopes, obtained by linear least squares method, along with correlation coefficients, of a plot of induced chemical shift versus equivalents of shift reagent added and Figure 8 is a graphical interpretation of the same.

The assignment of relative configuration of polycyclic systems from LIS data is rarely straightforward. In this case, analysis was rendered particularly difficult because the hydroxyl group did not appear to be the site of chelation, as might be expected. This assumption was based on the observation that the hydroxyl proton did not undergo the exceedingly large shifts attributed to pseudocontact with the lanthanide.

In order to determine first the site of chelation and then the relative configuration of the three chiral centers in **56**, the PDIGM

Table 3. Slopes from the Plot of $\Delta\delta$ vs. [Eu(fod)$_3$]/[Substrate]

	Furodysinin Lactone		Semisynthetic Furodysinin Lactone	
Proton (δ)	Slope	Correlation Coefficient	Slope	Correlation Coefficient
5.67	2.02	.986	2.11	.998
5.36	.35	.993	.35	.998
3.50	2.01	.992	2.12	.991
2.80	.65	.994	.70	.996
2.28	1.16	.990	1.22	.998
1.96	.20	.990	.21	.999
1.70	.26	.996	.25	.999
1.63	.54	.986	.50	.988
1.57	1.42	.985	1.50	.996
1.61	.09	.995	.10	.999
1.38	.47	.999	.50	.998
1.21	.28	.983	.31	.998
1.12	.89	.999	.89	.999

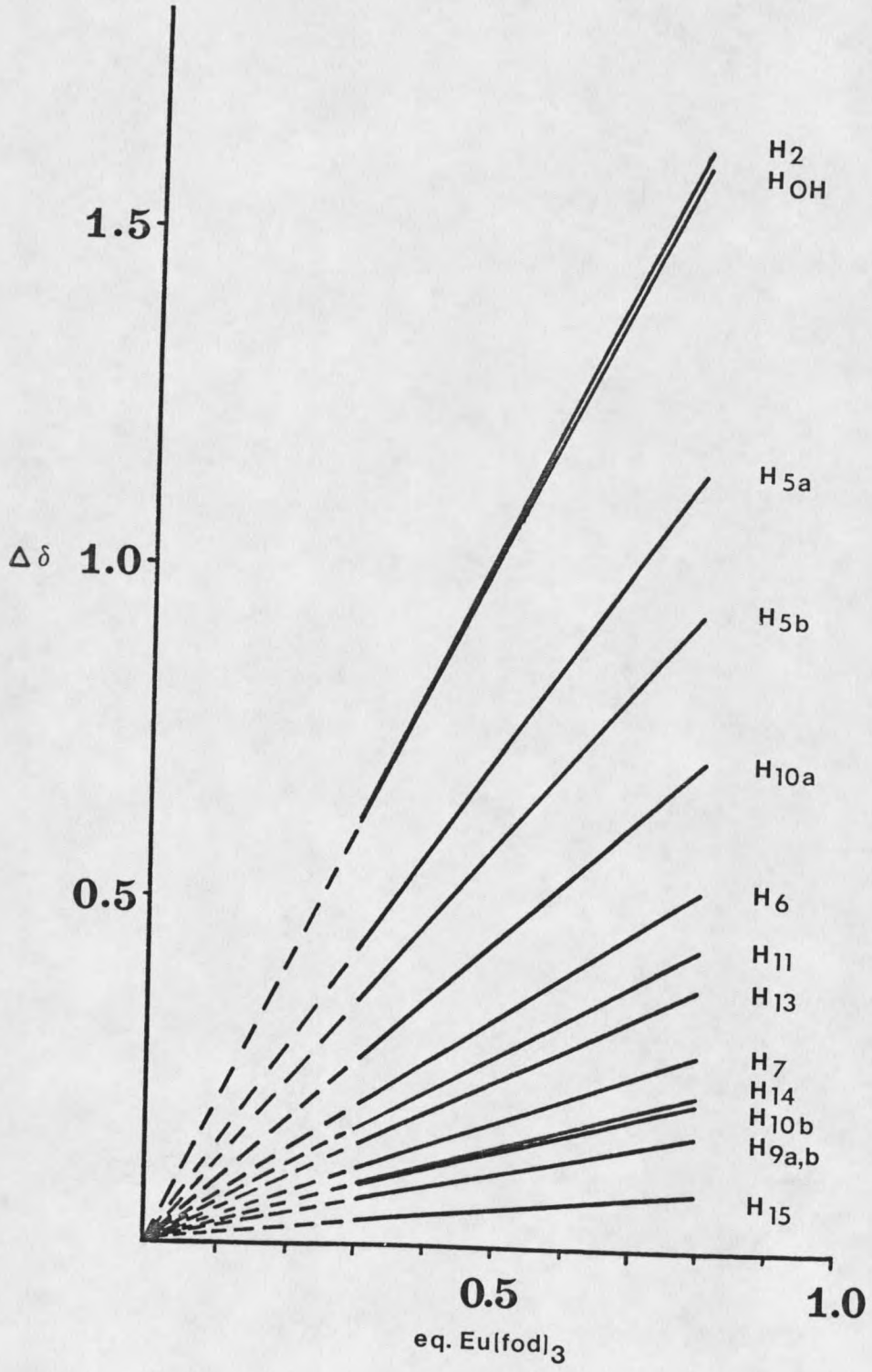


Figure 8. Plot of Change in Chemical Shift vs. Equivalents Eu(fod)3

program of Willcott and Davis (70) was utilized. The program calculates the expected induced shift for a given set of proton coordinates and compares them to the experimental induced shifts. The europium position is varied to obtain the lowest agreement factor, R.

$$R = \left[\frac{(\text{observed induced shift} - \text{calculated induced shift})^2}{\text{observed induced shift}^2}\right]^{1/2}$$

Since the cis ring juncture between the two cyclohexane rings was clearly defined from the chemical correlation with **27**, only the two diastereomers epimeric at the hemiketal carbon had to be compared. Dreiding models of the C-4 epimers of **56** were constructed and Cartesian coordinates were generated by projection onto the XY plane; Z coordinates were measured directly. Any of the three oxygens could have served as the site of chelation of the lanthanide with a lone pair of electrons. Each of these possibilities was evaluated and the best fit for the LIS data indicated complexation of the europium with the carbonyl oxygen at a distance of 2.4 Å in the isomer with the hydroxyl cis to the ring juncture protons as shown in **56**; the minimum R value was 7.98%. The C-4 epimer gave a minimum R value of 16.83%. No other combination of relative configuration and site of complexation resulted in minimum R values under 28.48% (see Table 4).

The R factor ratio test is often used to determine the confidence level with which one isomer may be favored over another

Table 4. Agreement Factors for Furodysinin Lactone and its C-4 Epimer.

Site of Complexation	Minimum R(%) for **56**	Minimum R(%) for the C-4 Epimer of **56**
OH	63.70	61.76
O	28.48	30.98
CO	7.98	16.83

(71). The thirteen protons used in the experiment leave nine degrees of freedom. Thus an R1/R2 of 2.11 (16.83/7.98) indicates we have a greater than 99.5% confidence level that **56** is the correct isomer. Figure 9 illustrates the graphical relationship of the R values obtained for **56** with the distance between the europium and carbonyl oxygen atom.

Consideration of steric effects provides a suitable explanation for the failure of the $Eu(fod)_3$ to chelate with the hydroxyl group. The molecular model suggested the likelihood of intramolecular hydrogen bonding of the hydroxyl with a non-bonding electron pair on the lactone oxygen. This idea was supported by the sharpness and constancy of chemical shift (no effect of concentration on δ value) exhibited by the hydroxyl hydrogen in the ^{1}H-NMR. Such a configuration would preclude access of the lanthanide to the lone pairs of the hydroxyl oxygen and to one pair on the lactone oxygen. The net result would be that the carbonyl oxygen should become the primary complexation site.

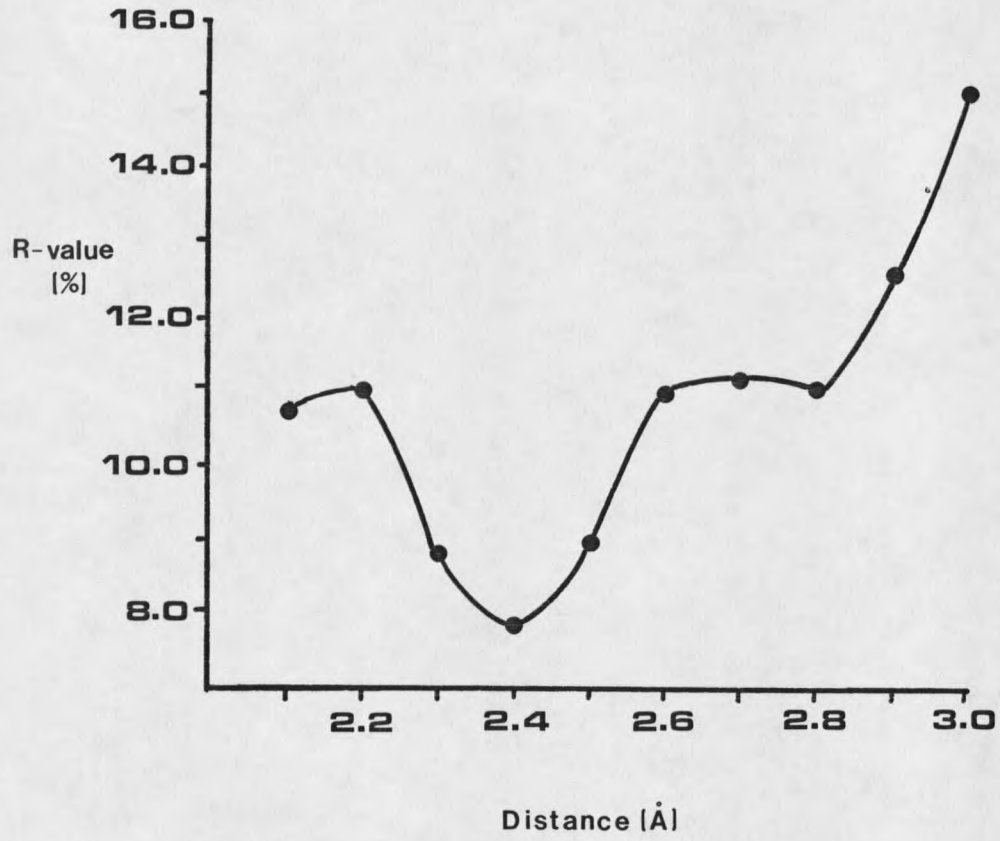
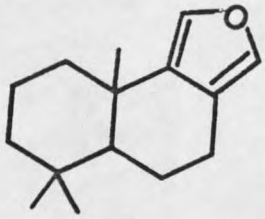


Figure 9. Plot of R Values vs. Europium-Oxygen Bond Distance

The $Eu(fod)_3$ shift reagent induced identical chemical shift changes in the lactone prepared by oxidation of furodysinin (see Table 3), confirming that a single diastereomer, identical to the natural product, was produced in the course of the oxidation.



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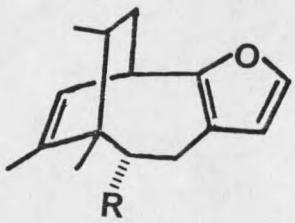


24 R = OAc
59 = OH
21 = H

Furanosesquiterpenes from Hypselodoris zebra

The nudibranch Hypselodoris zebra was frequently observed grazing on Dysidea etheria in Bermudian waters. When a single specimen of Hypselodoris zebra was dissected and offered in separate portions to Abudefduf saxatilis, an omnivorous scavenger fish common on Bermuda's reefs, only the gonads were consumed. The digestive tract and outer skin were categorically rejected by the fish. Extraction of several nudibranchs gave very limited quantities of extract; the extract, however, contained a significant percentage (36%) of sesquiterpenes.

Gel permeation chromatography of this extract through Bio-Beads S-X8 with cyclohexane-dichloromethane (2:3) yielded a mixture of furodysinin, **27**, euryfuran, **34**, and 5-acetoxy nakafuran-8, **24**, as major constituents and 5-hydroxy nakafuran-8, **59**, as a minor constituent of the extract. The identity of furodysinin and the two nakafuran-8 derivatives (31-33) were secured by direct comparison

with the furanosesquiterpenes isolated earlier from the sponge. Euryfuran was identified by comparison of its mass spectral and ^{1}H-NMR data with those reported in the literature (20,59,72). An apparent typographical error in reference 59 made the initial comparison to literature data confusing. We found chemical shifts of δ0.90 and 0.93 for two of the methyl groups in **61**, consistent with the data reported in reference 20 and 72 while reference 59 reports those signals at δ0.90 and 0.99.

Furodysinin (67,75) and nakafuran-8, **21**, and -9, **22**, (29) have previously been identified as feeding deterrents. Thus, it is likely that furodysinin serves the same function in Hypselodoris zebra. The large amount of 5-acetoxy nakafuran-8 and euryfuran concentrated in H. zebra suggest they also may be antifeedants. The structural similarity of nakafuran-8 to 5-acetoxy nakafuran-8 reinforces this view.

Euryfuran is conspicuously absent from D. etheria. Attempts will be made in the future to identify the dietary source of euryfuran in H. zebra.

Pharmacological Activity of Furodysinin and Furodysinin Lactone

Metabolites that are biological deterrents in nature often demonstrate in vitro biological activity. The biological activity of furodysinin and furodysinin lactone was assessed against a series of common plant pathogenic microbes. Both **56** and **27** were mildly active against Curvularia lunata and Rhodotorula glutinis and demonstrated no activity against Fusarium solani, Corynebacterium

michiganese, and Pseudomonas syringae. In addition, furodysinin lactone proved negative in tests against bacteria deficient in DNA repair capacity.

Ceramides from Dysidea etheria

The fractions which contained furodysinin lactone from the initial separation of D. etheria on Florisil, also contained, as revealed by subsequent purification via gel permeation chromatography, a white solid which ultimately proved to be a mixture of ceramides.

Ceramides are common isolates of many terrestrial fauna (74-78), fungi (79-82), and bacteria (83,84), and have recently been found in a variety of marine organisms, including the first isolation of such compounds from plants, red (85) and green (86,87) alga, as well as the electric organ of the ray Torpedo marmorata (88), the rectal gland of the spiny dogfish (89), the starfish Patiria pectinifera (90), octopus (91), and the shellfish Pinctada martensii (92), but they had previously not been reported from sponges.

Florisil chromatography of the dichloromethane soluble extracts of Dysidea etheria gave three polar fractions which, when further separated by gel permeation chromatography, yielded fractions (one each) which were appreciably soluble in tetrahydrofuran and pyridine only. Purification by flash chromatography yielded two fractions comprised of an amorphous white solid. The ^{1}H-NMR spectra (see Figures 10 and 11), recorded in deuterated tetrahydrofuran,

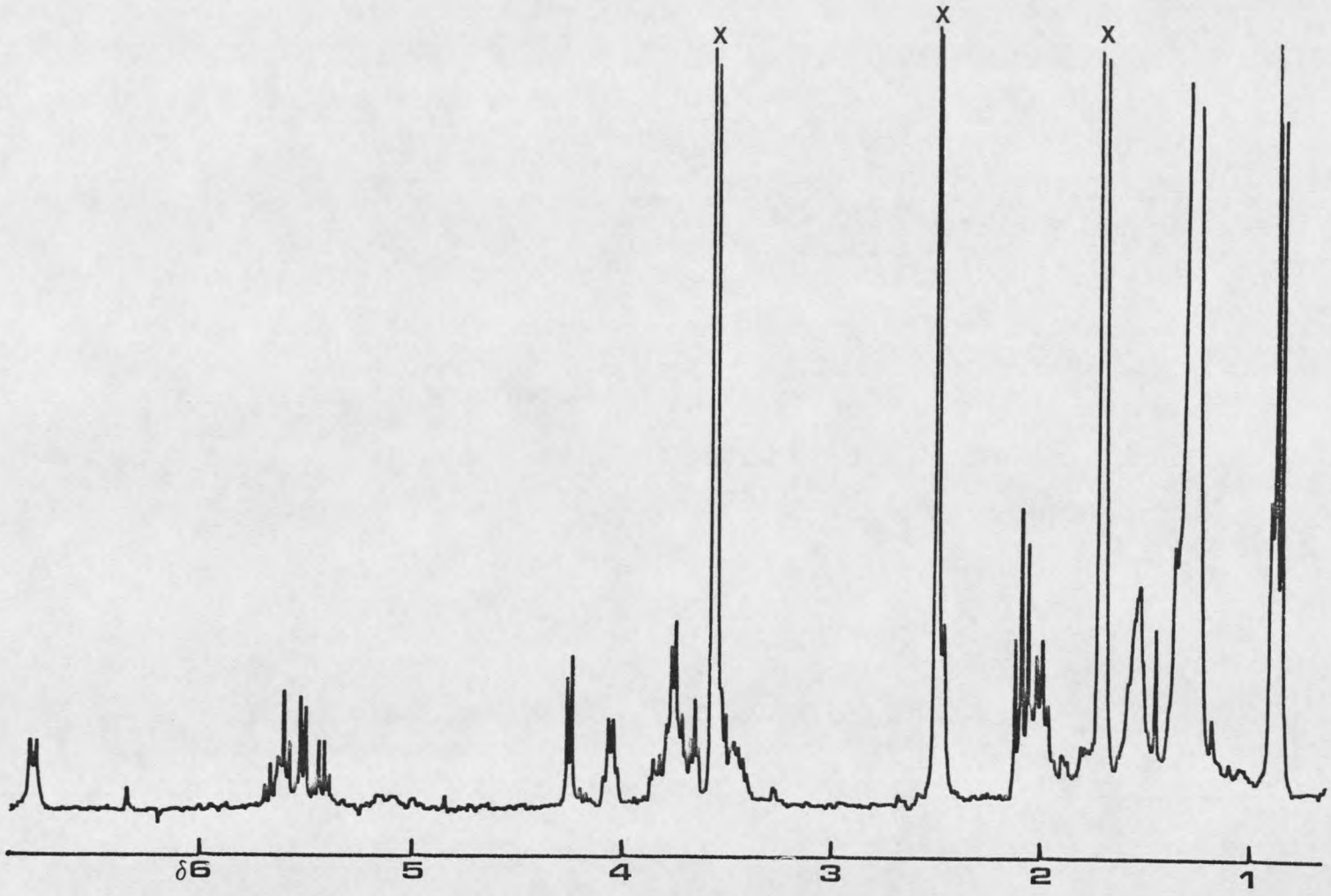


Figure 10. ^{1}H-NMR Spectrum of Ceramide 60.

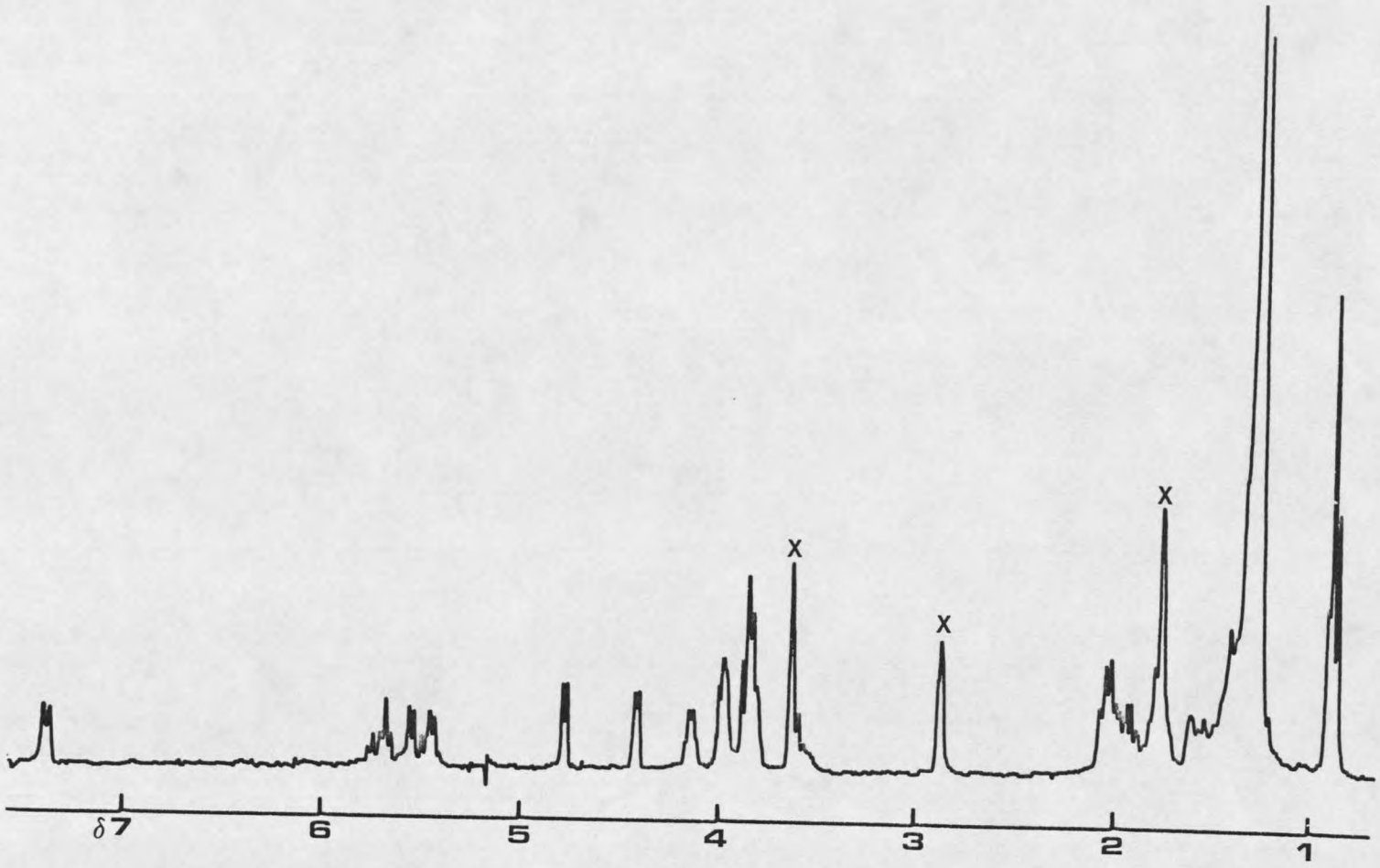


Figure 11. ^{1}H-NMR Spectrum of Ceramide 61.

suggested moderately functionalized molecules with a long aliphatic chain. The minor fraction, R_f 0.35 in silica gel TLC, appeared to contain three exchangeable protons, while the major fraction, R_f 0.26, had four such protons. However, acetylation of the minor fraction gave only a diacetate (see Figure 12), while the major fraction yielded a triacetate (see Figure 13). Absorption in the IR spectrum (KBr pellet) at 1623 cm^{-1} for the minor fraction and 1624 cm^{-1} for the major fraction suggested an amide functionality. Thus, in order to account for the number of deuterium exchangeable protons in each molecule, a secondary amide, along with a diol in the minor fraction and a triol in the major fraction, must be present.

The ^{1}H-NMR spectra of the two molecules, and their acetylated derivatives, had the typical paraffinic resonances at δ0.88 (broad triplet), 1.28 (broad singlet), and 1.45–1.70 (multiplet); additional straightforward information was precluded by the overlap of numerous midfield signals. ^{1}H-NMR decoupling experiments on the acetylated derivatives established part structure **60a** for the minor fraction and **61a** for the major fraction. The broad deuterium exchangeable resonance at δ5.58 was readily assigned to the amide proton in **60a** (see Table 5 for a complete list of ^{1}H-NMR assignments and coupling constants). It was coupled to a one proton multiplet at δ4.40 which in turn was vicinally coupled to three one proton doublet of doublets. Two of the doublets of doublets, δ4.29 and 4.02, demonstrated geminal coupling while the remaining doublet of doublets, δ5.26, was vicinally coupled to an olefinic proton at δ5.37. A coupling of 14.9 Hz to the remaining olefinic proton,

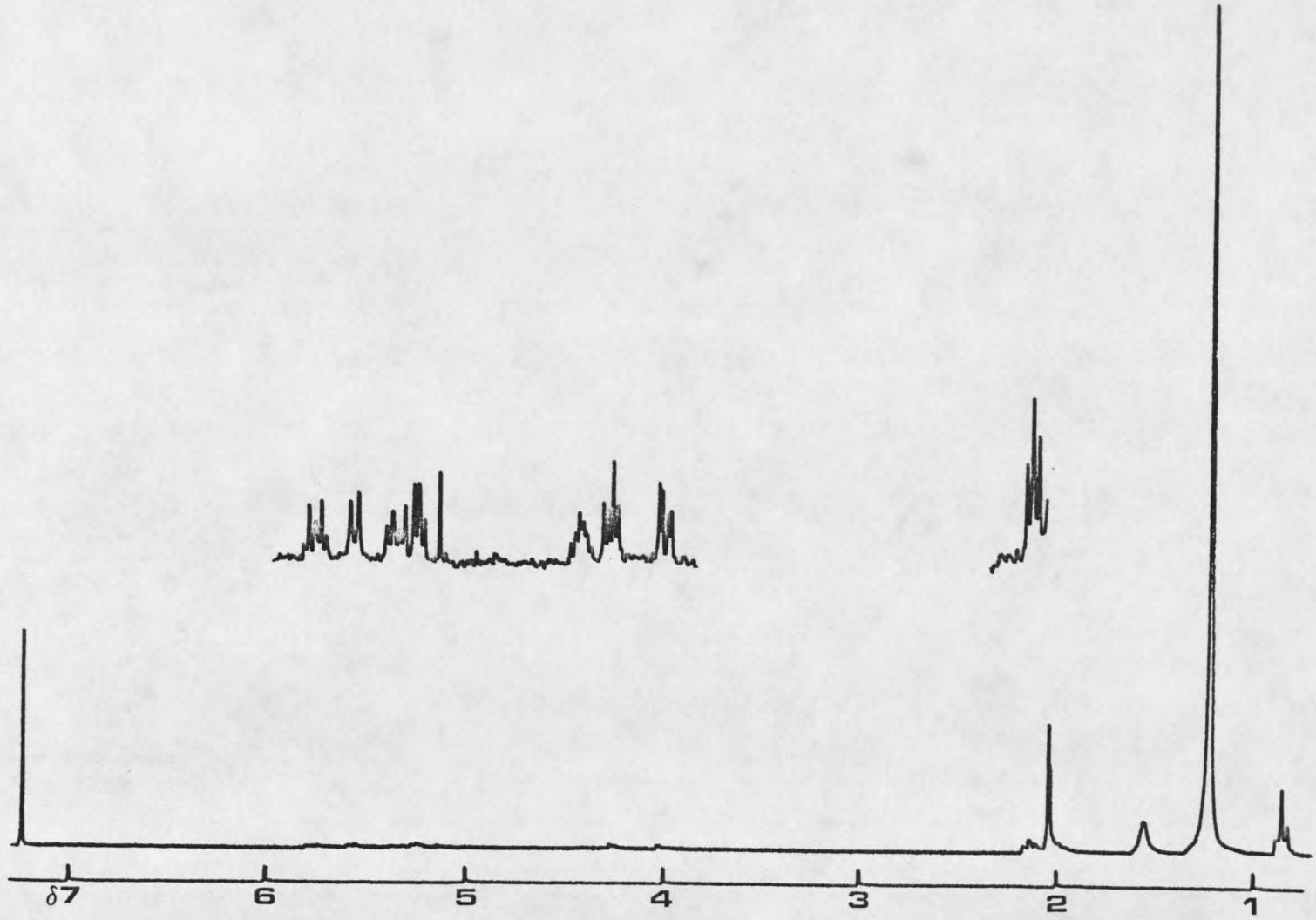


Figure 12. ^{1}H-NMR Spectrum of Ceramide Diacetate **60a**.

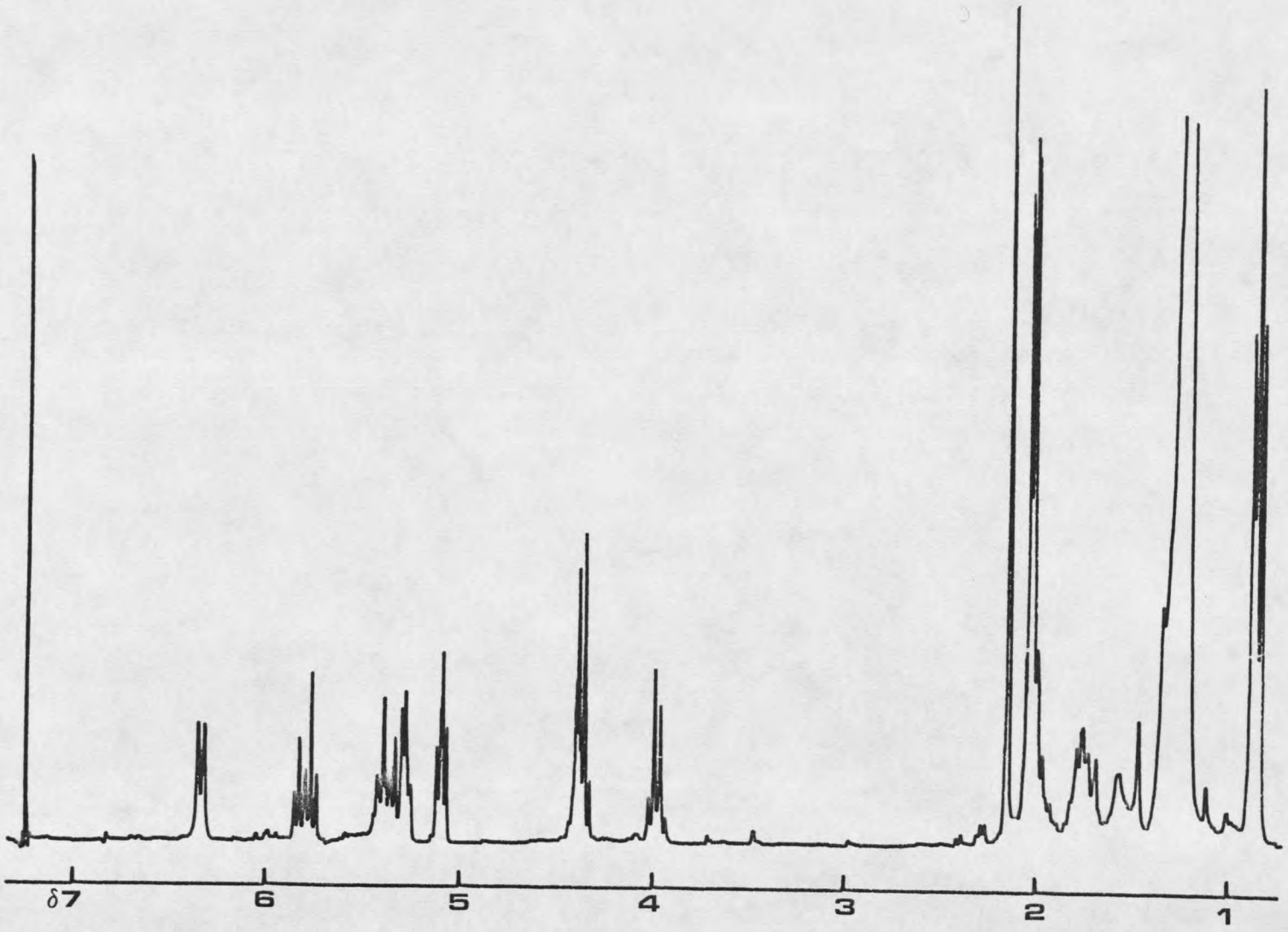


Figure 13. ^{1}H-NMR Spectrum of Ceramide Triacetate 61a.

Table 5. ^{1}H-NMR Assignments for the Ceramide Acetates.

	Chemical Shift (δ)		Multiplicity	Coupling Constants (Hz)	
H	**60a**[a]	**61a**[b]		**60a**	**61a**
1a	4.29	4.21	dd	12.3,6.2	11.6,7.9
1b	4.02	4.07	dd	12.3,3.9	11.6,4.7
2	4.40	4.35	m		
3	5.26	5.31	dd	7.0,6.5	6.7,6.5
4	5.37	5.46	dd	14.9,6.5	15.3,6.5
5	5.77	5.78	dt	14.9,6.2	15.3,6.5
6(2H)	2.00	1.97[c]	dt	6.2,6.8	6.5, unresolved
2'	2.15(2H,t)	4.95 (1H,br t)		7.1	6.7
3'		1.72(2H,m)			
$COCH_3$	2.05,2.07	2.13[c], 2.02[c], 2.00[c]	s		
NH	5.58	7.13	brd	8.6	8.5
CH_2[d]	1.45-1.65	1.45-1.65	m		
CH_2[d]	1.27	1.28	brs		
CH_3(6H)	0.84	0.83	brt	7.4	7.6

[a] $CDCl_3$ was used as solvent and internal standard.

[b] D_6-acetone was used as solvent and internal standard (see following note).

[c] The acetone impurity in d_6-acetone necessitated measurement in $CDCl_3$.

[d] The methylene protons of the alkyl chains. An indeterminant number due to the presence of a mixture of chain lengths.

δ5.77, suggested trans geometry. A two proton doublet of triplets at δ2.00 coupled to the olefinic proton at δ5.77 indicated an allylic methylene. Chemical shift considerations placed the two acetates on C-1 and C-3. ^{1}H-NMR assignments for **61a** followed from **60a**. The broad one proton triplet at δ4.95 necessitated placement of the third acetate on C-2'. The proton on C-2' was coupled to the two proton multiplet at δ1.72 which, in turn, was coupled to the methylene envelope, δ1.28.

The ^{13}C-NMR spectra of **60** and **61** indicated one carbonyl, two olefinic carbons and three heteroatom bearing carbons (two doublets and a triplet), for **60** (see Figure 14), and one carbonyl, two olefinic carbons and four heteroatom bearing carbons (three doublets and a triplet) for **61** (see Figure 15), which led to the 1,3-diacetoxy, 2 amido groupings, establishing both fractions as sphingosine type compounds. The upfield region of inverse gated (see Figure 15) and off resonance decoupled (see Figure 16) ^{13}C-NMR spectra of **61** in pyridine indicated approximately thirty-two methylenes (all but 7 between δ29.66 and 29.30) and two methyls.

After the structures of **60a** and **61a** were ascertained with certainty and the ^{1}H-NMR resonances were assigned it was possible, through spin-spin decoupling, to assign the resonances of **60** and **61** (see Table 6 for a list of ^{1}H-NMR assignments). Chemical shift considerations led to the assignment of the broad deuterium exchangeable proton at δ6.87 in **60**, which was coupled to the multiplet between δ3.78 and 3.90, to the secondary amide (N-H). The integration (2 protons), as well as the coupling to δ3.73, 3.53, and

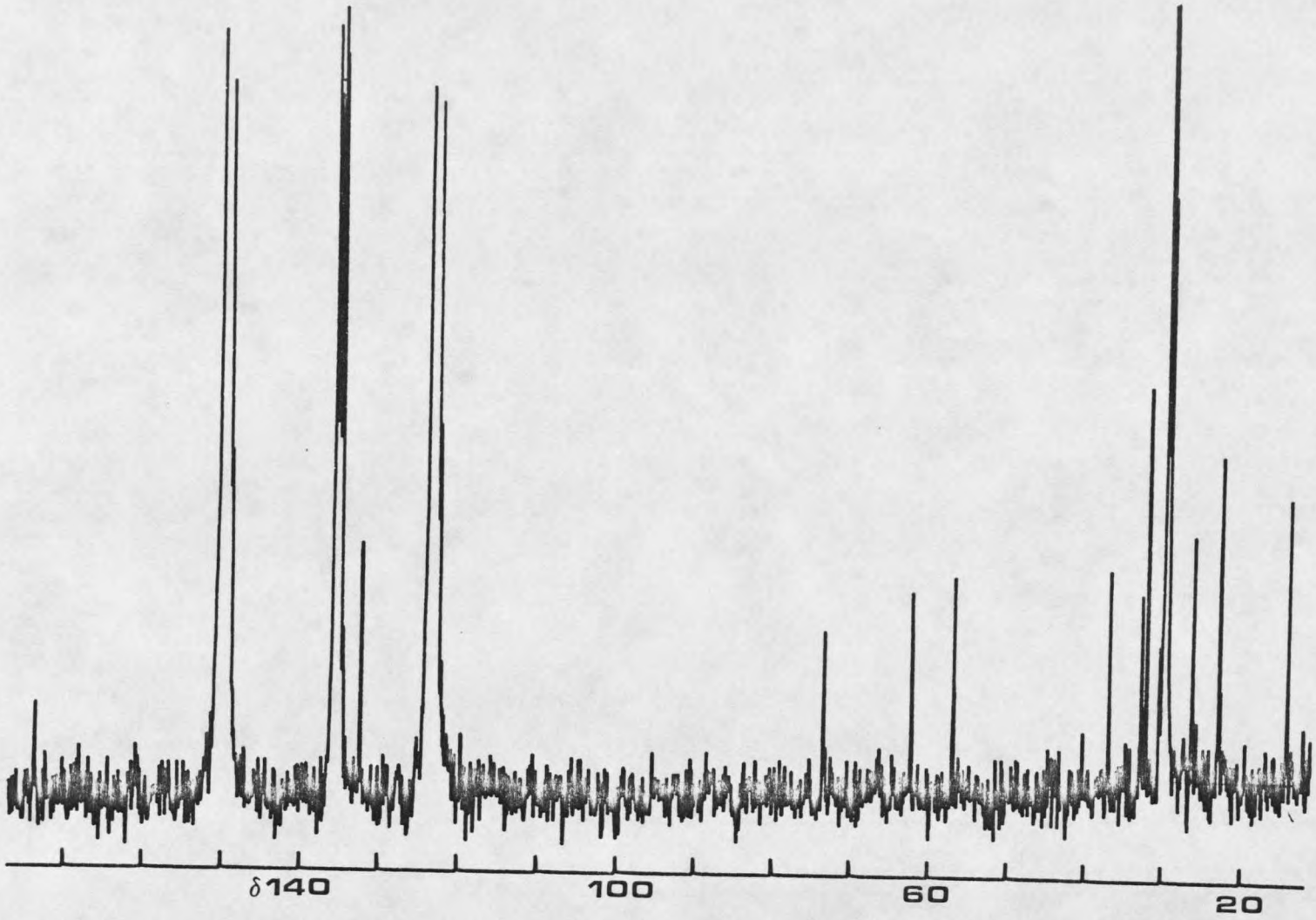


Figure 14. ^{13}C-NMR Spectrum of Ceramide 60.

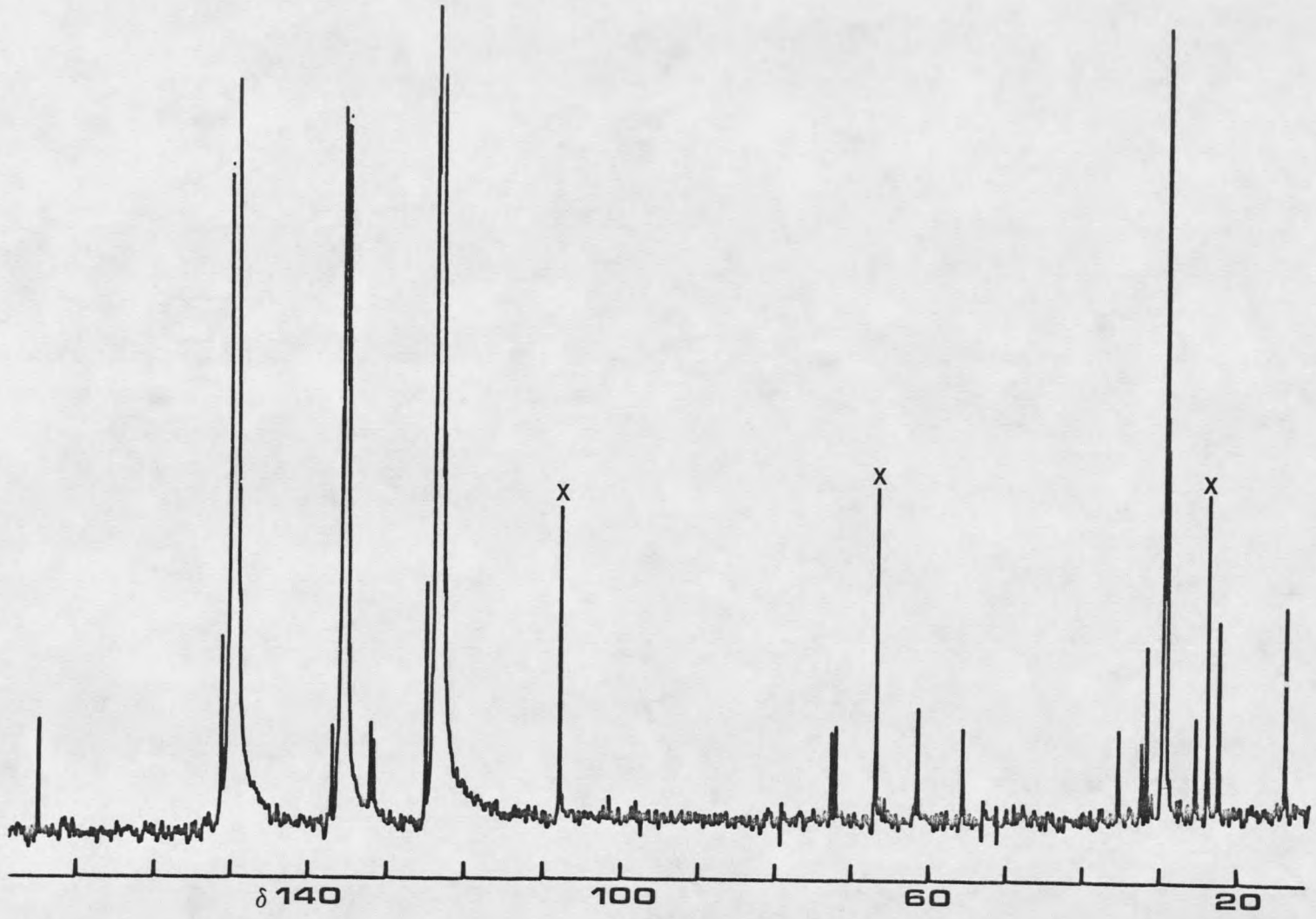


Figure 15. Inverse Gated ^{13}C-NMR Spectrum of Ceramide 61.

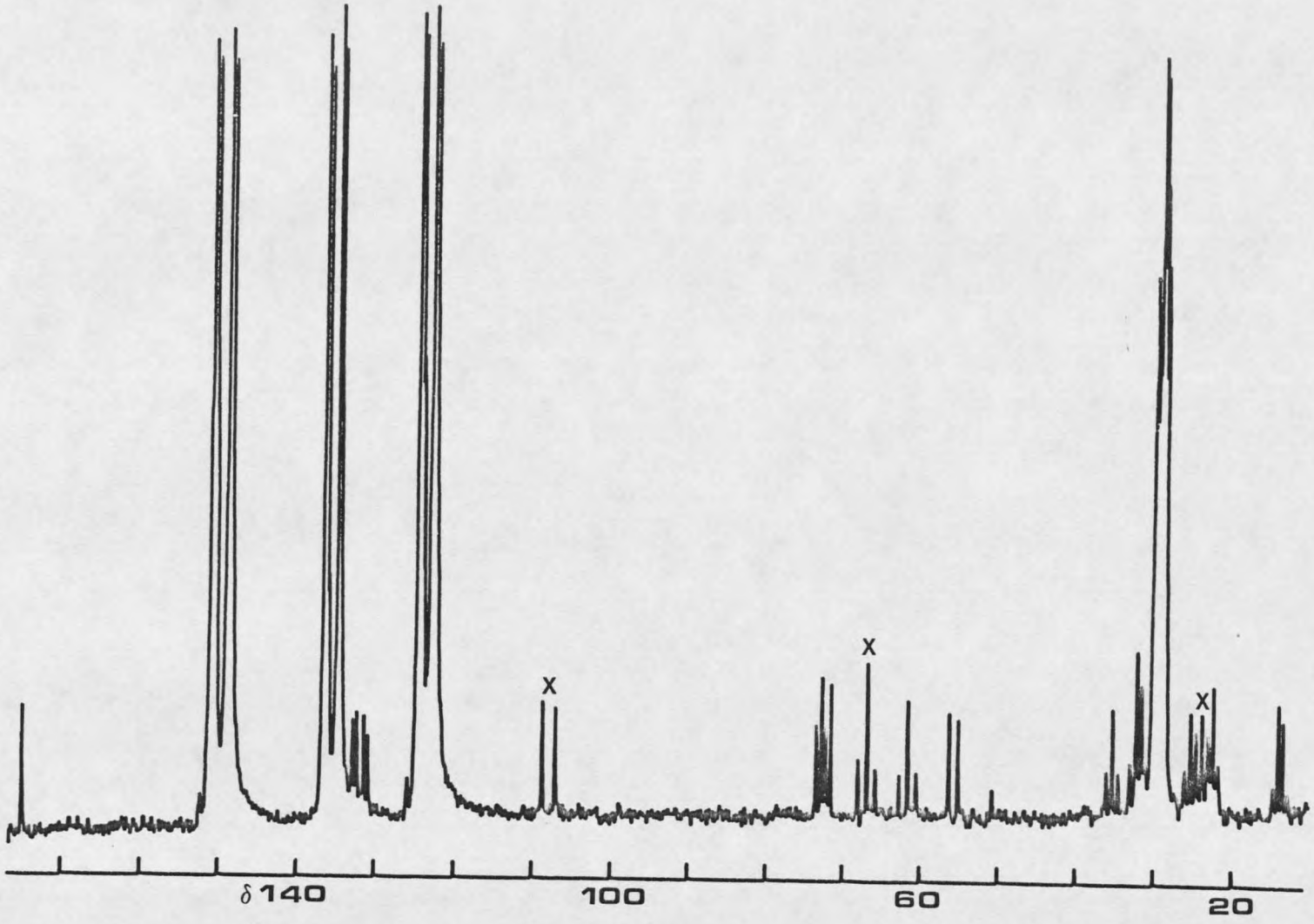


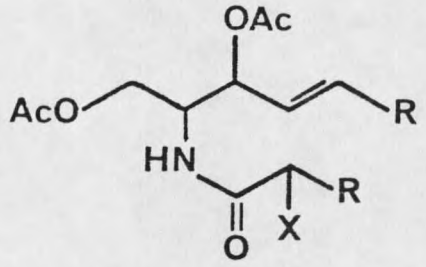
Figure 16. Off-Resonance Decoupled ^{13}C-NMR Spectrum of Ceramide 61.

Table 6. ^{1}H-NMR Assignments for the Ceramides.

	Chemical Shift (δ)		Multiplicity	Coupling Constants (Hz)	
H	60[a]	61[a]		60	61
1a	3.73(ddd)	3.77-3.90(m)		14.9,4.7,4.4	
1b	3.53	3.58	m		
2	3.78-3.90	3.77-3.90	m		
3	4.08	4.13	ddd	6.2,5.3,5.3	6.4,5.2,5
4	5.50	5.49	dd	15.5,6.2	15.5,6.3
5	5.65	5.70	dt	15.5,6.5	15.5,6.3
6(2H)	2.20	2.04	m		
2'	2.13(2H,t)	3.97(1H,m)		6.8	
3'		1.95(2H,m)			
OH(C-1)	3.78-3.90	3.77-3.90	m		
OH(C-3)	4.30	4.43	brd	5.3	5.2
OH(C-2')		4.79(d)			4.8
NH	6.87	7.39	brd	8.1	8.9
CH_2[b]	1.45-1.65	1.45-1.65	m		
CH_2[b]	1.30	1.30	brs		
CH_3(6H)	.88	.88	brt	8.1	8.1

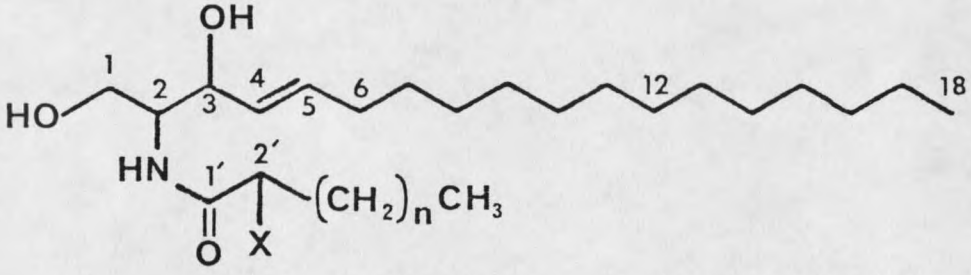
[a] D_8-THF was used as solvent and TMS as internal standard.

[b] The methylene protons of the alkyl chains. An indeterminant number due to the presence of a mixture of chain lengths.



60a R = alkyl, X = H

61a R = alkyl, X = OAc



60 X = H , n = 12,13,14,15,19,20,21

61 X = OH, n = 19,20,21

4.08 suggested the multiplet (δ3.78-3.90) constituted the primary alcohol proton and H_5. When THF-d8 was used as solvent, all the alcoholic protons demonstrated coupling to protons on neighboring carbons. This is primarily due to the decrease in the rate of exchange of the hydroxylic protons upon the use of a more polar solvent. The doublet of doublet of doublets at δ4.08 was coupled to the olefinic proton at δ5.50 (a doublet of doublets) and the hydroxyl proton at δ4.30 (a broad doublet). A coupling constant of 14.9 Hz between δ3.73 and 3.53 indicated geminal coupling, and one of 15.5 Hz between the olefinic protons at δ5.50 and 5.65 indicated trans geometry. The olefinic resonance at δ5.65 was coupled to the allylic methylene at δ2.02 and a two proton triplet at δ2.13 necessitated placement next to the carbonyl. Once again ^{1}H-NMR assignments for **61** followed from **60**. The additional alcoholic proton appeared as a broad doublet at δ4.79 coupled to a multiplet at δ3.97, which in turn was coupled to the methylene at δ1.95. Hydrolysis of both **60** and **61** gave sphingosine and a mixture of fatty acid methyl esters. The ^{1}H-NMR spectra of the sphingosine from both fractions were identical in all respects to that reported previously (85). The sphingosine was converted to its triacetate and identified by comparison with literature values {$[\alpha]_D$, ^{1}H-NMR (85,93)}. Fast atom bombardment mass spectral studies indicated an M+H ion at m/z 426 and diagnostic fragments at 366 (loss of O_2CCH_3) and 352 (loss of $CH_2O_2CCH_3$), to verify the presence of sphingosine triacetate. The fatty acid methyl esters of **61** were shown to be α-hydroxylated by the 1H-NMR (δ4.17, 1H, dd, J = 7.8, 3.6) and MS (M-

59, loss of CO_2CH_3) spectra and to be composed predominately of n-C_{22} acids with minor amounts of n-C_{23} and n-C_{24} acids. The fatty acid methyl esters of **60** were shown by mass spectrometry to be composed mainly of n-C_{22}, n-C_{23}, and n-C_{24} acids, with smaller amounts of n-C_{15}, n-C_{16}, n-C_{17} and n-C_{18} acids also present. The aliphatic chains of the sphingosinetriacetate and the fatty acid methyl esters of **60** and **61** were proven to be normal by virtue of the low mass fragmentation pattern of their mass spectra (m/z 29, 43, 57, 71, 85, etc.).

In spite of the ubiquitous nature of α-hydroxy fatty acids, relatively few stereochemical assignments have been made. This is due to the difficulty in assigning absolute configuration to compounds of weak optical activity (94-96). However, some configurations have been assigned and the subject has been reviewed (97). On the basis of comparison with literature data (see Table 7), the optical rotation $[\alpha]_D$ + 19.5 of the hydroxyacid methyl esters suggested an S configuration at C-2.

This isolation of sphingosine derivatives is the first reported occurrence of ceramides in marine sponges and is important for a number of reasons.

The biological function of free ceramides is not known (102). The α-hydroxy and non-hydroxy ceramides serve as precursors to cerebrosides (1-β-galactosyl ceramide) and sulfatides (sulfate in position 3 of the galactose) (103), both are abundant in mammalian nervous tissue (104). These sphingolipids are components of the

Table 7. Optical Rotations, Esters of 2-Hydroxyacids.

Compound[a]	$[\alpha]_D$	Solvent	Reference
S-methyl valerate	+16.6°	$CHCl_3$	98
S-methyl octanoate	+11°	$CHCl_3$	99
R-methyl tetradecanoate	-3.6°	$CHCl_3$	100
R-methyl hexadecanoate	-3.6°	$CHCl_3$	101
R-methyl octadecanoate	-2.1°	$CHCl_3$	101

[a]All esters have hydroxyl substituents at C-2.

surface membrane, the hydrogen bonding capabilities of the polar endgroups ensure cellular integrity. The function of the α-hydroxyl is unknown, however, it has been suggested that it serves to alter permeability and stability by creating a system of laterally oriented hydrogen bonds (105).

All known α-hydroxy fatty acids from ceramide sources are optically active (106). The stereochemistry about C-2 of α-hydroxy-n-tetracosanoic acid formed by the brain α-hydroxylating enzyme has always been the R configuration (i.e. D-hydroxyacids) (107-110). A synthetic mechanism for α-hydroxylation of ceramides from a cell free preparation of rat brain has been proposed to explain the reason for this preference (107). Hydroxylation of the fatty acid occurs by direct substitution of the pro R hydrogen (see Figure 17), with retention of stereochemistry. The fatty acid is subsequently incorporated into the ceramide (108). A similar explanation is

obtained from studies on the α-oxidation of fatty acids in plants (101,111-113). Fatty acid degradation is accomplished by successive α-oxidation. Studies on the α-oxidation system of pea leaf and germinating peanut seed demonstrated that only the D α-hydroxy fatty acid is formed and suggested that it must go through a D α-hydroperoxy intermediate (113).

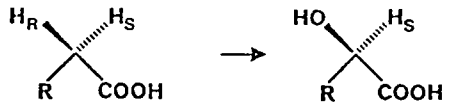


Figure 17. α-Hydroxylation of Fatty Acids

The S enantiomers found for the ceramide derived α-hydroxy fatty acids suggest an alternate pathway for the production or accumulation of the α-hydroxy fatty acids in Dysidea etheria.

The fatty acids, both hydroxylated and saturated, represented in the ceramides of Dysidea etheria were predominately C-22 or larger, in keeping with recently described profiles of fatty acids (114,115) and their derivatives (116) in the Porifera.

Sponges harbor and support considerable microbial communities; it is possible, therefore, that the ceramides could be of bacterial or fungal origin. However, the consistent quantities of these compounds found in several collections of D. etheria from numerous sites suggest that they are, in fact, produced by the sponge.

Pharmacological Activity of the Ceramides

Antimicrobial activity of 60 and 61 was assessed against the same series of plant pathogens as were furodysinin and furodysinin

lactone. The ceramide, **60**, demonstrated mild activity against C. michiganese and no activity against F. solani, C. lunata, R. glutinis, and P. syringae. The α-hydroxy ceramide, **61**, was negative against all microbes tested.

Diterpenes from Briareum polyanthes

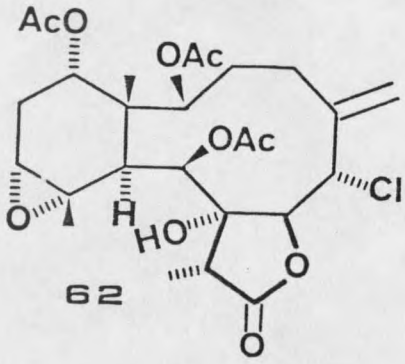
Along with the marine sponges, the soft corals are among the most primitive of animals. They are carnivorous invertebrates whose diet consists essentially of plankton. Their mouth, which is surrounded by eight pinulated tentacles, opens to an internal cavity that has no anus. The soft corals are widely distributed. The order Alcyonacea is especially prevalent in the South Pacific and Indian Oceans, and the order Gorgonacea is found in great abundance in the tropical western Atlantic.

While on a collecting expedition to the Bermuda Biological Station in 1979, Dr. John H. Cardellina II found a moderately large community of a soft coral from the genus Briareum at the eastern end of the Bermudian archipelago. Briareum species had not previously been reported from Bermudian waters and subsequent identification indicated a taxonomically confused species recently renamed B. polyanthes (117). Analysis of the organic metabolites revealed a series of highly functionalized diterpenes possessing the briaran carbon skeleton.

Prior to this work, compounds possessing the briaran ring skeleton have been found in one species of Briareum (B. asbestinum) and three different genera of the distantly related sea pens.

Briarein A, **49**, B, C, and D (see Introduction) have been isolated from B. asbestinin (47,48). Stylatulide, **62**, from Stylatula sp., was identified as the major component of a series of five metabolites by single crystal X-ray analysis (118). The structure of ptilosarcone, **63** from Ptilosarcus gurneyi, was identified by chemical degradations, comparison with NMR spectra of briarein A, and spin-spin decoupling experiments (119). A series of three diterpenes have been identified from Scytalium tentaculatum (120) and have been assigned structures **64**, **65**, and **66**. Structure elucidation was accomplished by high resolution mass spectrometry, ^{13}C- and ^{1}H-NMR, and chemical degradations.

Our collections on Briareum polyanthes were extracted with acetone, followed by dichloromethane. The dichloromethane extract was then equilibrated with the aqueous residue remaining after concentration of the acetone extract. The resulting organic phase was concentrated and then separated according to a partition scheme popularized by Kupchan (121). This scheme successively partitions the organic solubles against progressively more polar organic phases. Four organic phases were used; hexane, carbon tetrachloride, chloroform, and ethylacetate, which were partitioned against 10% aqueous methanol, 25% aqeous methanol, 35% aqueous methanol, and water respectively. The carbon tetrachloride and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OAc
OAc
Cl
O
H
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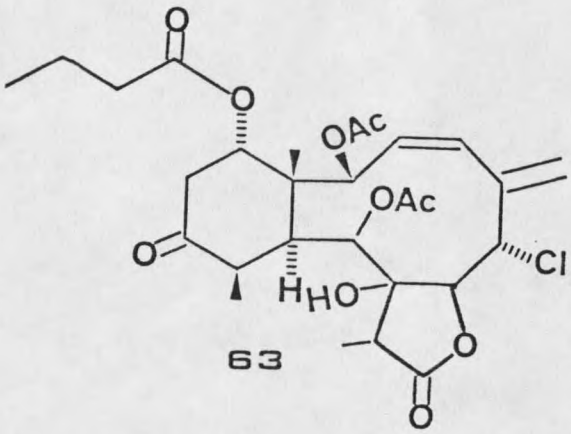
O

62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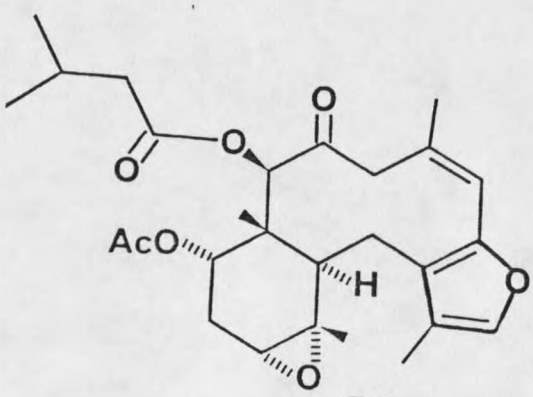
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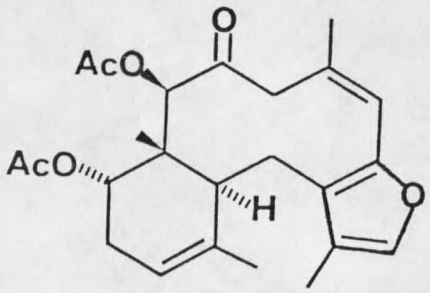
64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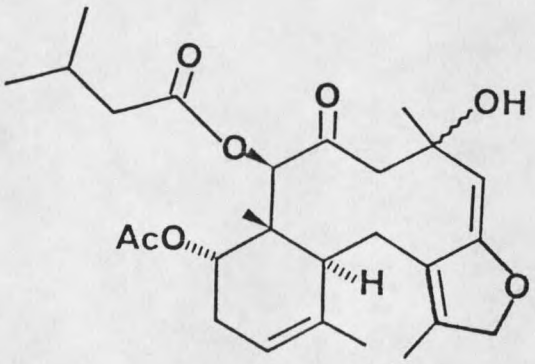
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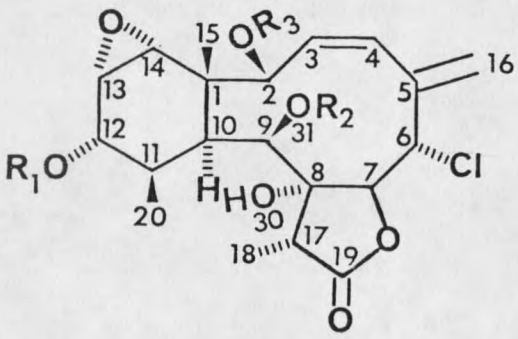
66

chloroform soluble extracts provided ^{1}H-NMR suggesting significant quantities of terpenoid components.

Crude separation of the chloroform residue was accomplished by step gradient gel permeation chromatography (122). This technique capitalizes upon the adsorption and partition effects of Sephadex LH-20 as well as the size separation which is expected of gel permeation. The gel was eluted successively with hexane-dichloromethane (1:4), acetone-dichloromethane (2:3), and acetone-dichloromethane (4:1). The first phase gave five fractions. ^{1}H-NMR analysis revealed the terpenoid components to be concentrated in fractions 2 and 4. Subsequent separation and purification was accomplished by gel permeation chromatography through Sephadex LH-60, then Biobeads S-X8 followed by HPLC on a nitrile-bonded phase column. Three diterpenes were obtained, briantheins Z, **67**, and Y, **68**, from fraction 2 and brianthein X, **69**, from fraction 4.



67 $R_1 = R_2 = R_3 = Ac$

68 $R_1 = R_2 = Ac$
$R_3 = COCH_2CH_2CH_3$

69 $R_1 = R_2 = Ac$
$R_3 = H$

The ^{13}C-NMR (see Figure 18) of **67** showed resonances for four carbonyls, four olefinic carbons (two doublets, one triplet, and one singlet), eight heteroatom bearing carbons (one singlet and seven doublets) and ten saturated carbons (six quartets, three doublets, and one singlet). An intense absorption at 1739 cm^{-1} in the IR spectrum, along with three singlets (each 3H) in the ^{1}H-NMR (see Figure 19) at δ2.15, 2.06, and 2.04, indicated the presence of three acetate groups and accounted for three of the four carbonyls, three of the eight heteroatom bearing carbons, and three of the ten saturated carbons. Another intense absorption in the IR at 1790 cm^{-1} suggested a γ-lactone and an absorption at 3540 cm^{-1} required a hydroxyl group. A positive Beilstein test then left two heteroatom bearing carbons, in addition to the seven saturated carbons, to be assigned.

A molecular ion was not obtained using electron impact or chemical ionization mass spectral techniques. However, fast atom bombardment in a glycerol-potassium iodide matrix gave an $(M+K)^+$ at m/z 579 and an $(M+K+2)^+$ at m/z 581 for **67** whose isotopic abundance indicated the presence of chlorine (see Figure 20). Fragments at m/z 519 (loss of HOAC) and m/z 561 (loss of H_2O) verified the acetyl and hydroxyl moieties respectively. A molecular weight of 540/542 is obtained upon subtraction of potassium from M+K. Addition of the number of carbons (obtained from the ^{13}C-NMR), hydrogens (obtained from ^{13}C- and ^{1}H-NMR data), oxygens mandated by the acetate, lactone, and hydroxyl moieties, and the single chlorine leave 16

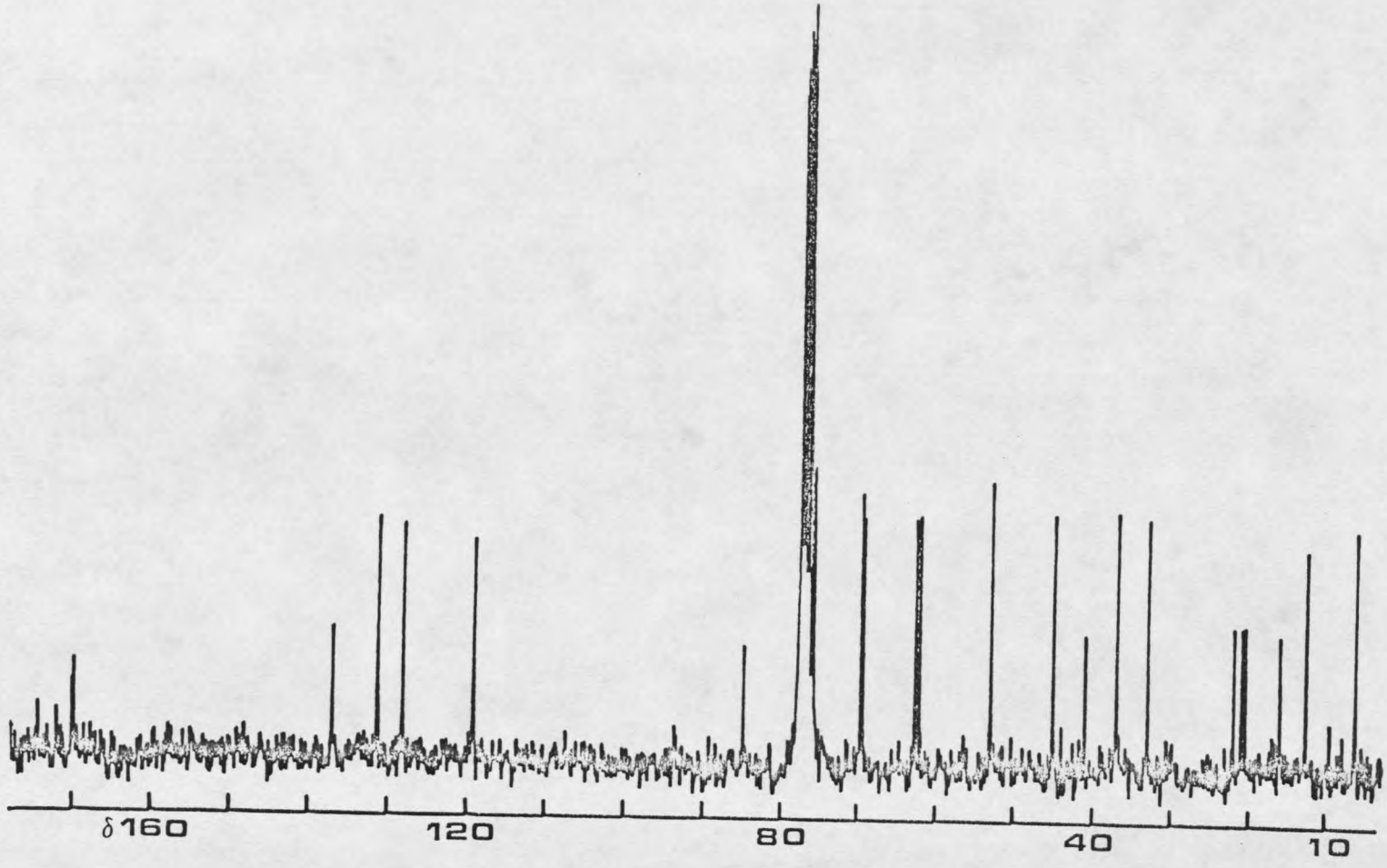


Figure 18. ^{13}C-NMR Spectrum of Brianthein Z.

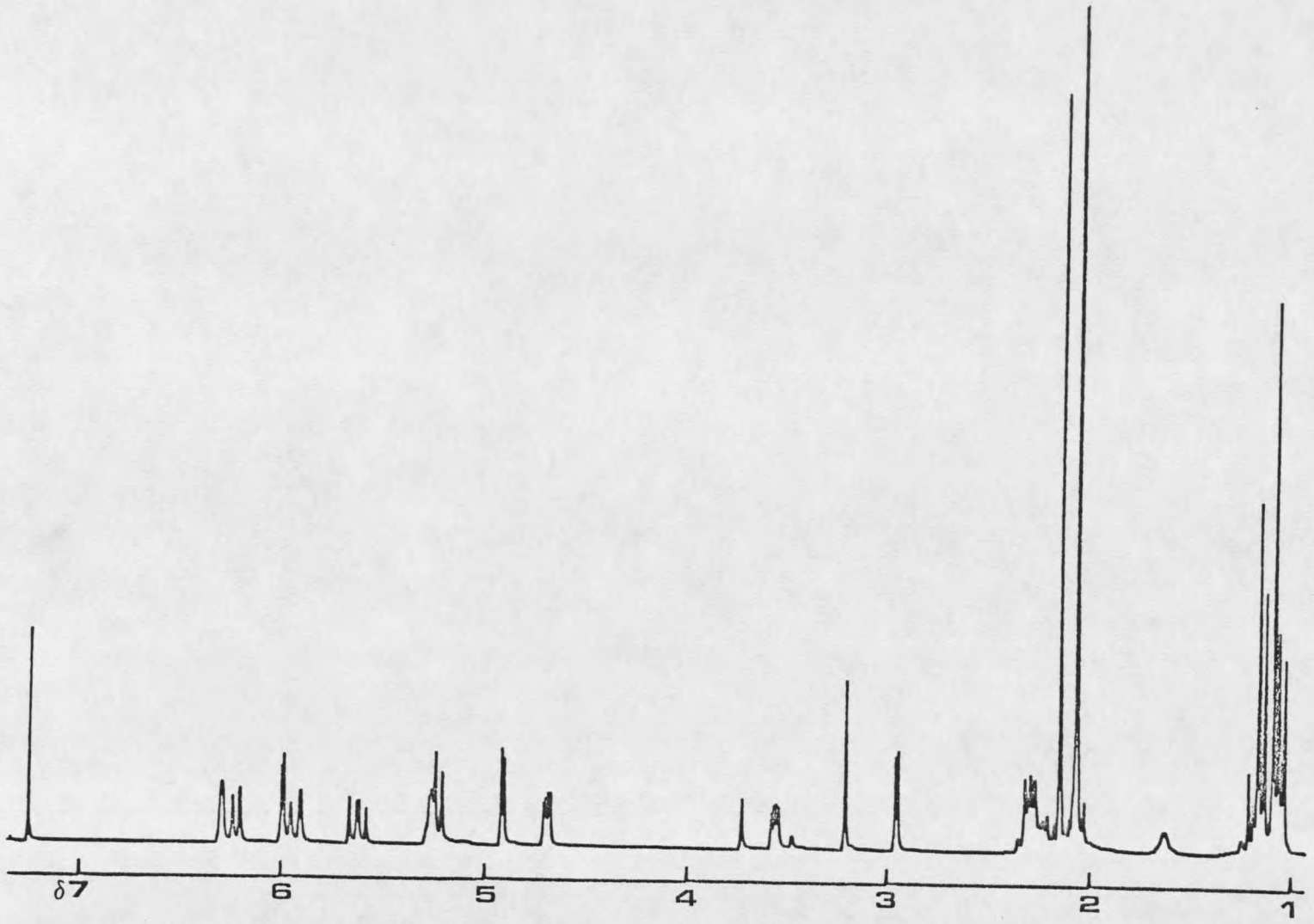


Figure 19. ^{1}H-NMR Spectrum of Brianthein Z.

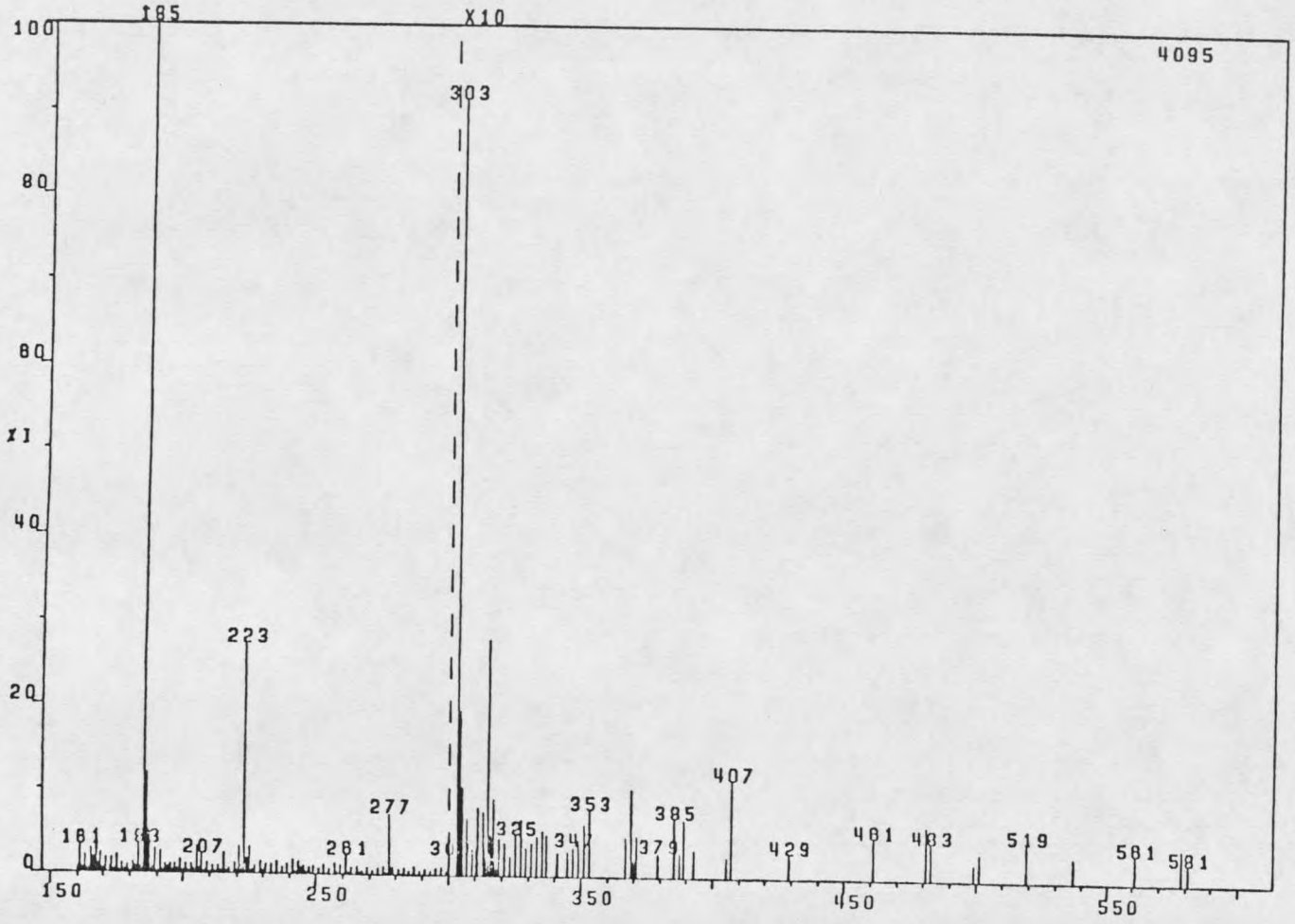


Figure 20. Mass Spectrum of Brianthein Z.

a.m.u. to account for. The inclusion of one more oxygen gave the molecular formula $C_{26}H_{33}ClO_{10}$.

^{1}H-NMR decoupling experiments (see Table 8 for a list of ^{1}H-NMR assignments and coupling constants for brianthein X, Y, and Z) on **67** revealed three isolated systems **67a-c**, assigning all resonances except a methyl singlet (δ1.09) and a one proton singlet at δ3.2 (OH). Irradiation of the one proton doublet of doublets at δ3.55 collapsed the sharp doublet at δ2.95 (1H) as well as eliminating the 5.8 Hz coupling of the doublet of doublets at δ4.68 (1H). The resonance at δ4.68 was coupled to the complex of signals between δ2.15 and 2.26 (2H) which in turn was coupled to the one proton doublet at δ5.22 and the methyl doublet at δ1.05. The 1,3-disubstituted butadiene of the second isolated system, **67b**, was formulated by consideration of the four sp^2 carbon resonances in the ^{13}C-NMR as well as ^{1}H coupling constants. Irradiation of the olefinic doublet of doublets at δ5.61 collapsed the doublet at δ6.22 (1H) as well as the broad 11.9 Hz doublet at δ5.93 (1H). The one proton broad doublets at δ6.30 and 5.99, upon irradiation, sharpened the signal at δ5.93 and each partially collapsed the one proton doublet of doublet of doublets at δ5.25. The one proton doublet at δ4.90 was then shown to be coupled to the signal at δ5.25. The final isolated system, **67c**, consisted simply of a methyl doublet (δ1.15) coupled to a one proton quartet at δ2.29.

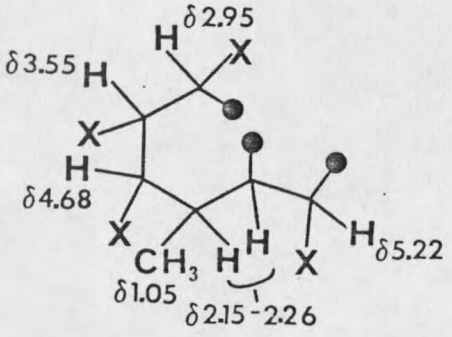
The unusual downfield chemical shift of many of the sp^3 protons in **67a** and **67b** suggested likely sites of heteroatom substitution. The resemblance of isolated spin system **67b** to briarein A prompted

Table 8. ^{1}H-NMR Assignments for Briantheins X, Y, and Z

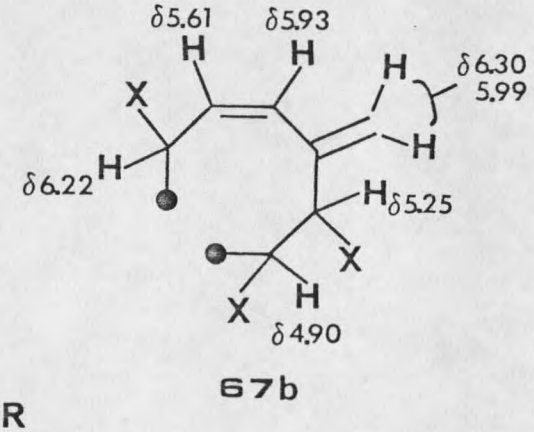
	Chemical Shift (δ)			Multiplicity	Coupling Constants (Hz)		
Proton	X	Y	Z		X	Y	Z
C_8-OH	3.47	3.18	3.20	s			
H_2	5.23	6.22	6.22	d[a]		9.1	8.8
H_3	5.21	5.60	5.61	dd[a]		11.8,9.1	11.9,8.8
H_4	5.83	5.91	5.93	brd	8,1	11.8,1	11.9,1
H_6	5.25	5.25	5.25	ddd	3.3,3,2.7	3.3,2.7,2.3	3,2.4,2
H_7	4.93	4.90	4.90	d	3	3.3	3
H_9	5.19	5.22	5.22	brd	8.2	8.5	8.5
H_{10}	2.18-2.27	2.15-2.26	2.15-2.26	m			
H_{11}	2.18-2.27	2.15-2.26	2.15-2.26	m			
H_{12}	4.73	4.68	4.68	dd	6.1,2.7	5.5,3.2	5.8,3
H_{13}	3.61	3.55	3.55	dd	6.1,3.1	5.5,3.3	5.8,3.2
H_{14}	3.14	2.92	2.95	d	3.1	3.3	3.2
H_{15}	1.04	1.07	1.09	s			
H_{16a}	5.59	5.99	5.99	brd	2.4	2.3	2
H_{16b}	5.90	6.32	6.30	brd	3.0	2.7	2.4
H_{17}	2.32	2.29	2.29	q	7.7	7.0	6.5
H_{18}	1.15	1.15	1.15	d	7.7	7.0	6.5
H_{20}	1.04	1.04	1.05	d	7.2	7.2	7.6
R_1 (3H)	2.15[b]	2.14[b]	2.15[b]	s			
R_2 (3H)	2.07[b]	2.05[b]	2.05[b]	s			
R_3	2.18(1H,s)	2.29(2H,t)	2.05(3H,s)			6.7	
R_3 (2H)		1.64		sextet		6.7	
R_3 (3H)		0.92		t		6.7	

[a]Overlapping multiplets in X.
[b]May be interchanged within each column.

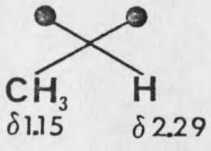
assemblage of the three substructures to give **67d**, which left just the heteroatom substitution about the cyclohexane ring to be assigned. The chemical shift of the proton residing on C-12 (δ4.68) necessitated placement of the remaining acetate. The two untendered heteroatom bearing carbons from the ^{13}C-NMR, the chemical shift of H_{13} and H_{14}, and the, as yet, unaccounted for oxygen from the molecular formula, suggested C-13 and C-14 as the site of an epoxide linkage, yielding structure **67** for brianthein Z.



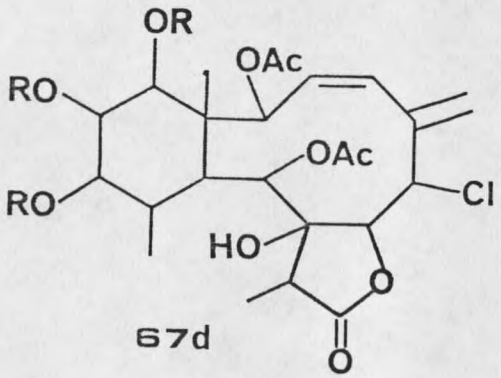
67a X = Cl or OR



67b



67c



67d

Brianthein Y, **68**, the most abundant diterpene showed a molecular weight 28 a.m.u. (M+K, m/z 607/609) greater than **67**.

Comparison of the ^{1}H- and ^{13}C-NMR (see Figures 21 and 22) of **67** and **68** were identical except for the highfield regions. Missing from the ^{1}H-NMR of **68** was an acetate methyl near δ2.0. The apparent replacement, a methyl triplet at δ0.92, a multiplet at δ1.64, and additional integral strength in the complex pattern of overlapping signals from δ2.18-2.27, suggested a butyrate ester. The upfield shift of a carbonyl from δ172.48 to 169.75 and the presence of two additional methylene carbons in the sp^3 region of the ^{13}C-NMR of **68** substantiated this conclusion.

Brianthein X, **69**, the most polar diterpene, showed a molecular weight 42 a.m.u. (M+K, m/z 537/539) less than **67** (see Figure 23). Their ^{1}H-NMR (see Figure 24) differed in that **69** showed only two acetate methyls near δ2.0, additional complexity and intensity between δ5.18 and 5.27, loss of the resonance at δ6.22, and an additional one proton singlet at δ2.18. The shift of the one proton resonance from δ6.22 to 5.23 and the appearance of the resonance at 2.18 suggested substitution of a hydroxyl for an acetate. The loss of a carbonyl and the shift of the heteroatom bearing carbon, C-2, from δ75.66 and 75.34 in brianthein Z and Y, respectively, to δ72.16 in **69** in the ^{13}C-NMR spectra (see Figure 25) verified this finding. The relationship of the two molecules was confirmed by acetylation of **69** in good yield to obtain a product identical to **67** (by ^{1}H-NMR and m.p.).

A series of ^{13}C-NMR single frequency off resonance decoupled experiments of **68** were untertaken to assist in the assignment of the

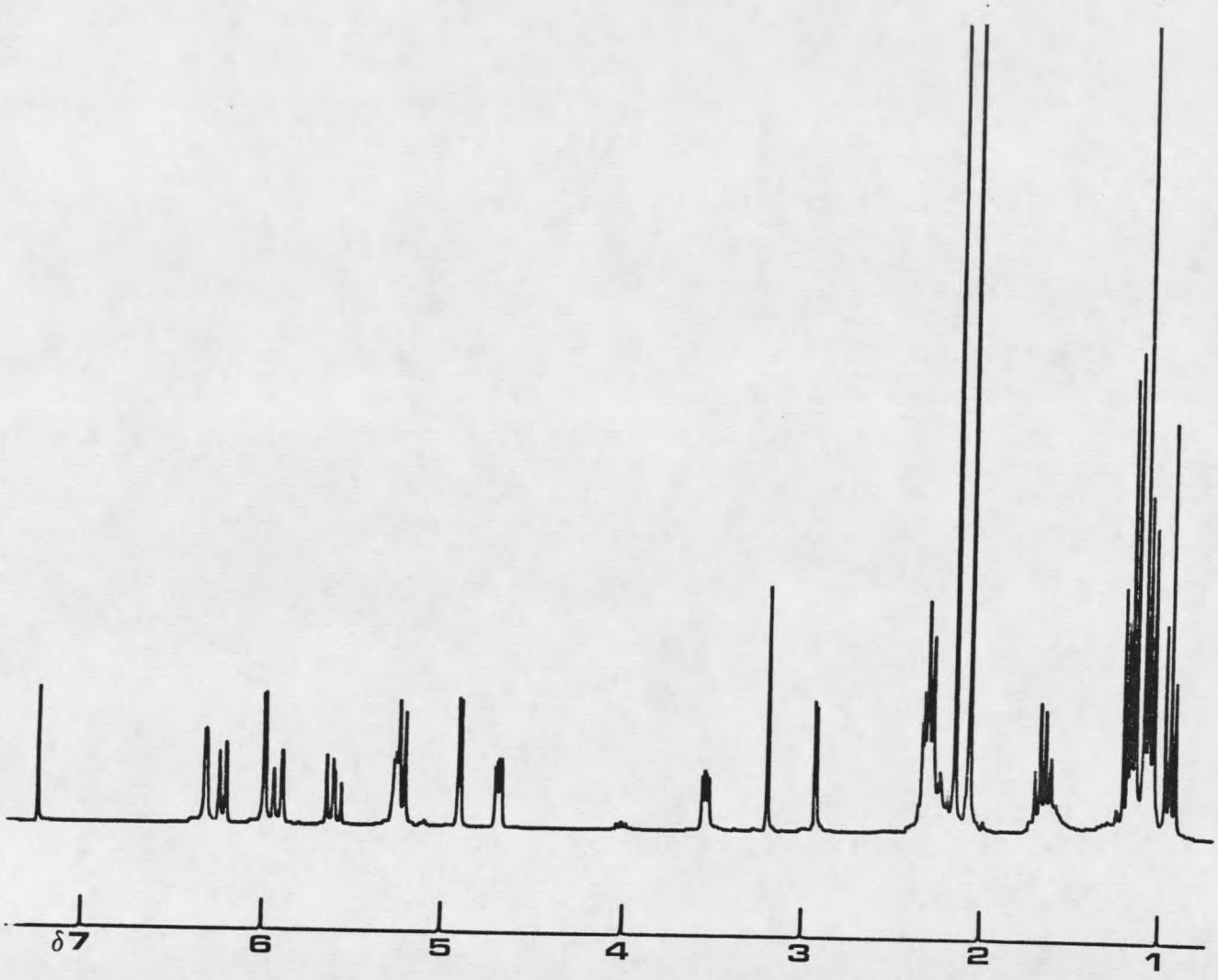


Figure 21. ^{1}H-NMR Spectrum of Brianthein Y.

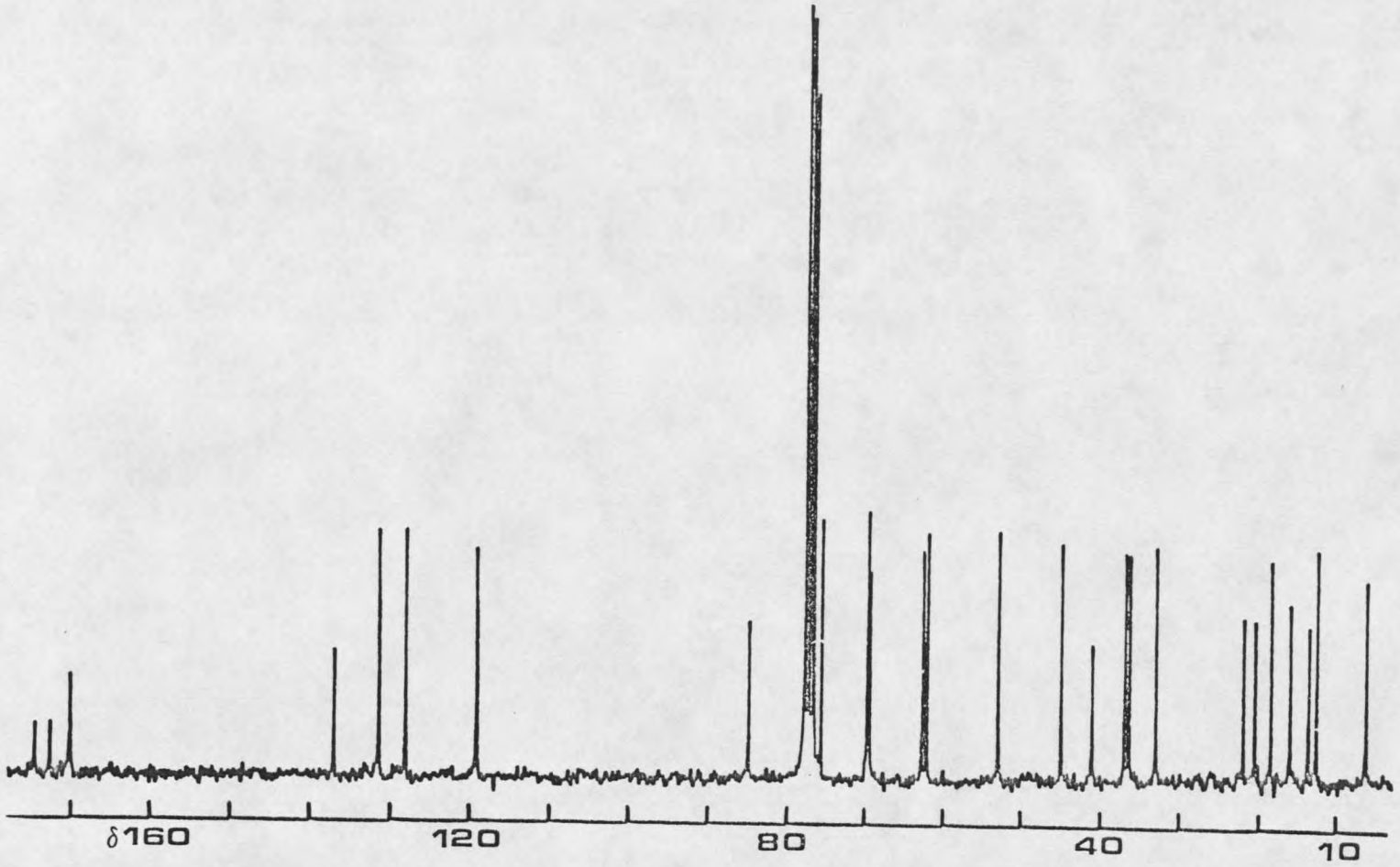


Figure 22. ^{13}C-NMR Spectrum of Brianthein Y.

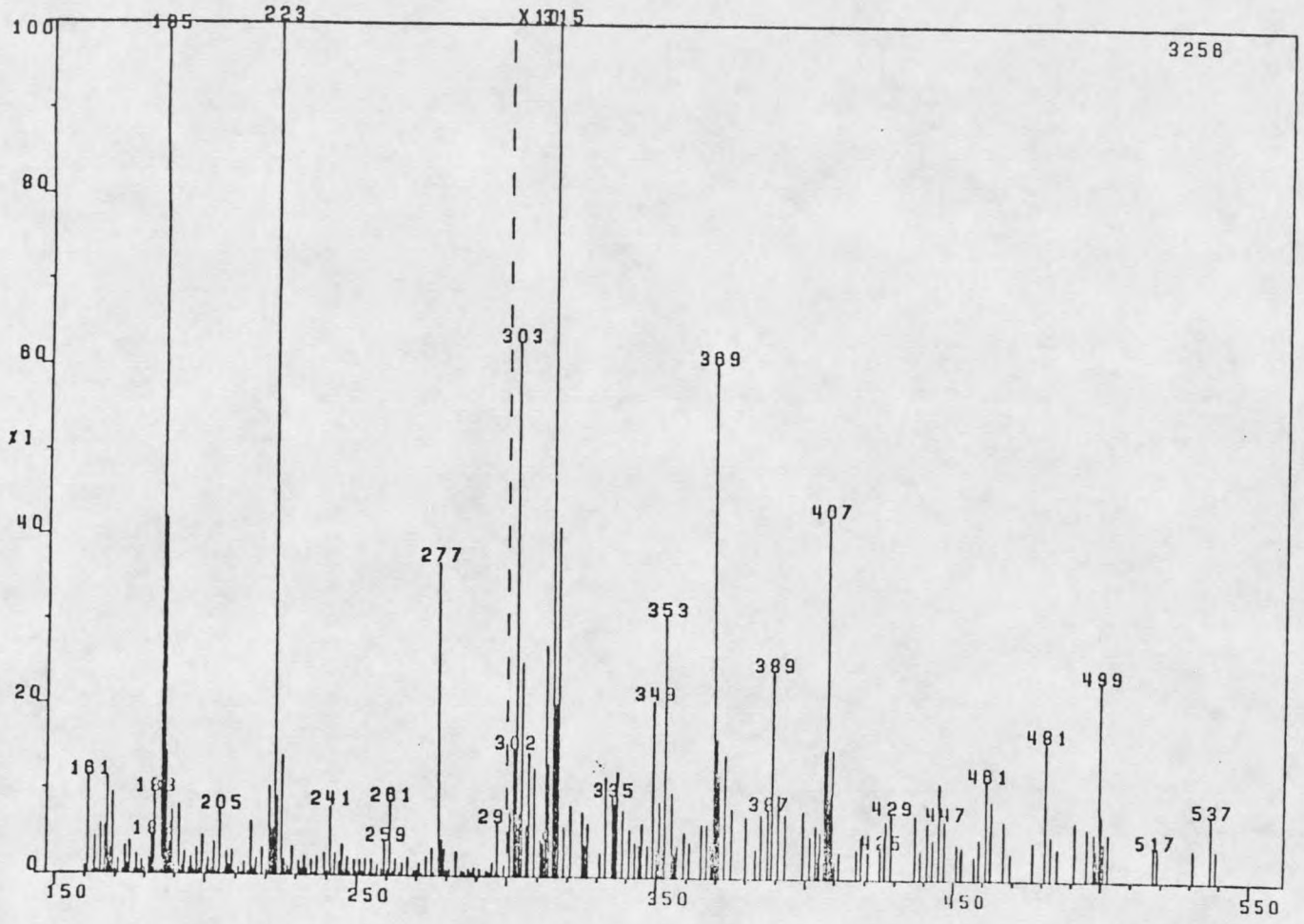


Figure 23. Mass Spectrum of Brianthein X.

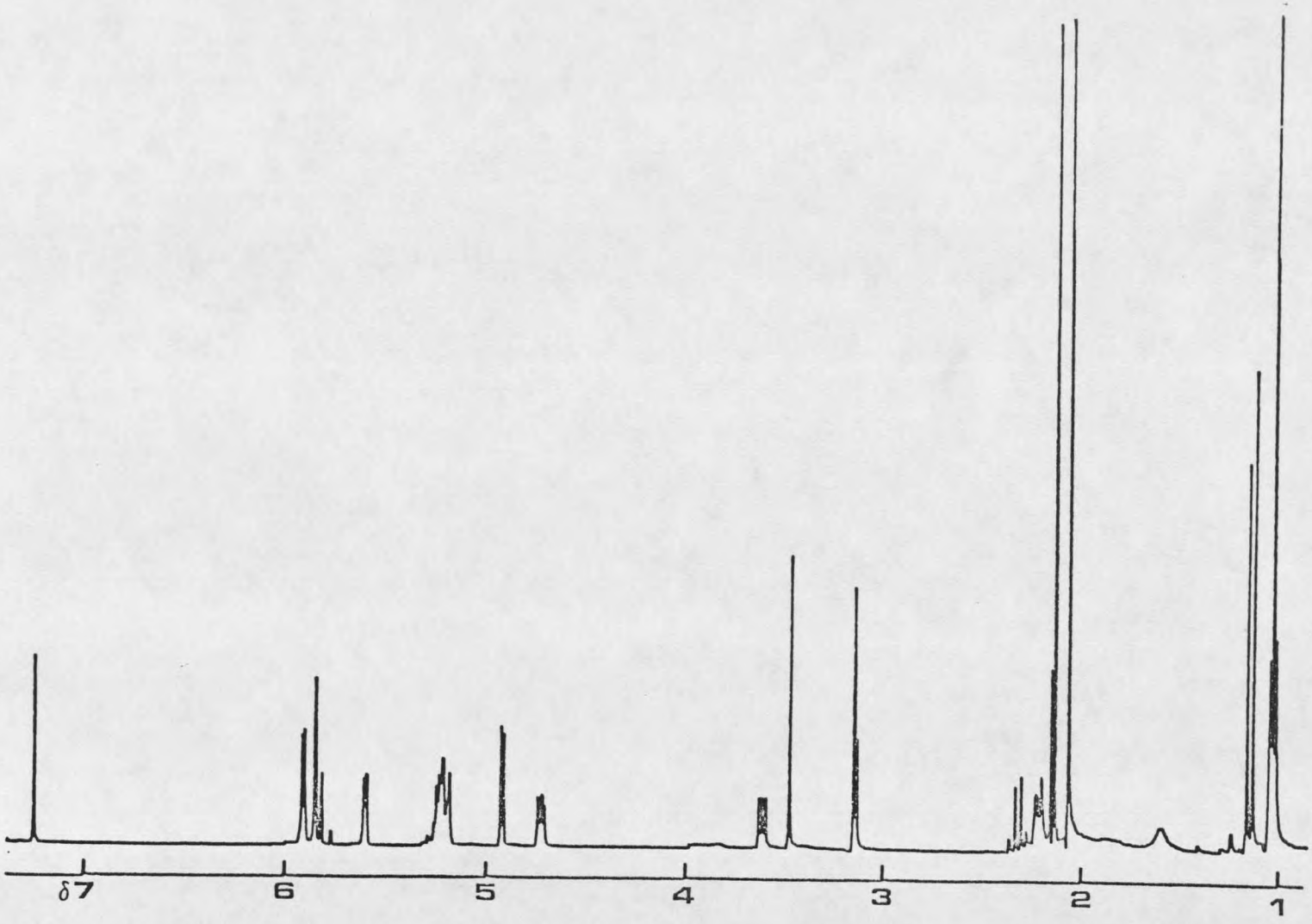


Figure 24. ^{1}H-NMR Spectrum of Brianthein X.

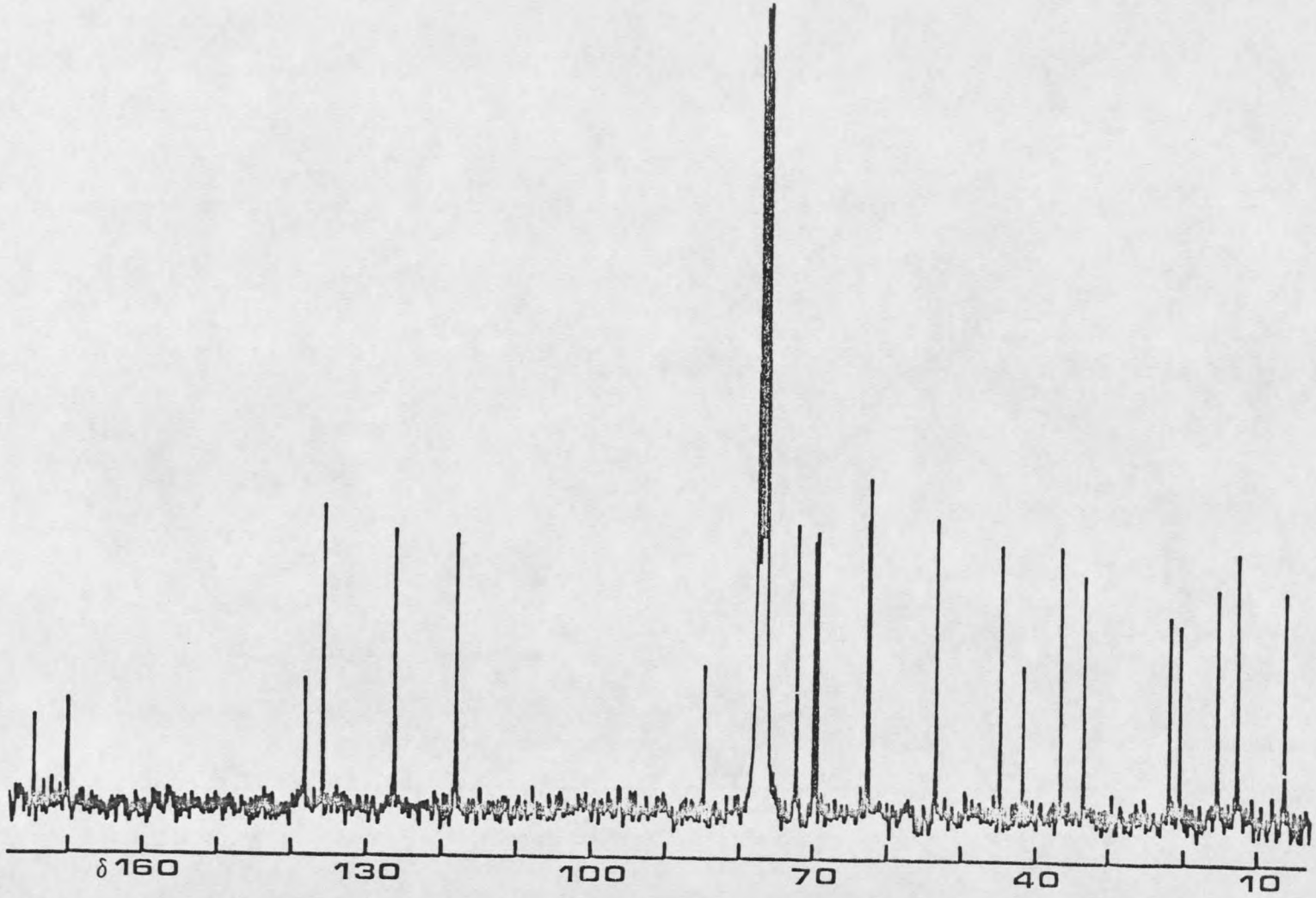


Figure 25. ^{13}C-NMR Spectrum of Brianthein X.

numerous heteroatom bearing carbons as well as to verify the position of the epoxide (see Table 9 for a list of ^{13}C-NMR assignments for brianthein X, Y, and Z). The chemical shift of sp^3 carbons roughly follows substituent electronegativity, as such, the five most deshielded heteroatom bearing carbons bore the three ester, one hydroxyl, and one lactone functionalities and the

Table 9. ^{13}C-NMR Assignments for Briantheins X, Y, and Z.

Carbon #	X	Y	Z	Multiplicity
1	41.54	40.78	40.81	s
2	72.16	75.34[j]	75.66	d
3	135.63	131.09	131.07	d
4	126.12	127.86	127.92	d
5	138.00	136.75	136.80	s
6	62.50	62.38	62.42	d
7	69.39	68.99[j]	69.18	d
8	84.61	84.58	84.64	s
9	77.66	77.41	77.10	d
10	33.24[h]	32.69[b]	32.86[e]	d
11	36.45[h]	36.71[b]	36.78[e]	d
12	69.84	69.38[j]	69.45	d
13	53.24	52.64[j]	52.70	d
14	62.36	61.80[j]	61.85	d
15	6.22	6.01	6.04	q
16	117.91	118.88	118.94	t
17	44.63	44.75	44.78	d
18	15.28	15.81	15.81	q
19	174.42	174.40	174.27	s
20	12.55	12.35	12.41	q
R_2	20.35[i]	20.30[c]	20.29[f]	q
R_1	21.70[i]	21.82[c]	21.78[f]	q
R_3	—	36.13	—	t
R_3	—	18.26	—	t
R_3	—	13.45	—	q
R_3(CO)	—	172.48	169.75	s
R_2(CO)	170.13[g]	170.18[a]	170.07[d]	s
R_1(CO)	169.98[g]	170.16[a]	169.98[d]	s

[a,b,c,d,e,f,g,h,i]—Assignments are interchangeable.
[j]Assignments verified by SFORD

relatively highfield resonance at δ62.38 bore the chlorine. Paralleling the chemical shift of cyclopropanes in respect to their acyclic equivalents, epoxides are notable for the relatively highfield chemical shift of their ^{13}C-NMR resonances. Irradiation of signals at δ2.92 and 3.55, as expected, collapsed the remaining two upfield doublets at δ61.80 and 52.64 respectively.

Left to be assigned were the configuration about the twelve chiral centers of the briantheins and the location of the butyrate ester in **68**. The conformation of brianthein Y will be discussed, however, it is assumed the conclusions will be the same for the entire brianthein series. Relative stereochemistry was suggested by an analysis of the ^{1}H-NMR coupling constants and by comparison with briarein A, whose absolute stereochemistry was determined by X-ray diffraction studies (46). The most striking characteristic of the substituents about the cyclohexane ring of **49** was the axial distribution of the two methyl groups and the acetate groups on C-14 and C-12. Assuming a similar conformation for the acetate and two methyl groups of the cyclohexane ring of **68**, steric considerations and coupling constants would then suggest that the epoxide bond between C-13 and C-14 would be α. The ^{1}H-NMR coupling constants of H_{13}, H_{12}, and H_{11} would then fit nicely for an axial arrangement of the C-12 acetate and the C-11 methyl. Construction of Dreiding models, examination of the dihedral angle between H_2 and H_3, and comparison with their coupling constants indicated a pseudoequatorial arrangement (β) for the acetate on C-2. C-9 of

briarein A contains an axially disposed acetoxyl group and a proton with a coupling constant less than 0.5 Hz. The equivalent proton in **68** has a coupling constant of 8.5 Hz, suggesting opposite stereochemistry. A 3.3 Hz coupling constant for H_6 and H_7 mandated an axial disposition (α) for the chlorine atom and examination of Dreiding models suggested that the hydroxyl and C-17 methyl should also be axial (α).

The location of the butyrate ester in brianthein Y remained to be assigned. Comparison with ptilosarcone, **63**, suggested a position on the cyclohexane ring, in this case on C-12. The absence of a brianthein with two hydroxyls, one acetate, and one butyrate (brianthein X, substituting a butyrate for an acetate) argued against that proposal and suggested instead the butyric acid ester resided on C-2. Brianthein X could then serve as a precursor to **67** and **68**. In either case, spectral evidence was insufficient for assignment.

An X-ray diffraction analysis of brianthein Y was undertaken which proved the location of the butyrate to be C-2 and determined the absolute stereochemistry of the brianthein series to be C-1(S), C-2(S), C-6(S), C-7(R), C-8(R), C-9(S), C-10(S), C-11(R), C-12(S), C-13(R), C-14(R), and C-17(R). A computer generated perspective drawing of **68** is presented in Figure 26.

The most notable observation concerning the crystallographic data is that the configuration about C-9 is not opposite of that in briarein A, **49**, as suggested by the ^{1}H-NMR decoupling experiments. The difference in coupling constants is readily explained by the

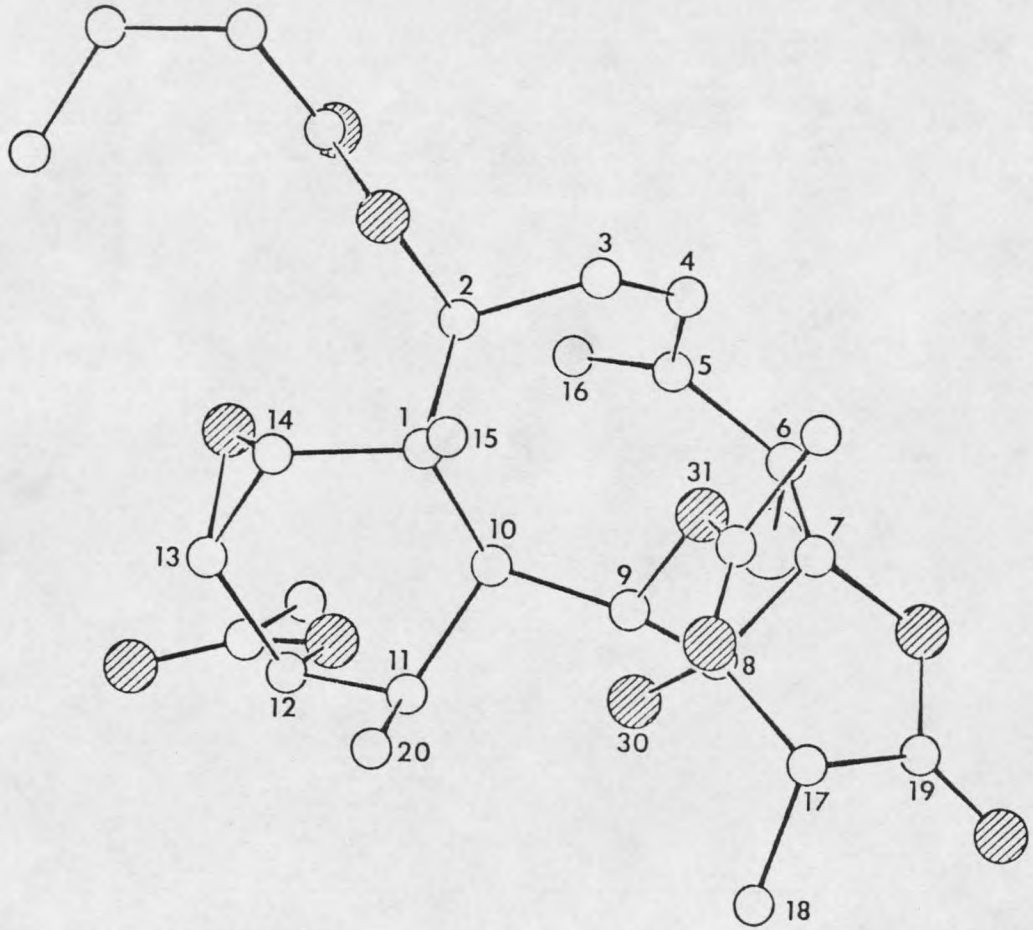


Figure 26. Computer Generated Perspective Drawing of Brianthein Y.

difference in the C-9 - C-10 torsional angle between the two molecules, 45.1°. The ten membered ring in **68** has adopted a much more open conformation due to the absence of an acetoxyl substituent on C-11. In **49** the transannular interaction of the C-11 substituent (α) with the oxygen of the hydroxyl and the exomethylene is greater than in **68**. Indeed, the internuclear distance of C-16 - O-30 and C-20 - O-31 are respectively 3.35 and 3.35 A in **49** and 3.82 and >4.0 A in **68**. Also, as a result of a more relaxed cyclodecane ring, the inclination of the olefinic bonds decrease from 68.9° (planar being 0°) in briarein A to 48.7° in brianthein Y.

The absolute stereochemistry about C-9 of briarein A and brianthein Y are the same, S. The absolute stereochemistry of stylatulide, **62**, was not determined, however, the relative stereochemistry was proven to be identical to that of briarein A (118). Of the diterpenes with the briaran ring skeleton only ptilosarcone, **63**, has been assigned C-9 stereochemistry opposite to that of **49** (119). Their assignment was based on the observation that the ^{1}H-NMR coupling constant at H_9 in **63** was 5.5 Hz while that of **49** was <0.5 Hz. Briarein A, brianthein Y, and ptilosarcone, all possess the same array of substituents about the cyclodecane ring, their only difference being the substituent array about the cyclohexane ring. As discussed previously, the small coupling constant in **49** is due to the steric congestion of the C-11 acetyl with the C-8 hydroxyl which consequently effects the torsional angle between C-9 and C-10. The absence of an acetoxyl substituent on C-11 in **63** provides for a less constrained 10 membered ring which, as in **68**, changes the C-9 - C-10

torsional angle and necessarily increases the ^{1}H-NMR coupling constant of H_9. This suggests the stereochemistry of C-9 in ptilosarcone is identical to that of briarein A and brianthein Y and not of opposite stereochemistry as previously assigned.

The chemotaxonomic significance of the characterization of the brianthein series lies in the confusion surrounding the identity of the producing organism. Dr. Frederick M. Bayer of the Smithsonian Institution attests to B. polyanthes having been variously described as Ammothea polyanthes Duchassaing and Michelotti (1860), Erythropodium polyanthes Deichmann (1936) and synonymous with Briareum asbestinium Bayer (1961). The absence of briarein A and the presence of large amounts of brianthein X, Y, and Z, diterpenes possessing the briaran ring skeleton, but with a different array of functionalities, lends credence to the current classification of Briareum polyanthes as a distinct species.

Pharmacological and Insecticidal Activity of the Briantheins

The pharmacological activity of diterpenes possessing the briaran carbon skeleton is well documented. Ptilosarcone, **63**, inhibited acetylcholinesterase and serum cholinesterase and was just mildly toxic to mice, LD_{50} 7.4 mg/kg (119), whereas stylatulide, **66**, showed toxicity (LD_{100}) to larvae of the marine copepod Tishbe furcata johnsonii at concentrations of 0.5 ppm (118). One of the three diterpenes from Scytalium tentaculatum, **65**, was measured for its effect on the cardiac output of anaesthetized cats. Interperitonal injection (150 mg/kg) produced increased blood

pressure and heart rate for 30–40 minutes followed by a decrease to levels below normal (120).

Field tests on freshly prepared extracts of E. polyanthes, using the same microbes as described previously for D. etheria, were negative. Brianthein X, Y, and Z were also tested for activity against C. lunata, R. glutinis, F. solani, P. syringae. and C. michiganense. Each brianthein showed no activity against any of the plant pathogenic microbes. In addition 68 exhibited no mutagenicity in Salmonella strains TA 97, TA 98, and TA 100. The onset of toxicity to the bacteria occured at concentrations of 7 μg/ml; however, mutagenicity was not observed. Also, 68 had no effect in tests for selective toxicity to bacteria deficient in DNA repair capacity.

The lack of activity against any of the microbes tested suggested the briaran diterpenes had a more sophisticated mode of action.

The grasshopper Melanoplus bivittatus is a major source of agricultural crop loss in the plain and plateau states. Brianthein Y, because it was the most abundant diterpene, was chosen to determine if the briantheins have insecticidal or insect repellant activity by observing its effect on the feeding behavior of the grasshopper.

Approximately 3 mg of 68 were coated, in an ethanol–water solution, onto freshly cut squares (2x2 cm) from leaves of sunflower plants. Three sets of grasshoppers were maintained. One set was

given the treated squares, one set squares treated with just an ethanol-water solution, and the final set of controls untreated sunflower leaves. All grasshoppers were given water regularly. The insects exposed to 68 expired within five days, while all but one (of eight) controls survived. Before death it was observed that the exposed insects did not respond to stimuli as the controls did (i.e. knocking or shaking of their beaker). They appeared to be in a stupor with concomitant loss of equilibrium and coordination, suggesting perhaps, the mode of action of brianthein Y is through the central nervous system. In support of this view is the inhibitory activity demonstrated by ptilosarcone on acetylcholinesterase and serum cholinesterase, enzymes which control the breakdown and biosynthesis of the well known neurotransmitter acetylcholine (123).

CONCLUSION

The isolation and characterization of the novel diterpenes, brianthein X, **69**, Y, **68**, and Z, **67**, from the gorgonian soft coral Briareum polyanthes is significant for two reasons. From a taxonomic perspective, their presence supports the new classification of B. polyanthes as a taxonomically distinct species. From a biochemical perspective, the absence of inhibitory activity demonstrated by the antimicrobial and DNA repair assays in conjunction with the demonstrated insecticidal activity against Melanoplus bivittatus suggested a mode of action involving the central nervous system. The inhibitory action of ptilosarcone on acetylcholinesterase and serum cholinesterase supports this view.

The characterization of these compounds utilized, by necessity, a relatively novel mass spectral technique. Fast atom bombardment of a glycerol matrix containing dissolved potassium iodide and one of the briantheins gave $(M+K)^+$ and $(M+K+2)^+$ ions from which the molecular formulas could be obtained. ^{1}H-NMR decoupling experiments and ^{13}C-NMR allowed structure elucidation (except for the placement of the butyrate in **68**). The correct relative stereochemical assignments of all but one chiral center were afforded by examination of ^{1}H-NMR coupling constants and comparison with briarein A. An X-ray diffraction analysis of brianthein Y placed the butyrate and assigned the absolute configuration in the series. The stereochemistry of ptilosarcone **63** and **68** were discussed which led to the suggested reassignment of the C-9 configuration in **63**.

The characterization and presence of the discussed isolates of Dysidea etheria were important for predominantly one reason. A source for the reported antibiotic activity of D. etheria (66) was suggested by the weak activity demonstrated by ceramide, **60**, furodysinin, **27**, and furodysinin lactone, **56**, against some plant pathogenic microbes.

The characterization of **56** was accomplished by spin-spin decoupling experiments, by comparison with **27**, and by a lanthanide induced shift experiment, which utilized the calculations of the PDIGM program to determine the best fit. The unusual preference of the carbonyl oxygen over the hydroxyl oxygen as site of europium-oxygen chelation was discussed and explained. The assignment of the ^{13}C-NMR resonances of **27** was realized by a series of heteronuclear J-modulated spin echo experiments which differentiated a group of four overlapping highfield signals (in the off-resonance decoupled spectrum).

Analysis of the nudibranch Hypselodoris zebra, found feeding on D. etheria, revealed high concentrations of furanosesquiterpenes. Furodysinin and 5-acetoxy nakafuran-8, **24**, were identified by direct comparison with those previously isolated from D. etheria (^{1}H-NMR and MS), 5-hydroxy nakafuran-8, **59**, by direct comparison with the hydrolysis product of **24** (^{1}H-NMR and MS), and euryfuran, **34**, by comparison with the physical constants reported in the literature (^{1}H-NMR and MS). The antifeedant nature of furodysinin was discussed (67,73) and a similar deterrent role was suggested for the remaining furanosesquiterpenes.

Structure elucidation of the ceramides was afforded by spin-spin decoupling experiments on the acetylated derivatives and by analysis of the products obtained by acid hydrolysis. The sphingosine hydrolyzate was converted to its triacetate and identification accomplished by comparison with literature reported physical data. The methyl esters of the fatty acids obtained by hydrolysis were analyzed by MS and revealed a profile typical of fatty acids isolated from marine sponges (114,115). The optical rotation of the α-hydroxy fatty acid methyl esters suggested an S configuration about C-2. The importance of this finding, which suggests a different biosynthetic mechanism for the production of α-hydroxy fatty acids in D. etheria, was discussed.

EXPERIMENTAL

Physical Measurements

NMR spectra were obtained on a Brucker WM-250 (250 MHz) multinuclear Fourier Transform Spectrometer, using $CDCl_3$, d_6-acetone, d_5-pyridine, pyridine, or d_8-tetrahydrofuran as solvent and internal standard. Chemical shifts are reported in ppm (δ units) relative to tetramethylsilane (δ=0). Mass spectra were obtained at Montana State Unversity on a VG MM-16F operating at 70 eV in the electron impact mode or chemical ionization mode or on a VG 7070HE operating in the fast atom bombardment mode (FAB) or at the Midwest Center for Mass Spectrometry on a Kratos MS-50 high resolution mass spectrometer. IR spectra were determined on a Beckman IR-20 spectrophotometer, UV spectra on a Varian G34 spectrophotometer, and optical rotations with a Carl Zeiss Circle Polarimeter. GC separations were obtained on a Varian 3700 Gas Chromatograph with an injection port temperature of 230°C, utilizing a 5% SE-30 packed column (6'x 1/8") whose temperature was programed to rise from 150 to 220°C at a rate of 10°C/min. The carrer gas was helium and the flow rate was maintained at 30 mL/min; detection was accomplished by the MM-16F mass spectrometer with a source temperature of 200°C. Column chromatography was accomplished with the packings listed in Table 10 and eluants were monitored on an ISCO UA-5 UV monitor operating at 254 and 280 mn. The Beckman-Altex component system, consisting of the model 110A pump and the model 153 UV monitor (operating at 254 nm), was used for separations requiring high performance liquid

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

Table 10. Chromatography Packing Material.

Material	Mesh	Manufacturer
Florisil	60-200	Floridin Corp.
Silica gel	70-230	EM Reagents
Silica gel	37-53 μm	Whatman Inc.
Sephadex LH-60	25-100	Pharmacia Fine Chemicals
Sephadex LH-60	40-120	Pharmacia Fine Chemicals
Biobeads S-X8	200-400	Bio-Rad Laboratories

Pharmacological and Insecticidal Screening

Assays for mutagenicity and selective toxicity to bacteria deficient in DNA repair capacity were conducted by Dr. Samuel J. Rogers of this department.

Qualitative antimicrobial assays were conducted on freshly collected organisms and on their purified isolates. Each freshly collected organism was chopped and extracted briefly with acetone. Sterile paper discs were dipped in the extract and placed on agar plates which were subsequently streaked with a test microbe. The test microbes were Bacillus subtilis, Staphylcoccus aureus, Escherichia coli, Pseudomonas aeruginosa, and Candida albicans

The purified isolates were assessed for activity against a series of plant pathogens. Table 11 lists the microbes used, their

appropriate growth media, and the diseases they inflict. The isolated metabolites were each dissolved in an appropriate solvent; tetrahydrofuran for the ceramides, dichloromethane for furodysinin, furodysinin lactone, and the briantheins. A sterile paper disc was dipped into the solution and placed onto an agar plate. The test microbes were prepared four to six days in advance by transferring and streaking onto fresh growth media. Transfer of the test microbe was accomplished by immersion in distilled water, vigorous scraping and shaking, pouring into a glass jar, and finally spraying onto the agar plates containing the test compounds.

The insecticidal activity of brianthein Y was tested in the following way. Approximately 20 grasshoppers, Melanoplus bivittatus, were collected on the day the test began. A total of eight insects were isolated in separate 500 mL beakers enclosed with a sheet of aluminum foil that had been punctured with 0.5 cm holes to allow for respiration. Three of the isolated grasshoppers were fed untreated sunflower leaves to ensure their eating habits had not changed in the new environment. All demonstrated hearty appetites. Brianthein Y was dissolved in an ethanol-water solution and applied to a sunflower leaf such that 3 mg of compound resided on each cut 2x2 cm square. The treated squares were placed with three of the isolated grasshoppers and two squares treated with just the ethanol-water solvent system, were placed with the remaining grasshoppers. All the insects were fed water regularly. Two of the treated grasshoppers

Table 11. Test Organisms for Antimicrobial Assays.[a]

Microbe	Growth medium	Disease Name
Pseudomonas syringae	Kings medium B[b]	Bacterial canker of stone fruits, Citrus blast, Lilac blight.
Corynebacterium michiganese	Nutrient agar[b]	Bacterial canker of tomato.
Fusarium solani	Potato dextrose agar[b]	Fusarium wilt of chili pepper.
Curvularia lunata	Potato dextrose agar[b]	Gladiolus flower blight and leaf spot, Curvularia disease.
Rhodotorula glutinis	Potato dextrose agar[b]	Contaminating organism found in microbiological laboratories, no economic significance.

[a]Information on these plant pathogenic microbes was obtained from references 124 and 125.

[b]Obtained from Difco Laboratories, Detroit, Michigan.

died within 36 hours, the third after being fed an additional square treated with 3 mg of **72** on the third day, died on the fifth day. After 48 hours one of the controls being fed untreated sunflower leaves expired.

Collection and Extraction of Dysidea etheria

Dysidea etheria was collected from a variety of shallow water (2–8 m deep) habitats in Bermuda, primarily in Harringon Sound, in October, 1979, and August, 1982, and stored in acetone prior to extraction.

The 1979 collection (dry weight 210.7 g) was homogenized in a Waring Blender and extracted with acetone (twice, 24 hours each), then with dichloromethane (thrice, 24 hours each). The aqueous suspension remaining after evaporation of the acetone extract was equilibrated with the dichloromethane extracts. The organic phase was concentrated to yield 17.5 g of a brown oil (8.3% of dried weight).

The acetone extracts from the 1982 collection (dry wt. 350 g) were decanted and filtered. The sponges were then ground in a Waring blender and extracted with acetone (twice, 24 hours each), then with dichloromethane (thrice, 24 hours each). The combined acetone extracts were reduced, *in vacuo*, to an aqueous suspension. The dichloromethane extracts were then equilibrated with the aqueous suspension which gave, upon subsequent evaporation of the

dichloromethane phases, 17.4 g of crude extract (4.9% of dried weight).

Crude Separation of 1979 Collection

Initial separation was accomplished by chromatography of 7.3 g of the crude extract on 275 g of Florisil with a hexane-ethyl acetate-methanol gradient which gave fifteen fractions. Fraction 1, eluted with hexane and hexane-ethyl acetate (49:1), was a nearly colorless oil with a pleasant aroma (912 mg) and fraction 10, eluted with ethyl acetate-methanol (97:3) was a brown colored gum (272 mg).

Isolation of Furodysinin

A portion of Florisil fraction 1 (210 mg) was further purified on silica gel (30g, 70-230 mesh). Elution with hexane gave 146 mg of a colorless oil. Additional separation was accomplished by gel permeation through Bio-Beads S-X8 (2 x 125 cm). Elution with dichloromethane-cyclohexane (3:2) yielded one major fraction (118 mg), which was then submitted to flash chromatography (100 g silica gel, 37-53μm). Hexane eluted one major fraction (109 mg),whose ^{1}H-NMR and ^{13}C-NMR (see Table 2) spectra were identical with those reported (32) for furodysinin, **27**; $\gamma_{max}^{CHCl_3}$ 2861,2838,1632,1377,1361 cm^{-1}; λ_{max}^{EtOH} 223 nm (ε =8300); ^{1}H-NMR ($CDCl_3$): δ7.21 (1H, br s), 6.22 (1H, d J=1.8), 5.62 (1H, dd J=4.7,1.3), 2.70 (1H, dd J=19.5,6.5), 2.65 (1H, m), 2.28 (1H, dd J=19.5,13), 2.03 (2H, m), 1.71 (1H, m), 1.66 (3H, br s), 1.55 (1H, ddd J=10.3,3.7,1.6), 1.28 (1H, m), 1.21 (3H, s), 1.20(3H, s); MS: m/z (rel int) 216(33), 201(15), 122(100), 107(11), refer to Figure 5 for additonal fragments; HRMS: m/z

216.1510 (M^+, $C_{15}H_{20}O$ requires 216.1506), 122.0742 ($C_8H_{10}O$ requires 122.0751).

Isolation of Furodysinin Lactone

Florisil fraction 10 was permeated through Sephadex LH-20 (2 x 125 cm) with dichloromethane-methanol (1:1) to give six fractions. The sixth fraction was further separated by HPLC on an Ultrasphere-ODS column. Elution with acetonitrile-water (3:2) yielded 12 mg of furodysinin lactone, **56**: λ_{max}^{EtOH} 221 nm (ε=8700); γ_{max}^{CHCl3} 3580,3330,2925,2890,2857,1745,1640 cm^{-1}; MS: m/z (rel int) 248 (M^+, 6), 230(95), 215(66), 202(30), 187(25), 159(20), 140(63), 112(97), 93(100), 79(66), 67(71); HRMS: m/z 248.1404 (M^+, $C_{15}H_{20}O_3$ requires 248.1412), 93.0715 (C_7H_9 requires 93.0705).

Oxidation of Furodysinin

To a stirring mixture of 113 mg furodysinin, **27**, in 2.5 mL dichloromethane, 120 mg m-chloroperbenzoic acid were added over ten minutes at room temperature. The mixture was then heated to 35°C for thirty minutes. The dichloromethane was evaporated and the residue was taken up in 2 mL acetone. Jones reagent (1.5 mL) was then added over twenty five minutes at 0°C. After the mixture was heated to 30°C and stirred for an additional half hour, the reaction was quenched with isopropanol. The mixture was then filtered twice through Hyflo-Super Cel and evaporated. The residue was suspended in dichloromethane, washed with 5% $NaHCO_3$, dried over $MgSO_4$, and concentrated again to yield 39 mg of a light brown gum, which was

purified by gel permeation chromatography through Bio-Beads S-X8 with dichloromethane-cyclohexane (3:2) to yield 27 mg of furodysinin lactone, **56** (21% yield), identical in all respects with the natural product.

Photooxidation of Furodysinin in Methanol

In a test tube, 188 mg of furodysinin, **27** were dissolved in 2.5 mL methanol and 3.5 mL dichloromethane to which was added a catalytic amount of Rose bengal. Oxygen was bubbled through the mixture which was immersed in cold water during illumination by a Sylvania 300 watt bulb for 4 hours (68). The mixture was then reduced *in vacuo*, dissolved in dichloromethane and washed with distilled water. The organic phase was evaporated and permeated through Sephadex LH-20 with dichloromethane-methanol (1:1) to yield 148 mg of the 4-*O*-methyl-furodysinin lactone, **59**; ^{1}H-NMR ($CDCl_3$): δ 5.78 (1H, s), 5.30 (1H, br dd, J=5.7,1.5), 3.12 (3H, s), 2.70 (1H, m), 2.30 (1H, dd, J=13.8,4), 1.96 (2H, m), 1.69 (1H, br ddd, J=12.6,3.7,3.1), 1.59 (3H, br s), 1.55 (1H, dd, J=14,14), 1.31 (3H, s), 1.20 (3H s) 1.10 (1H, br ddd, J=12.6,12,9.5).

Photooxidation of Furodysinin in Tetrahydrofuran

In a test tube immersed in cold water, 121 mg of **27** was dissolved in 3 mL THF to which was added a catalytic amount of Rose bengal. Distilled water was added dropwise (0.4 mL) over ten minutes as oxygen was bubbled through the mixture during illumination by a Sylvania 300 watt bulb for 1.5 hours (68). The mixture was then reduced *in vacuo*, leaving an aqueous residue which was diluted and

then extracted three times with dichloromethane. The dichloromethane extract was evaporated and analysis by ^{1}H-NMR revealed mostly starting material.

LIS Study of Furodysinin Lactone

Furodysinin lactone, **56** (3 mg) was dissolved in 0.5 mL $CDCl_3$ and transferred to a 5 mm NMR tube. One equivalent of $Eu(fod)_3$ (12.4 mg) was added to the solution in approximately 0.1 equivalent increments. ^{1}H-NMR spectra were recorded after the addition of each equivalent. The change in chemical shift for each distinguishable proton was then determined for each recorded spectrum.

Linear least squares slopes of the change in chemical shift versus equivalents $Eu(fod)_3$ added were obtained on a Texas Instrument TI-55 hand calculator by consideration of the points between 0.3 and 0.8 equivalents. Coordinate files were obtained by projecting a Dreiding model of **56** and its C-4 epimer onto graph paper containing 1 mm grids. The Dreiding models were secured to the graph paper by tape. X and Y coordinates were then obtained by projection onto the graph paper and Z coordinates were obtained directly by a centimeter ruler. All coodinates were then converted to angstrom units.

The coordinates thus obtained were entered into the PDIGM program (70) along with the slopes obtained from the change in proton chemical shift versus equivalents $Eu(fod)_3$ added. The agreement factors (R values) and europium-oxygen distances were then calculated. The R values versus sites of chelation for **56** and its C-4 epimer were tabulated and are presented in Table 4.

LIS Study of Semisynthetic Furodysinin Lactone

Semisysnthetic furodysinin lactone (5.2 mg) was dissolved in 0.5 mL $CDCl_3$ and transferred to a 5 mm NMR tube. One equivalent (21.8 mg) of $Eu(fod)_3$ was added in approximately 0.1 equivalent intervals. The ^1H-NMR spectra were recorded, changes in proton chemical shift calculated, slopes of change in proton chemical shift versus equivalents $Eu(fod)_3$ added were determined, and R values obtained, in the exact manner as previously described. The only site of europium chelation considered for the PDIGM calculations was the carbonyl oxygen.

Collection and Extraction of Hypselodoris zebra

Five specimens of Hypselodoris zebra were collected in Shark's Hole and Church Bay, Harrington Sound, and under the airport causeway in Castle Harbour, Bermuda. The nudibranchs were stored in acetone prior to extraction. The animals (dry weight 1.5 g) were extracted with acetone (twice, 24 hours), then with dichloromethane (thrice, 24 hrs). The aqueous suspension remaining after evaporation of the acetone extracts was equilibrated with the dichloromethane extracts. The organic phase was then concentrated to yield 192 mg of a pleasant smelling orange oil.

Isolation of Furanosesquiterpenes

The organic extract (192 mg) was initially separated by gel permeation chromatogrphy on Bio-Beads S-X8 (125 x 2 cm). Elution with cyclohexane-dichloromethane (2:3) yielded seven fractions. The

fifth fraction (71 mg) was further purified by permeation through Sephadex LH-20 (125 x 2 cm). Elution with dichloromethane-methanol (1:1) yielded five fractions. The third fraction's (54 mg) ^{1}H-NMR spectrum indicated a mixture of furodysinin, **27**, euryfuran, **34**, and 5-acetoxy-nakafuran-8, **24**. Analysis by GC-MS indicated that the ratio of furodysinin (m/z 216, 122 base peak) to euryfuran (m/z 218, 201 base peak) to 5-acetoxy-nakafuran-8 (m/z 274, 232, 43 basepeak) was 2:1:1. The sixth fraction (16 mg) from the initial Bio-Beads S-X8 separation had an NMR spectrum identical to that of a mixture of furodysinin, 5-hydroxy-nakafuran-8, **59**, and euryfuran. Analysis by GC-MS indicated that the ratio of euryfuran to furodysinin to 5-hydroxy-nakafuran-8 (m/z 232, 136 base peak) was 3:2:0.1.

Isolation of Ceramides

The dichloromethane soluble extract (17.4 g) from the 1982 collection was chromatographed on Florisil (350 g). Elution with combinations of hexane, ethyl acetate, and methanol of gradually increasing polarity gave 13 fractions. ETOAc-MeOH (97:3), ETOAc-MeOH (95:5) and ETOAc-MeOH (90:10) eluted three fractions (302 mg, 276 mg, and 51 mg, respectively), each of which contained the sphingosine derivatives as detected by TLC (SiO_2, THF-hexane, 1:1) R_f 0.26 and 0.35. Detection was best achieved with phosphomolybdic acid. The crude fractions were individually submitted to gel permeation on Sephadex LH-20 (125 x 2 cm), using CH_2Cl_2-MeOH (1:1), to give 64 mg, 160 mg, and 23 mg fractions, respectively, each highly enriched in sphingosine derivatives (as indicated by TLC). These three fractions

were combined, washed with cold methanol and then purified by flash chromatography (100 g, SiO_2, 37-53μm), using hexane-tetrahydrofuran (1:1) to obtain two fractions. The minor fraction (32 mg, R_f 0.35) contained a mixture of N-acylspingosines whose spectral data were similar to those previously reported (87): γ_{max}^{KBr} 3300,2912,2845,1623,1525,1453 cm^{-1}; ^{13}C-NMR (pyridine-d_5): δ 173.54(s), 132.38(d), 132.33(d), 73.36(d), 62.17(t), 56.84(d), 36.82(t), 32.64(t), 32.03(2C, t), 30.37-29.50(XC, t), 26.33(t), 22.83(2C, t), 14.14(2C, q). The major fraction (44 mg, R_f 0.26) contained a mixture of α-hydroxy-N-acylsphingosines, **61**, γ_{max}^{KBr} 3330,2912,2842,1624,1517,1447 cm^{-1}; ^{13}C-NMR (pyridine): 175.00(s), 132.15(d), 131.58(d), 72.50(d), 71.94(d), 61.32(t), 55.46(d), 35.18(t), 32.16(t), 31.53(2C, t), 29.41-29.03(XC, t), 25.26(t), 22.32(2C, t), 13.66(2C, q). There was an indeterminant number of carbons between δ30.37-29.50 in **60** and 29.41-29.03 in **61** due to the presence of a mixture of ceramides. ^1H-NMR data has been tabulated and reported for **60** and **61** in Table 6.

Acetylation of α-Hydroxy-N-acylsphingosines

A mixture of α-hydroxy-N-acylsphingosines (11 mg) was added to 0.5 mL pyridine, 2 mg 4-N,N-dimethylaminopyridine and 0.5 mL acetic anhydride. The flask was flushed with nitrogen, heated to 35°C for 1.5 hours and left at room temperature overnight. The organic solvents were evaporated and the residue separated on Sephadex LH-20 (125 x 2 cm) by elution with CH_2Cl_2-MeOH (1:1) to yield 11 mg of the triacetylated N-acylsphingosine, **61a**; $\gamma_{max}^{CHCl_3}$ 3409,2887,2839,1723,

1668,1347 cm^{-1}. ^{13}C-NMR ($CDCl_3$): δ171.45(s), 169.96(s), 169.91 (s), 169.77(s), 130.77(d), 124.01(d), 74.16(d), 73.74(d), 62.13 (t), 50.98(d), 32.26(t), 31.88(2C, t), 29.66-29.30(XC, t), 28.82 (t), 24.79(t), 22.63(2C, t), 21.12(q), 21.10(q), 20.77(q), 13.99 (2C, q). There was an indeterminant number of carbon resonances between δ29.66 and 29.30 due to the presence of a mixture of ceramides. The ^1H-NMR data was tabulated and presented in Table 5.

Acetylation of N-Acylsphingosine

A mixture of N-acylsphingosine (4 mg) was added to 0.5 mL pyridine, 1 mg 4-N,N-dimethylaminopyridine and 0.5 mL acetic anhydride. The flask was flushed with nitrogen heated to 35°C for 1 hour and left at room temperature overnight. The organic solvents were evaporated and the residue separated on Sephadex LH-20 (125 x 2 cm) by elution with CH_2Cl_2-MeOH (1:1) to yield 3 mg of the diacetylated N-acylsphingosine derivative, **60a**. The ^1H-NMR data has been tabulated and recorded in Table 5.

Hydrolysis of α-Hydroxy-N-acylsphingosines

A portion of the mixture of α-hydroxy-N-acylsphingosines (28 mg), in 7 mL of THF, was hydrolyzed with 7 mL 1.2 M H_2SO_4 in 85% MeOH at gentle reflux for 4 hours (85). The organic solvents were then evaporated, yielding an acidic aqueous suspension which was extracted twice with hexanes to remove the α-hydroxy-fatty acid methyl esters (16 mg). The aqueous phase was then basified with 2 M KOH and extracted twice with hexane-CH_2Cl_2 (1:1) to remove the sphingosine (8

mg). The fatty acid esters were separated by gel permation on Sephadex LH-20 with CH_2Cl_2-MeOH (1:1) to yield one major fraction (14 mg) which contained n-C_{22} (MS: m/z 370, 311), n-C_{23} (MS: m/z 384, 325) and n-C_{24} (MS: m/z 398, 339) acid methyl esters in ca. 9:1.5:1 ratio, $[\alpha]_D$+19.5 ($CHCl_3$, c 0.61).

Hydrolysis of N-Acylsphingosine

A portion of the mixture of N-acylsphingosines (27 mg) was hydrolyzed exactly as above. The fatty acid methyl esters (10 mg) were separated on Sephadex LH-20 by CH_2Cl_2-MeOH (1:1) to yield three major fractions. The first two (2 mg and 4 mg, respectively) contained n-C_{22} (MS: m/z 354, 311), n-C_{23} (MS: m/z 368, 325), n-C_{24} (MS: m/z 382, 229) in an overall ratio of ca. 8:1.5:1. The third fraction (1 mg) contained pentadecanoic (MS: m/z 256, 213), hexadecanoic (MS: m/z 270, 227), heptadecanoic (MS: m/z 284, 241), and octadecanoic (MS: m/z 298, 255) acid methyl esters in ca. 1:11:5:3 ratio.

Acetylation of Sphingosine

Sphingosine (8 mg) was added to 0.5 mL pyridine, 0.5 mL acetic anhydride and 1 mg 4-N,N dimethylpyridine. The mixture was sealed under nitrogen and then heated to 40°C for two hours. After cooling overnight, the solvent and excess reagent were evaporated and the residue separated by gel permeation on Sephadex LH-20 with CH_2Cl_2-MeOH (1:1) to yield one major fraction, 6 mg of sphingosine triacetate, $[\alpha]_D$-8.3 ($CHCl_3$, c 0.58) [lit. 93 $[\alpha]_D$-11.7 ($CHCl_3$, c 1.0)], ^{1}H-NMR ($CDCl_3$): δ5.76 (1H dt, J= 15.3,6.9), 5.63 (1H, br d

J=9.0), 5.37 (1H, dd, J=15.3,6.5) 5.25 (1H, dd, J=6.5,6.9), 4.32 (1H m), 4.27 (1H, dd, J=11.7,5.9), 4.02 (1H, dd, J=11.7,3.7), 2.05 (3H, s), 2.04 (3H, s), 1.96 (3H, s), 1.32 (2H, m), 1.24 (22H, br s), 0.86 (3H, br t, J=7.2), MS m/z (rel int): 426(3), 370(4), 369(4), 366(2), 352(2), 338(7), 277(30), 75(100), 57(100).

Collection and Extraction of Briareum polyanthes

Briareum polyanthes was collected in October, 1979, and September, 1981, at depths of 4 to 7 meters in the channel between Governor's Island and Smith's Island. The specimens were chopped and stored in acetone at -5°C prior to extraction. The acetone was decanted and the gorgonian pieces were homogenized in a Waring blender with fresh acetone. The combined filtered extracts were then reduced to an aqueous suspension. The soft coral marc was extracted twice with dichloromethane, after which the dichloromethane extracts and the aqueous suspension were equilibrated. Evaporation of the dichloromethane phase, in vacuo, gave 25.4 g of a thick brown oil (from 482.3 g dry weight).

Partitioning and Fractionation of Crude Extract

The crude extract was distributed between hexane and 10% aqueous methanol. The polar phase was increased to 25% water and then extracted with carbon tetrachloride. Finally, the upper phase was increased to 35% water and extracted with cholorform. The methanol was then evaporated from the aqueous phase and the remaining aqueous solution was then extracted with ethyl acetate. Evaporation

of the hexane phase gave 18.62 g extract, the carbon tetrachloride 3.75 g, the chloroform 2.27 g, and the ethyl acetate 0.13 g.

The chloroform soluble extract was applied to a 2.2 x 195 cm column of Sephadex LH-20 and elution with hexane-dichloromethane (1:4) gave five fractions. Subsequent elution with acetone-dichloromethane (2:3), then (4:1), yielded three fractions with each solvent combination.

Isolation of Briantheins Z and Y

Fraction 2 from the step gradient gel permeation chromatography (565 mg) was permeated through Sephadex LH-60 (column 2 x 168 cm) with dichloromethane-acetonitrile (3:2). Two fractions were obtained. The first, 492 mg, was further separated by Bio-Beads S-X8 (column 1.7 x 126 cm) with cyclohexane-dichloromethane (2:3) to give five fractions. The third fraction, 451 mg, was subjected to HPLC on an Ultrasphere-Cyano column (0.9 x 25 cm). Seven fractions were obtained by elution with hexane-isopropanol (2:1). The fourth was brianthein Y, **68**, 129 mg, and the fifth was brianthein Z, **69**, 30 mg.

Isolation of Brianthein X

Fraction 4 from the step gradient gel permeation chromatography (528 mg) was permeated through Sephadex LH-20 (column 2 x 122 cm) with dichloromethane-methanol (1:1). The second of three fractions, 310 mg, was filtered next through Sephadex LH-60 with dichloromethane-acetonitrile (3:2). The second of three fractions, 241 mg, was submitted to HPLC on an Ultrasphere-Cyano column (0.9 x

25 cm). Elution with hexane–isopropanol (2:1) gave brianthein X, 69, 105 mg, as the second of three fractions.

Characterization of Briantheins X, Y, and Z

Brianthein X (69): mp 230–243°C (dec); $\lambda_{max}^{CH_2Cl_2}$ 231 nm (ε=6900); $\gamma_{max}^{CHCl_3}$ 3566,2963,1788,1739,1363 cm^{-1}; MS (rel int) $(M+K)^+$ 537(.7), $(M+K+2)^+$ 539(.4), 519(.4), 502(.6), 477(.4). For additional fragments, see Figure 24.

Brianthein Y(68): mp 233–235°C (dec); $\lambda_{max}^{CH_2Cl_2}$ 229 nm (ε=6500); $\gamma_{max}^{CHCl_3}$ 3575,2964,1788,1739,1364 cm^{-1}; MS (rel int) $(M+K)^+$ 607(2.7), $(M+K+2)^+$(1.2), 587(2.1), 567(3.9), 566(2.3), 565(3.9), 549(.7), 519(.9), 517(2.7).

Brianthein Z(67): mp 240–242°C (dec); $\lambda_{max}^{CH_2Cl_2}$ 230 nm (ε=6700); $\gamma_{max}^{CHCl_3}$ 3540,2930,1790,1739,1365 cm^{-1}; MS (rel int) $(M+K)^+$ 579 (.4), $(M+K+2)^+$ 581(.2), 561(.3), 537(.3), 519(.4). For additional fragments see Figure 21.

An MS of the glycerol-KI matrix is provided (Figure 27) for comparison with Figures 21 and 24. The ^1H- and ^{13}C-NMR data are tabulated and presented in Tables 8 and 9.

Acetylation of Brianthein X

Brianthein X, 5 mg, was dissolved in 0.5 mL dry pyridine. Acetic anhydride (0.5 mL) was added and the mixture was allowed to stand in a sealed flask at 50°C for one hour. Solvent and excess reagent were removed, in vacuo, and the residue was permeated through Bio-Beads S-X8 with dichloromethane–cyclohexane (3:2) to give, in

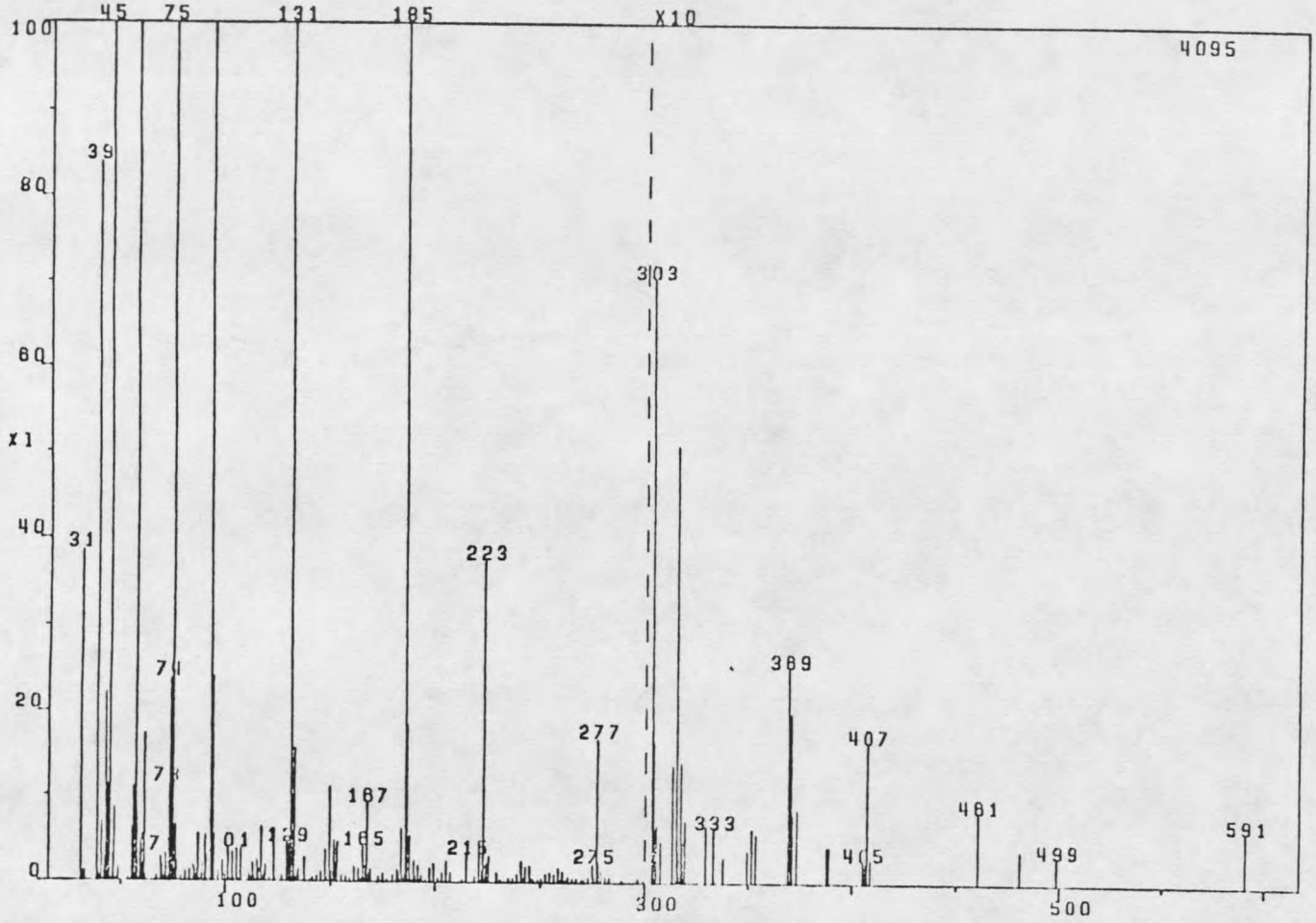


Figure 27. Mass Spectrum of the Glycerol-KI Matrix.

quantitative yield, a crystalline solid, mp 243–246°C, whose ^{1}H-NMR spectrum was superimposable on that of brianthein Z, 71.

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