ANTIFEEDANT DITERPENES FOR THE GYPSY MOTH LARVAE FROM *KALMIA LATIFOLIA*: ISOLATION AND CHARACTERIZATION OF TEN GRAYANOIDS[1]

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ABSTRACT.—A systematic fractionation of the antifeeding ethanolic residue of *Kalmia latifolia* leaves gave ten grayanoid diterpenes. Four of these, lyoniol-A (**3**), grayanotoxin-XVIII (**4**), -II (**7**), and -III (**11**) are known compounds but not previously reported from this source. The other six were new natural products and were given the names kalmitoxin-I (**10**), -II (**5**), -III (**9**), -IV (**6**), -V (**2**), and -VI (**8**). Characterization was by spectral and chemical methods. Based on the concentration of these constituents, kalmitoxin-I is the major antifeedant, with kalmitoxin-IV and grayanotoxin-III also appearing significantly deterrent at the levels present.

As part of a program designed to uncover possible new control measures for the gypsy moth larvae (*Lymantria dispar* L.)—a serious defoliator established in the northeastern USA—a search for feeding deterrents from plant sources was undertaken. Laboratory studies of the larval feeding characteristics led to the development of a screening test which allowed classification of plant extracts into four categories (1). Briefly, the test consisted of measuring the extent of feeding by third-instar insects on a diet composed of the ethanolic plant extract residue admixed with cellulose, agar, and water. The extracts that gave diets acceptable to the insects corresponded to host plant material. For example, red oak (*Quercus rubra* L.) leaves, which are readily fed on in the field, gave acceptable extracts. Residues yielding unaccepted diets could be divided into three distinct groups on the basis of the feeding response observed when the extract was tested in combination with the red oak diet. Those that did not inhibit feeding were classified as neutral and parenthetically indicated the requirement of feeding stimulants for the insect. Extracts that reduced feeding were classified as inhibitory or deterrent, while the third group which produced an increase in diet consumption above the red oak response were called synergistic.

The leaves of the mountain laurel, *Kalmia latifolia* L. (Ericaceae), from field studies are reported not to be a favorite food of the gypsy moth larvae (2, 3). Extracts from collections made in Connecticut and Ohio were reproducibly deterrent in the laboratory test and a study was undertaken to isolate the active constituents. This report is on the isolation of ten grayanotoxin-type diterpenes from one fraction of the ethanolic extract known as the ethyl acetate solubles. Six of these constituents are new natural products; the others are known compounds but not previously isolated from this source. In fact, there is no literature report to our knowledge on the isolation of any single well-characterized grayanoid from *Kalmia latifolia*. In 1957, grayanotoxin I (**1**) (andromedotoxin or

[1]Taken in part from the Ph.D. dissertation of S.F.E., which was accepted by Ohio State University, June 1979.

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acetylandromedol) was reported to be present from paper electrophoretic studies in sodium tetraborate solutions on the basis of migration similarities with a known sample (4). Very recently, a similar identification was made by thin layer chromatography (5).

RESULTS AND DISCUSSION

The residue (figure 1, Fraction A) from the percolation by ethanol of *K. latifolia* leaves significantly reduced the feeding of gypsy moth larvae as shown in table 1; and is dose dependent. Partitioning the residue between chloroform and water (figure 1) resulted in the distribution of activity into both phases (Fractions B and C) with the water solubles (Fraction B) considered more potent. Further partitioning of the water solubles between ethyl acetate and water, followed by extraction of the aqueous layer with *n*-butanol yielded three fractions of which the ethyl acetate solubles (Fraction F) and the *n*-butanol solubles (Fraction E) were deterrent. Partitioning of the chloroform solubles (Fraction C) between hexane and 10% aqueous methanol divided the activity about equally between the two phases (Fraction G and H). Since the ethyl acetate solubles (Fraction F) represented the largest fraction and was highly potent, it was examined first. Column chromatography on silicic acid provided dihydrochalcones, already reported from this source, and other phenolics (6); but none showed significant antifeeding activity. The activity appeared to reside with a minor fraction that lacked phenolic characteristics; and, as will be shown, consisted of polar diterpenes. An efficient separation method was therefore required to yield the minor constituents.

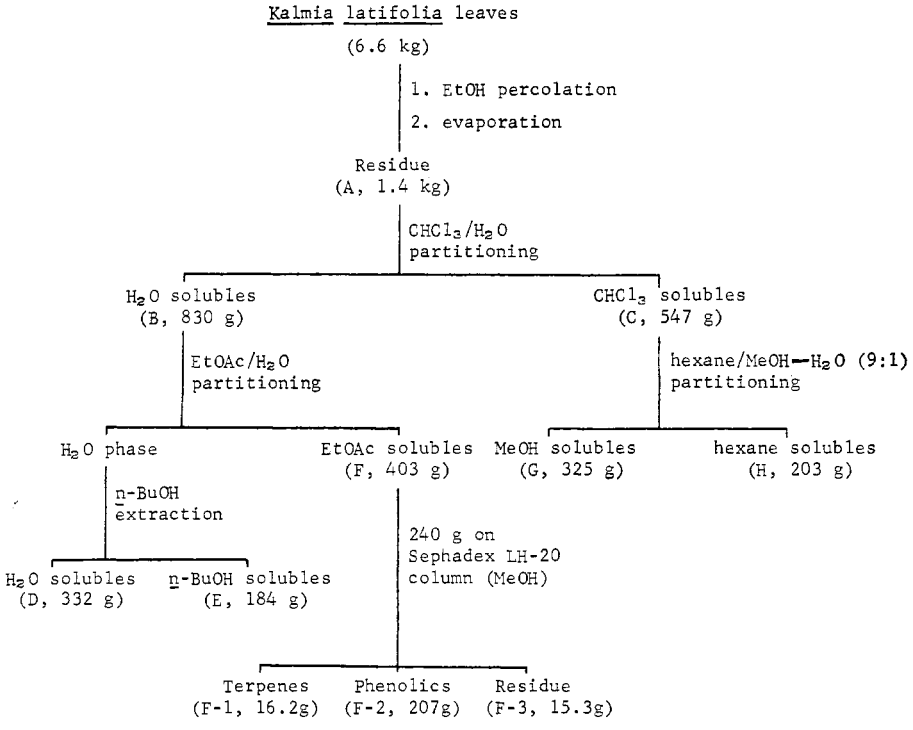


FIG. 1. Separation scheme for dividing the extract residue of *K. laitfolia* leaves into fractions of similar polarity.

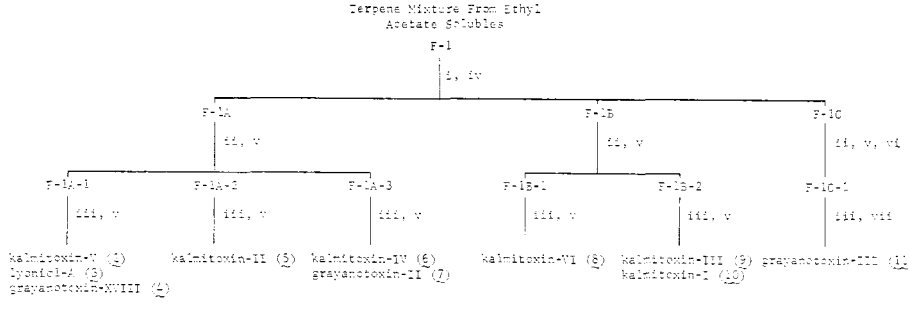
TABLE 1. Activity of fractions from *K. latifolia* leaves in the gypsy moth larval feeding test.[a]

Fraction	Concentration[b] mg/ml	Respone in %[c]		Compound	Concentration μg/ml	Response in %	
		Mean	Standard Error			Mean	Standard Error
A	6.25	38	2	K-V (**2**)	2.5	92	3
A	25.0	18	2	L-A (**3**)	7.5	95	3
B	17.5	8	1	G-XVIII (**4**)	20.0	121	4
C	7.5	59	7	K-II (**5**)	17.5	86	3
D	5.0	100	7	K-IV (**6**)	30.0	47	1
E	5.0	21	3	G-II (**7**)	15.0	65	3
F	10.0	11	3	K-VI (**8**)	45.0	106	4
G	2.5	48	5	K-III (**9**)	27.5	99	2
H	3.75	53	6	K-I (**10**)	60.0	23	0.4
F-1	2.25	17	0.3	G-III (**11**)	32.5	49	1
F-2	11.0	50	3				
F-3	0.6	101	2				
F-4	4.25	160	5				

[a]Details of the bioassay are given in reference 1.
[b]Test concentrations were chosen roughly on the basis of weight distribution into the various fractions.
[c]The feeding response was compared to the red oak control taken as 100% with the standard error ranging between 1% and 3% for different runs.

The use of a Sephadex LH-20 column conveniently divided the phenolic constituents of the ethyl acetate solubles (Fraction F) from the polar terpenic fraction. These were designated Fractions F-2 and F-1, respectively. The terpenes (~6%) appeared to be little delayed by passage through the column, whereas the phenolics were effectively adsorbed. A third fraction, F-3, still more strongly held, consisted of a tarry residue. The column wash, Fraction F-4, was in subsequent separations combined with Fraction F-3. Bioassay showed the activity to be concentrated in the F-1 terpenic fraction. The phenolics were significantly reduced in potency, while the remaining two fractions, F-3 and F-4 were not inhibitory, with F-4 possibly even stimulatory.

Chromatography of Fraction F-1 on silicic acid provided three subfractions, F-1A, F-1B, and F-1C, based on dry weight determination and thin layer chromatographic analysis of effluent fractions (see figure 2). Further resolution of

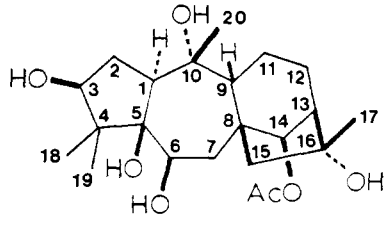


Conditions: i, silicic acid column: ii, silica gel column: iii, silica gel-silver nitrate column; iv, solvent B to C: v, solvent B; vi, solvent C: vii, solvent D.

FIG. 2. Summary of the isolation procedure for the grayanoid diterpenes from the ethyl acetate soluble fraction (F-1) of *Kalmia latifolia* leaves.

these fractions required chromatography in sequence on two columns; the first was prepared with silica gel, and the second, with silica gel containing 5% silver nitrate. Without silver nitrate, separation of the complex mixtures would not have been possible. Although the fractionation was still monitored by the feeding test, it was decided that development of a systematic separation scheme for the terpenes would not only yield the active ones, but others of more general interest, considering little work on these constituents has been reported from this source. The ten diterpenes obtained as crystalline products are listed in order of appearance from Fraction F–1: kalmitoxin-V (**2**), lyoniol-A (**3**), grayanotoxin-XVIII (**4**), kalmitoxin-II (**5**), -IV (**6**), grayanotoxin-II (**7**), kalmitoxin-VI (**8**), -III (**9**), -I (**10**), and grayanotoxin-III (**11**). The general name kalmitoxin for the grayanoids from *Kalmia latifolia* was chosen in accord with the practice established by investigators of related compounds from other members of the Heath family.

The four known compounds were identified by comparison of physical properties with those reported in the literature. Lyoniol-A (lyoniatoxin) (**3**) was originally obtained from *Lyonia ovalifolia* Drude var. *elliptica* Handel-Mazzetti and its properties (7, 8, 9) are in agreement with those of the *Kalmia* product. Grayanotoxin-XVIII was recently reported from *Leucothoe grayana* (10) and our compound gave comparable physical constants. The ^{13}C-nmr peaks were identical, and those reported from the ^{1}H-nmr and ir spectra were also in agreement. Preparation of the diacetate derivative with physical properties as recorded (10) con-



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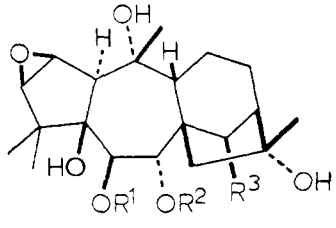
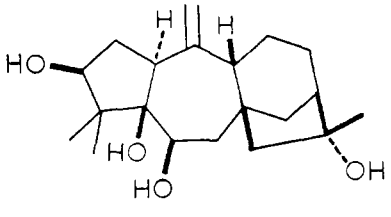


2; $R^1=R^2=Ac$, $R^3=OH$

3; $R^1=Ac$, $R^2=R^3=H$

6; $R^1=Ac$, $R^2=H$, $R^3=OH$

16; $R^1=R^2=H$, $R^3=OH$



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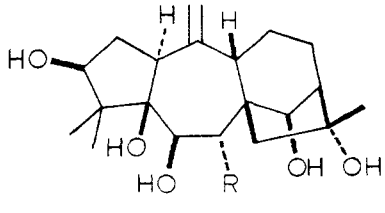


5; R = OH

7; R = H

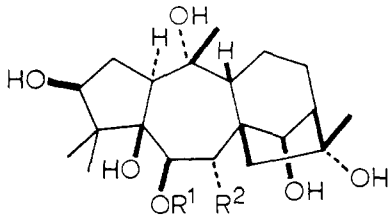
firmed the assignment. Grayanotoxin-II (**7**) was identified on the basis of comparison of the ir spectrum and other physical data with those in the literature (11, 12), as was grayanotoxin-III (**11**) (13). Furthermore, direct comparison of the last two compounds with authentic samples confirmed their presence.

The first new natural product was kalmitoxin-I (**10**). The mass spectrum and elemental analyses supported the molecular formula $C_{20}H_{34}O_7$; and the ^{13}C-nmr spectrum showed absorption for twenty carbons divided rather cleanly into three sets. The set between 75 and 85 ppm—a region for tetrahedral carbons bearing an oxygen—contained seven peaks, which under off-resonance decoupling conditions appeared as three singlets and four doublets. Since kalmitoxin-I (**10**) contains one additional oxygen than grayanotoxin-III (**11**) and lacks carbonyl absorption in the ir spectrum, a close relationship between the two was suspected. Comparison of their nmr spectra was informative. For example, the ^{13}C-nmr spectrum of grayanotoxin-III contained six peaks in the oxygenated carbon region; and the expected off-resonance pattern would be three singlets and three doublets. Kalmitoxin-I with an additional doublet in this region was viewed as a grayanotoxin-III with the seventh hydroxyl a secondary alcohol. Of the five possible positions to locate this hydroxyl, C-7 was chosen from analysis of the ^1H-nmr spectrum. A clean AB quartet with δ_A 4.05 and δ_B 4.25 (J 9.5 Hz) is present for kalmitoxin-I, and was assigned to H–6 and H–7, whereas grayanotoxin-III contains a one-proton double doublet at δ 4.55 (J 4 and 11 Hz) for H–6. Other relevant peaks (H–3, H–14, and the four methyls) did not differ greatly in the two spectra.

Acetylation of kalmitoxin-I produced two products; the major one, a tetraacetate, was formulated as **12** on the basis that the protons assigned to carbons bearing secondary alcohols in the starting material all showed downfield shifts greater than 1 ppm in the ^1H-nmr spectrum (14). The minor product, a pentaacetate, gave a ^1H-nmr spectrum similar to **12** but had one of the methyls associated with a tertiary alcohol shifted 0.28 ppm downfield. This supported placing the acetate at C–10 or C–16, preferably the latter, since anchimeric assistance from the C–14 functionality (acetate or hydroxyl) would be possible in its formation. The pentaacetate is therefore formulated as **13**.

Treatment of kalmitoxin-I with acidic acetone gave as homogeneous products two isomeric compounds, dianhydrokalmitoxin-I acetonide-A (**14**) and -B (**15**). Their structures were assigned from analysis of spectral data. Elimination of water from the two methyl-bearing tertiary alcohol centers is supported in the ^1H-nmr spectra by the presence of olefinic methylene peaks and an olefinic methyl. Location of the acetonide was indicated from the ^1H-nmr spectrum of acetylated acetonide-B (**15**). First, the formation of a diacetate rather than a triacetate eliminated the C–5, C–6 acetonide, leaving C–6, C–7 or C–7, C–14 as the other possibilities. Second, the AB quartet for the H–6 and H–7 protons was not affected significantly by acetylation (an upfield shift of 0.2 ppm occurred) but the H–3 and H–14 protons moved downfield by at least 1.1 ppm, in accord with the acylation shift expected for secondary alcohols (14). This made the C–7, C–14 acetonide untenable, and required that acetonide-B have structure **15**. It follows that acetonide-A should have structure **14**.

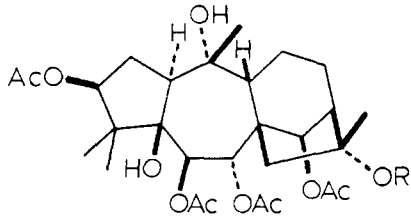
The accumulated data supported structure **10** for kalmitoxin-I, a new natural product but a known heptaol obtained on alkaline hydrolysis of the grayanoid esters asebotoxin-IV, -V, -VI, and -VIII (15, 16). Comparison of the ir and ^1H-nmr spectra of deacetylasebotoxin-IV with those of kalmitoxin-I showed them to be identical, and confirmed our spectral analysis.



9; R^1=Ac, R^2=OH

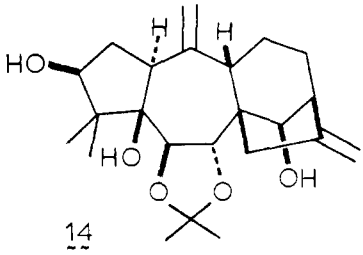
10; R^1=H, R^2=OH

11; R^1=R^2=H



12; R=H

13; R=Ac



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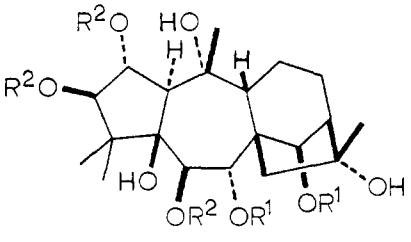
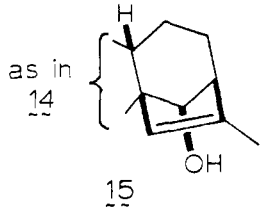


8; R^1=Ac, R^2=H

17; R^1=R^2=H



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Kalmitoxin-II (**5**) was assigned a structure from analysis of spectral data, aided by comparison with those of grayanotoxin-II (**7**) and kalmitoxin-I (**10**). The highest mass peak at *m/e* 368 in the mass spectrum corresponds to formula $C_{20}H_{32}O_6$; and the ir spectrum contains peaks for hydroxyl and olefinic groups. The ^{13}C-nmr spectrum contains absorption for twenty carbons, six of which appear in the region characteristic of oxygen-bearing tetrahedral carbons (72–84 ppm). In addition, an exocyclic double bond was indicated by the peaks at 112.3 and 153.2 ppm; for grayanotoxin-II (**7**) the values are 112.0 and 152.8 ppm. Assuming kalmitoxin-II (**5**) possesses the same A-*nor*-B-*homo*-*ent*-kaurane skeleton as the other diterpenes already considered, its structure is most likely a grayanotoxin-II with an additional hydroxyl or specifically 10,20-anhydrokalmitoxin-I. The ^{1}H-nmr spectrum of kalmitoxin-II (**5**) with the expected major peaks for the proposed structure also contained a relevant two proton singlet at 4.48 ppm that changed to an AB quartet δ_A 4.39 and δ_B 4.46 (J 8 Hz) on D_2O exchange. This could be accommodated only by a *trans* C–6, C–7 diol, and locates the sixth hydroxyl at C–7 on a grayanotoxin-II skeleton. Kalmitoxin-II has therefore struc-

ture **5**. The spectral properties of the tetraacetate derivative, in which all of the secondary hydroxyls are acetylated, are in accord with the proposed structure.

Kalmitoxin-III (**9**) exhibited spectral properties that suggested it was an acetate derivative of kalmitoxin-I. The ^{1}H-nmr spectrum contains a three-proton singlet at 2.20 ppm; and the ir spectrum shows carbonyl absorption. The chemical ionization mass spectrum shows a very weak quasimolecular ion peak that corresponds to the molecular formula $C_{22}H_{36}O_8$, but the electron impact mass spectrum shows only the M–18 peak. Alkaline hydrolysis of kalmitoxin-III (**9**) afforded kalmitoxin-I (**10**) in excellent yield, thereby establishing the nature of the alcoholic unit. Since kalmitoxin-III gave a negative periodate test (17)—kalmitoxin-I was positive—the acetate function must be at C–6, and kalmitoxin-III has structure **9**.

Kalmitoxin-IV (**6**), like kalmitoxin-III (**9**), is a monoacetate exhibiting a three-proton singlet at 2.14 ppm in the ^{1}H-nmr spectrum and a strong carbonyl peak in the ir spectrum. It does not give a molecular ion peak in the electron impact mass spectrum; but does show a quasimolecular ion peak in the chemical ionization mass spectrum. The difference of two mass units between the molecular weight of kalmitoxin-IV (**6**) and kalmitoxin-III (**9**) is neither due to the presence of an olefinic nor a carbonyl function, since the ir spectrum lacks characteristic double bond absorption and that of the hydrolysis product, deacetylkalmitoxin-IV (**16**), is devoid of a carbonyl peak. An oxirane ring was suggested by the pair of one-proton doublets at 3.25 and 3.82 ppm (J 3 Hz); and double irradiation studies confirmed their spin relationship. These peaks occur in the ^{1}H-nmr spectrum of lyoniol-A (**3**) for H–2 and H–3 (8, 9) and in other epoxide-containing grayanoids such as rhodojaponin-I (17), and pieristoxin-G (18). Lithium aluminum hydride reduction of kalmitoxin-IV gave kalmitoxin-I (**10**). This established for deacetylkalmitoxin-IV (**16**) the carbon skeleton and oxygenation positions except for the second epoxide carbon. The epoxide must be between C–2 and C–3 and with β-stereochemistry (2*S*, 3*R*) to fit the spectral data and the nature of the reduction product. Location of the acetate in kalmitoxin-IV (**6**) at C–6 was made possible by the negative sodium metaperiodate test (19).

Kalmitoxin-V (**2**), the least abundant of the isolated terpenes, exhibits spectral properties similar to kalmitoxin-IV (**6**). There are four three-proton singlets in the ^{1}H-nmr spectrum for methyls on quaternary carbons, and a pair of doublets (J 3 Hz) for the epoxide protons H–2 and H–3. In addition, the two acetate methyls suggested that kalmitoxin-V was likely an acetylkalmitoxin-IV. This was confirmed when hydrolysis of kalmitoxin-V (**2**) yielded deacetylkalmitoxin-IV (**16**). The presence of an AB quartet at the low field positions of 5.25 and 5.44 ppm for the C–6 and C–7 protons in the ^{1}H-nmr spectrum of kalmitoxin-V (**2**) requires that the acetates be located at these positions. Therefore, kalmitoxin-V must have structure **2**.

Kalmitoxin-VI (**8**) has the formula $C_{24}H_{38}O_{10}$, supported by elemental analyses. The mass spectrum does not show a molecular ion peak but one for M–H_2O at *m/e* 468. The chemical ionization mass spectrum likewise lacked the peak for the quasimolecular ion. The ^{1}H-nmr spectrum contained two acetate peaks and four methyl singlets as would be expected for a fully saturated grayanoid. That ten oxygen-bearing carbons were present was seen from the ^{13}C-nmr spectrum. The two acetate carbonyls appeared at 170.4 and 171.3 ppm, and eight peaks were located between 75 and 87 ppm, the region for oxygen-bearing tetrahedral carbons. The ^{1}H-nmr spectrum contained an AMX pattern that was also observed for *bis*-deacetylkalmitoxin-VI (**17**) and was unraveled by double irradiation experiments.

The doublet at 3.27 ppm (*J* 5.7) of kalmitoxin-VI (**8**) was coupled to a double doublet at 5.18 ppm (*J* 5.7, 3) which in turn was coupled to a doublet at 4.11 ppm (*J* 3). This relationship has been described for the *trans*-2,3-diols, rhodojaponin-VI (20) and -VII (21), and suggested the same diol structure for kalmitoxin-VI. Ring opening of the epoxide of kalmitoxin-IV (**6**) produced a compound that showed the same tlc mobility in four solvent systems as *bis*-deacetylkalmitoxin-VI (**17**), but insufficient quantities of material prevented a complete characterization.

Location of the acetate functions in kalmitoxin-VI (**8**) was aided by comparison of ^{1}H-nmr chemical shifts with those of the hydrolysis product **17**. Two significant upfield shifts had occurred on deacetylation; the singlet at 6.33 ppm assigned to H–14 had moved to 5.11 ppm and the doublet (one-half of an AB quartet pattern at 5.73 ppm for H–6 or H–7 shifted to 4.41 ppm. These changes of over 1 ppm, as already stated, are expected for α-protons of secondary acetates on conversion to secondary alcohols (14). One acetate must be located at C–14; and the results from periodate cleavage of kalmitoxin-VI (**8**) require that the second acetate be placed at C–7. Kalmitoxin-VI consumed 1.7 times as much reagent as grayanotoxin-III (**11**), a standard possessing only one susceptible diol. Apparently the 2,3-diol is cleaved much slower than the 5,6-diol of kalmitoxin-VI and accounts for the lower value obtained in the fixed time experiment.

The antifeeding activity of the diterpenes as listed in table 1 was determined at a dose that reflects the relative concentration. Therefore, kalmitoxin-I (**10**) must be the constituent most responsible for the deterrency, while kalmitoxin-IV (**6**), grayanotoxin-II (**7**) and -III (**11**) are also significantly active. It is of special note that the phenolic constituents (e.g. phoridzin) are not deterrent to the gypsy moth larvae, yet are effective against host-unassociated aphids (23). For host-associated aphids, the phenolics act as probing stimulants (24).

The toxic nature of members of the Ericaceae to livestock and man is well known (25), and the responsible constituents in those species that have been studied were identified as grayanoids. However, *Kalmia latifolia* has not been systematically investigated prior to our work. Although insects were our test animals, we believe they indicate in a qualitative matter at least a toxic response that would be observed with laboratory mammals. A full pharmacological evaluation of these compounds in laboratory animals is planned, as well as the isolation of additional compounds from the other partition fractions.

EXPERIMENTAL[3]

PLANT MATERIAL.—The leaves of *Kalmia latifolia* L. for large scale isolation were collected in September 1976 in Fairfield County, Ohio, and were dried in a forced draft oven at 40°, then powdered in a Wiley mill.

INSECT FEEDING BIOASSAY.—The details of the bioassay are published (1). The test consists of ten third-instar larvae (*Lymantria dispar* L.) placed in a petri dish containing diet pellets prepared from the plant extract (or fractions thereof), red oak leaf extract, cellulose, agar,

[3]Melting points are uncorrected. Nmr spectra were determined in stated solvents with tetramethylsilane as internal standard on a Bruker HX–90E instrument (proton at 90 MHz and carbon at 22.63 MHz) equipped for pulse mode Fourier transform analysis. Chemical shifts (δ) are reported in ppm and coupling constants (*J*) in Hz. Ir spectra were taken in KBr pellets or in chloroform on a Beckman IR 4230 instrument. Uv spectra were taken in methanol on a Cary 15 or Beckman 5260 instrument. Mass spectra were obtained on AEI MS–9 and DuPont 21–491 instruments by direct inlet probe at 70 eV. *i*-Butane was used for chemical ionization. Optical rotations were determined on a Perkin-Elmer 241 photoelectric polarimeter. Silica gel G for thin layer chromatography and silica gel for column separations were from E. Merck (Darmstadt). Silicic acid was from Mallinckrodt Chemical Company (St. Louis), and Sephadex LH–20 was obtained from Pharmacia (Piscataway, N.J.).

and water. After a 24- or 48-hour feeding period, the amount of frass produced is dried to constant weight and weighed. The extent of feeding is reported as a percentage based on the frass produced from the control diet containing red oak leaf extract. Ten replicates are run and the standard error is recorded. By reporting the results in this manner, the lower the value, the greater the inhibition of feeding.

EXTRACTION AND PARTITIONING OF EXTRACT RESIDUE.—A 6.6 kg sample of powdered leaves was extracted by percolation with ethanol at room temperature. The percolate (about 150 liters) was evaporated at reduced pressure and 40° to give 1.4 kg (Fraction A) of a dark viscous residue. The residue (1.3 kg) was partitioned (see figure 1) between 3 liters each of chloroform and water. After separation of the layers, the aqueous phase was reextracted twice with chloroform. Evaporation of the combined chloroformic extract left 547 g of residue (Fraction C) which was partitioned between 2 liters each of hexane (3 times) and methanol-water (9:1). Evaporation of the solvents left 325 g of methanol solubles (Fraction G) and 203 g of hexane solubles (Fraction H). The water solubles (830 g, Fraction B) from the chloroform/water partitioning were repartitioned between 3 liters each of ethyl acetate (3 times) and water. The aqueous phase was then extracted with 3 liters of *n*-butanol (3 times). Evaporation of solvents gave the ethyl acetate solubles (403 g, Fraction F), the *n*-butanol solubles (184 g, Fraction E) and the water solubles (332 g, Fraction D).

CHROMATOGRAPHIC SOLVENT SYSTEMS AND SPRAY REAGENTS.—A spray reagent prepared from 5 ml of *p*-anisaldehyde, 90 ml of ethanol and 5 ml of sulfuric acid, when applied to tlc plates followed by heating at 110° for 5 min, gives red to dark brown colors for the terpene constituents. The solvent systems (lower phase only) employed were prepared from chloroform-methanol-water in the following ratios: A. 60:15:4; B. 37:3:2; C. 17:3:1; and D. 35:5:2. Solvent systems E and F were prepared from benzene-ethyl acetate (1:1) and (3:1), respectively.

SEPARATION OF THE TERPENIC COMPONENTS FROM THE ETHYL ACETATE SOLUBLES (FRACTION F).—A column of Sephadex LH-20 (100 g, 2.8 x 55 cm) was poured as a slurry in methanol. Ethyl acetate solubles (Fraction F) in 33 g quantities were dissolved in 50 ml of methanol, passed into the column and eluted with methanol. Effluent fractions of 2.5 ml were collected and every fifth fraction was analyzed by tlc using solvent system A. Fractions 1–80 (Fraction F-1) contained the terpenes, fractions 81–175 (Fraction F-2) the phenolics, and fractions 176–200 (Fraction F-3) a tarry residue. A fourth fraction (Fraction F-4) was obtained as a final column wash with 2 liters of methanol. From a 240 g sample of ethyl acetate solubles the following quantities were collected: 16.2 g of F-1, 207 g of F-2, and 15.3 g of combined F-3 and F-4. Bioassay results are found in table 1.

Passage of the terpenic fraction F-1 (21 g) through a short column (6.8 x 12.5 cm) of silicic acid (100 gm) containing 6.5% water and poured as a slurry in chloroform removed tarry materials which improved subsequent separations. The sample was dissolved in 8 ml of methanol, mixed with 100 g of silicic acid containing 6.5% water, and dried in a rotary evaporator at reduced pressure. The total weight of the adsorbent plus sample was adjusted to 121 g with water, mixed thoroughly and applied to the top of the column. Elution with 5 liters of chloroform removed 1 gm of a nonterpenic oil, then 5% methanol in chloroform gave 10.7 g of mixed terpenes. A final wash with methanol yielded 8 g of a black tarry residue that was not further investigated.

CHROMATOGRAPHY OF TERPENIC FRACTION F-1.—A column (6.5 x 89 cm) was prepared from 1.45 kg of silicic acid activated at 120° for 24 hours and poured as a slurry in solvent system B. The clarified terpenic fraction F-1 (10.6 g) was dissolved in 15 ml of methanol, mixed with 100 g of silicic acid, and dried under reduced pressure for 3 hours in a rotary evaporator. The adsorbed mixture was placed on top of the column and elution was started with solvent system B. Effluent fractions of 90 ml were collected after passage of the holdup volume. The polarity of the solvent system was changed in five steps (~3 liters each) by increasing the ratio of methanol to chloroform but keeping the combined volume constant and the water proportion unchanged until the composition of solvent system C was reached. Effluent fractions were evaporated to dryness. The residues were weighed and analyzed by thin layer chromatography using solvent A. Three major pooled fractions were formed; F-1A (3.16 g) with two zones R_f 0.45 and 0.50, F-1B (3.59 g) also with two zones R_f 0.30 and 0.35, and F-1C (5.8 g) with four main spots, R_f 0.08, 0.10, 0.15 and 0.27.

CHROMATOGRAPHY OF FRACTION F-1A.—A sample (3.16 g) of Fraction F-1A was dissolved in 2.5 ml of methanol, diluted with 27.5 ml of chloroform and applied to a column (4.7 x 75 cm) of silica gel (500 g) poured as a slurry in solvent system B. The column was eluted with solvent B. Effluent fractions of 85 ml were collected, evaporated to dryness, and the residue weights determined. From the elution diagram and thin layer chromatographic analysis on silica gel G containing 5% silver nitrate and solvent system B, three fractions were formed. Fraction F-1A-1 (272 mg) showed three spots R_f 0.38, 0.57 and 0.62 (major). (Without silver nitrate and solvent A, only one spot, R_f 0.50 was observed.) Fraction F-1A-2 (233 mg) exhibited four zones R_f 0.30 (major), 0.48, 0.50 and 0.52. (Without silver nitrate and solvent A,

one spot, R_f 0.45, was obtained.) Fraction F-1A-3 (1.13 g) contained two spots, R_f 0.3 and 0.5, also running as a single zone (R_f 0.45) without silver nitrate. These fractions were resolved by chromatography on a silver nitrate-impregnated silicic acid column as follows.

Silica gel 60 (120 g) was mixed with a solution of 6 g of silver nitrate in 50 ml of water. The solvent was removed by evaporation in a rotary evaporator followed by heating at 120° for one-half hour to reduce the weight to 126 g. A column (2.6 x 50 cm) was poured from this material as a slurry in solvent B. Separations were performed with the same solvent, and effluent fractions of 17 ml were analyzed by thin layer chromatography as given above. Fractions were pooled according to these results. Any contaminating silver nitrate was removed by dissolving the residue in methanol (20 ml), adding 2 ml of saturated aqueous sodium chloride to form silver chloride as a flocculant precipitate, and centrifuging the mixture following the addition of 10 ml of chloroform. Removal of the supernatant, washing of the precipitate with methanol-chloroform (2:1), and evaporating the combined extracts left the terpenes as clean foamy solids ready for crystallization. The same column could be used repeatedly after complete elution of the sample.

Fraction F-1A-1 (272 mg) was dissolved in 0.75 ml of methanol, diluted with 9.25 ml of chloroform and passed through the silica gel-silver nitrate column. Effluent fractions containing R_f 0.62 material yielded kalmitoxin-V (**2**); R_f 0.57 fractions gave lyoniol-A (**3**); and R_f 0.38 fractions afforded grayanotoxin-XVIII (**4**).

Fraction F-1A-2 (233 mg) was dissolved in 0.1 ml of methanol diluted with 14.9 ml of chloroform and passed into the silica gel-silver nitrate column. Fractions with R_f 0.30 material gave kalmitoxin-II (**5**).

Fraction F-1A-3 (1.13 g) was dissolved in 1.5 ml of methanol, diluted with 18.5 ml of chloroform and separated on the silica gel-silver nitrate column. Fractions with R_f 0.5 material gave kalmitoxin-IV (**6**), while the R_f 0.28 zone yielded grayanotoxin-II (**7**).

CHROMATOGRAPHY OF FRACTION F-1B.—A sample (3.59 g) of Fraction F-1B was separated on a column (4.7 x 73 cm) of 500 g of silica gel with solvent system B. Addition to the column was as a solution in 2.3 ml of methanol diluted with 27.5 ml of chloroform. The effluent fractions (85 ml) were evaporated to dryness, and the residues were examined by thin layer chromatography with solvent system A. Fractions F-1B-1 (689 mg) contained mainly material with R_f 0.35, while Fractions F-1B-2 (710 mg) showed a major spot at R_f 0.30.

Further separation of Fraction F-1B-1 on a silica gel-silver nitrate column with solvent system B, as described above for the F-1A fractions, resulted in 358 mg of residue, R_f 0.35, from which was obtained kalmitoxin-VI (**8**).

Separation of Fraction F-1B-2 in a similar manner gave one fraction with R_f 0.35 from which kalmitoxin-III (**9**) was obtained, and another with R_f 0.28 which gave kalmitoxin-I (**10**).

CHROMATOGRAPHY OF FRACTION F-1C.—A sample (5.8 g) of Fraction F-1C was chromatographed on a column (4.7 x 75 cm) of silica gel with solvent system B. The material was placed on the column as a solution in 4.5 ml of methanol and 55.5 ml of chloroform. Effluent fractions of 85 ml were collected and analyzed by thin layer chromatography. After approximately 700 ml was collected, solvent system C was used. Fraction F-1C-1 gave a zone at R_f 0.27 (1.7 g) on silver nitrate-impregnated thin layer plates and solvent system A. Other column fractions were not further studied.

Chromatography of F-1C-1 on a silica gel-silver nitrate column as described for Fraction F-1A with solvent system D afforded two zones. The first emerging zone gave grayanotoxin-III (**11**), the second, a glycoside whose structure is not yet established.

LYONIOL-A (LYONIATOXIN) (**3**).—The R_f 0.57 material from separation of column Fraction F-1A-1 was crystallized from acetone to give 38 mg (10^{-3}% of dried leaves) of lyoniol-A (**3**) as tiny colorless prisms: mp 253–4°; $[\alpha]^{24}D$ −34° (*c* 0.28, MeOH); ir (KBr) ν max 3490 (hydroxyl), 1720 (acetate), 1375, 1255 and 1095 cm^{-1}; ^1H-nmr (pyr-d_5) δ 1.27 and 1.51 (2s, 2 tertiary Me), 1.60 and 1.91 (2s, 2 tertiary Me on hydroxyl-bearing carbon), 2.18 (s, acetate Me), 2.92 (s, H-1), 3.27 (d, H-3, *J* 3), 3.91 (br t, H-7, *J* ~10, changing to doublet *J* 9 with D_2O), 4.22 (d, H-2, *J* 3), 5.71 (d, H-6, *J* 9), with three one-proton singlets at 4.07, 5.12 and 5.92 ppm for tertiary hydroxyl, and a one-proton multiplet for secondary hydroxyl at 5.45 ppm all exchanged with D_2O; and mass spectrum *m/e* 410 (0.1%, M^-, $C_{22}H_{34}O_7$), 393 (0.8), 374 (7), 356 (3), 332 (15), 314 (14), 289 (7), 271 (9), 258 (9), 248 (100), 230 (18), 205 (23), 193 (18), 169 (22), 163 (39), 148 (54), 135 (38) and 116 (38). [Lit. (7, 8, 9) values: mp 250–253°; $[\alpha]^{15}D$ −41° (MeOH); and ^1H-nmr spectrum with peaks as given above.] A negative result was obtained in a periodate test for α-glycols.

GRAYANOTOXIN-XVIII (**4**).—The R_f 0.38 material from Fraction F-1A-1 separated on the silver nitrate-containing column was crystallized from isopropyl ether to give glistening needles (147 mg, 3 x 10^{-3}% of dried leaves) of grayanotoxin-XVIII (**4**): mp 162–3°; $[\alpha]^{22}D$ −22° (*c* 0.83, MeOH); ir (KBr) ν max 3350, 1624, 1370, 1025 and 892 cm^{-1}; ^1H-nmr ($CDCl_3$) δ 1.02 and 1.25 (2s, 2 Me), 1.38 (s, Me on carbon with hydroxyl), 3.00 (dd, H-1, *J* 8.3, 10.5), 3.60 (d, H-3 or H-6, *J* 6.4), 3.77 (dd, H-6 or H-3, *J* 3.0, 10.6), 4.96 and 5.12 (2 br s, H-20); (pyr-d_5, 90 MHz)

δ 1.14, 1.57 and 1.59 (3s, 3 Me), 3.18 (dd, H-1, *J* 11, 11), 3.95 (dd, H-3 or H-6, *J* 2, 7), 4.20 (br d, H-6 or H-3, *J* 9), and 5.20 (br s, H-20); (pyr-d_5, D_2O exchanged) δ 1.14 (s, Me), 1.58 (s, 2 Me), 2.98 (dd, H-1, *J* 10, 10), 3.97 (dd, H-3 or H-6, J 3, 7), 4.14 (br d, H-6 or H-3, *J* 9), 5.14 and 5.20 (2 br s, H-20) ppm; ^{13}C-nmr (pyr-d_5) δ_c 19.3, 24.2 (two carbons), 25.5, 25.9, 36.5, 39.5, 44.5, 44.8, 46.8, 48.0, 50.6, 52.5, 62.6, 70.8, 79.6, 81.3, 83.6, 112.4 and 153.1 ppm; and mass spectrum *m/e* 336 (3%, M^-, $C_{20}H_{32}O_4$), 318 (47), 300 (56), 282 (30), 257 (35), 239 (29), 203 (27), 174 (31), 145 (42), 135 (46), 119 (51), 109 (64), 107 (64), 93 (82), 69 (100) and 55 (72).]Lit. (10) values: mp 162–4°, $[\alpha]^{25}D$ −6.81 (*c* 2.2, MeOH).] The ^{1}H and ^{13}C-nmr spectra contain the same peaks as reported (10) for grayanotoxin-XVIII.

Acetylation of grayanotoxin-XVIII (25 mg) with acetic anhydride in pyridine gave a product that crystallized from methanol as tiny needles (16 mg): mp 112–3° [Lit. (10) values: mp 120–122° (*n*-hexane-diethyl ether)]; $[\alpha]^{23}D$ −13° (*c* 0.26, MeOH), R_f 0.26 on tlc with solvent E and spectral (ir and ^{1}H-nmr) peaks as reported (10).

GRAYANOTOXIN-II (7).—Fraction F-1A-3 after separation on the silver nitrate-containing column gave a zone that yielded 200 mg (2 x 10^{-3}%) of grayanotoxin-II from acetone as large prisms: mp 199–200°; $[\alpha]^{21}D$ −45° (*c* 0.81, MeOH); ir (KBr) ν max 3380, 1613, 1405, 1230, 1055, 1025 and 890 cm^{-1}; ^{1}H-nmr (pyr-d_5-D_2O exchanged) δ 1.07, 1.51 and 1.55 (3s, 3 Me), 3.95 (br dd, H-3, *J* 2, 7), 4.31 (s, H-14, without D_2O a doublet, *J* 7), 4.48 (br dd, H-6, *J* 3, 7), 5.19 and 5.12 (2s, H-20, without D_2O br s at 5.20); ^{13}C-nmr (pyr-d_5) δ_c 18.6, 23.9, 24.6 (3 carbons), 39.4, 42.0, 45.7, 50.5 (2 carbons), 51.1, 54.2, 60.7, 69.5, 81.0, 81.22, 81.23, 83.8, 112.0, 152.8; and mass spectrum *m/e* 352 (6%, M^-, $C_{20}H_{32}O_5$), 334 (32), 316 (75), 298 (88), 280 (23), 270 (43), 255 (43), 241 (39), 227 (41), 215 (35), 201 (46), 159 (68), 135 (69), 107 (58), 91 (84), 69 (100), and 55 (78). [Lit. (11) values: mp 198°, $[\alpha]^{16}D$ −43°.] The ir spectrum is comparable to that illustrated in the literature (12), and the ir and ^{1}H-nmr spectra were identical to those of an authentic sample.

GRAYANOTOXIN-III (**11**).—Chromatography of Fraction F-1C-1 on a silica gel column containing silver nitrate gave effluent material (5 x 10^{-3}% yield) which crystallized from ethyl acetate as colorless prisms (180 mg): mp 218–22° (d) with final rapid decomposition at ~255°, $[\alpha]^{24}D$ −16° (*c* 0.45, MeOH), −13° (*c* 0.45, EtOH); ir (Nujol) ν max 3280, 1453, 1373, 1030, 870 and 710 cm^{-1}; ^{1}H-nmr (pyr-d_5, D_2O exchanged) δ 1.17, 1.51, 1.67 and 1.87 (4s, 4 Me), 3.20 (m, 1H), 3.87 (br s, H-3), 4.55 (br dd, H-6, *J* 4, 11) and 5.00 (s, H-14) ppm; ^{13}C-nmr (pyr-d_5) δ_c 19.7, 22.4, 23.3, 23.8, 27.0, 28.3, 35.7, 44.2, 51.6, 51.7, 52.4, 55.1, 56.3, 60.3, 74.0, 78.1, 79.3, 79.8, 82.6 and 84.6 ppm; and mass spectrum *m/e* 352 (3 ,M−H_2O, $C_{20}H_{34}O_6$ requires 370), 334 (38), 316 (67), 298 (53), 273 (44), 205 (50), 163 (55), 137 (70), 119 (93) 93 (100), 69 (79) and 55 (74). [Lit. (13) values: mp 218° (d), $[\alpha]^{15}D$ −12° (*c* 2.3, EtOH).] The tlc mobility, ir and ^{1}H-nmr spectra were identical with those of an authentic sample.

KALMITOXIN-I (**10**).—Chromatography of Fraction F-1B-2 on a silica gel-silver nitrate column gave R_f 0.28 material that crystallized from acetone as colorless needles (330 mg, 6 x 10^{-3}% yield) of kalmitoxin-I: mp 245–6°; $[\alpha]^{22}D$ −16° (*c* 0.25, MeOH): ir (KBr) ν max 3420, 1420, 1110 and 1038 cm^{-1}; ^{1}H-nmr (methanol-d_4) δ 1.07, 1.26, 1.34 and 1.35 (4s, 4 Me), 2.68 (dd, H-1, *J* 5, 10), 3.17 (d, H-15, *J* 15), 3.42 and 3.76 (ABq, H-6 and H-7, *J* 9.5), 3.58 (d, H-3, *J* 3) and 4.54 (s, H-14); (pyr-d_5, D_2O exchanged, 315°K) δ 1.17, 1.52, 1.69 and 1.80 (4s, 4 Me), 3.20 (dd, H-1, *J* 6.4, 10.1), 3.65 (d, H-15, *J* 15),[4] 3.85 (dd, H-3, *J* 2), 4.05 and 4.25 (ABq, H-6 and H-7, *J* 9.5) and 5.17 (s, H-14); ^{13}C-nmr (pyr-d_5) 21.2, 23.6, 24.6, 25.0, 27.7, 29.6, 37.1, 53.3, 53.4 (hidden), 53.5, 55.0, 57.1, 57.7, 78.9, 80.3, 80.7, 81.2, 82.9, 84.0 and 84.5: (D_2O with methanol as std) 19.0 (q), 21.6 (t), 22.6 (q, 2 carbons), 25.8 (t), 27.1 (q), 34.0 (t), 50.0 (d), 50.9 (t), 51.9 (s), 52.9 (d), 55.0 (s), 55.4 (d), 78.0 (d), 78.4 (s), 78.6 (d), 80.7 (s), 81.0 (d): 82.8 (d) and 83.5 (s); and mass spectrum *m/e* 386 (1%, M^+, $C_{20}H_{34}O_7$), 368 (2), 354 (1), 341 (2), 327 (0.7), 325 (0.7), 279 (5), 256 (22), 185 (19), 149 (26), 129 (41), 97 (43), 83 (48), 73 (88), 69 (100), 60 (57) and 55 (76).

Anal. Calcd for $C_{20}H_{34}O_7$: C, 62.15; H, 8.87. Found: C, 61.86; H, 8.81%.

Comparison of the ir and ^{1}H-nmr spectra of kalmitoxin-I with those of deacetylasebotoxin-IV (15) showed them to be the same.

ACETYLATION OF KALMITOXIN-I (**10**).—Kalmitoxin-I (25 mg) was heated 3 hours on the steam bath with 1 ml each of pyridine and acetic anhydride, then treated with 1 ml of methanol. After 30 min, the mixture was evaporated to dryness and the residue chromatographed on 6 g of silica gel with solvent system E. The major product (R_f 0.1 on tlc with solvent E), kalmi-

[4]This doublet with *J*~15 Hz is also present in the spectra of kalmitoxin-II, III and VI, as well as the parent alcohol of kalmitoxin-VI. Double resonance studies with kalmitoxin-I using the INDOR technique and monitoring each peak of the doublet indicated only two other peaks, at 1.81 and 1.99 ppm, as part of an AX spin system. Irradiation at these positions in a spin-tickling experiment confirmed the relationship, and irradiation at 1.90 with increased power collapsed the doublet at 3.65 ppm to a singlet. The two protons at C-15 appeared to be the most likely candidates for this pattern, with H-α (pro-*R*) placed at the downfield position, since the hydroxyls at C-7, C-14 and C-16 are properly located for deshielding.

toxin-I tetraacetate (**12**, 17 mg) was crystallized from ether-hexane: mp 135–6°; $[\alpha]^{21}D+61°$ (*c* 0.17, MeOH); ir ($CHCl_3$) ν max 3575, 1747, 1375, 1210–1255, 1032 and 908 cm^{-1}; ^1H-nmr ($CDCl_3$, D_2O exchanged) 0.98, 1.03, 1.31, 1.35 (4s, 4 Me), 2.02, 2.05 (double intensity) and 2.21 (3s, 4 Ac), 3.28 (dd, H-1, *J* 4, 11), 4.79 (d, H-3 *J* 8), 5.21 and 5.30 (ABq, H-6 and H-7, *J* 10) and 5.81 ppm (s, H-14); (pyr-d_5, D_2O exchanged) δ 1.19, 1.32, 1.43 and 1.82 (4s, 4 Me), 1.94, 2.10, 2.14 and 2.30 (4s, 4 Ac), 3.76 (dd), H-1, *J* 6, 10), 5.01 (br d, H-3, *J* 4), 5.71 and 5.87 (ABq, H-6 and H-7, *J* 9.5) and 6.45 (s, H-14); and mass spectrum *m/e* 554 (3%, M^+, $C_{28}H_{42}O_{11}$), 536 (3), 521 (7), 494 (3), 476 (14), 434 (25), 418 (43), 374 (93), 356 (52), 314 (86), 296 (46), 213 (68), 162 (75), 153 (82), 109 (100).

A minor amorphous product (8 mg, R_f 0.4 on tlc with solvent E) was also eluted from the column and was formulated as kalmitoxin-I pentaacetate (**13**) on the basis of the ^1H-nmr spectrum ($CDCl_3$) δ 0.99, 1.03, 1.34 and 1.54 (4s, 4 Me), 1.99, 2.02 and 2.05 (double intensity) and 2.14 (4s, 5 Ac), 3.33 (dd, H-1, *J* 4, 11), 4.79 (d, H-3, *J* 5), 5.26 (s, H-5 and H-6) and 5.69 (s, H-14).

ACETONIDES FROM KALMITOXIN-I (**10**).—A solution of kalmitoxin-I (50 mg) in 5 ml of dry acetone containing 5 μl of 60% perchloric acid was allowed to react for 30 min, then treated with 2 ml of saturated sodium bicarbonate. The mixture was filtered, and the filtrate was evaporated under vacuum. The residue was triturated with benzene-ethyl acetate (3:1) to give 60 mg of an acetonide mixture after evaporation of solvent. Chromatography of the product on a 24 g column of silica gel with solvent F with analysis of 1 ml effluent fraction gave in Fraction No. 31–35, 10 mg of a diacetonide mixture (^1H-nmr results) which was not further studied.

The residue from Fraction No. 69–79 contained dianhydrokalmitoxin-I acetonide-A (**14**) which crystallized from acetone-hexane as large needles (10 mg): mp 203–4°; ir ($CHCl_3$) ν max 3480, 1655, 1638, 1383, 1370, 1200–1235, 1077, 895 and 883 cm^{-1}; ^1H-nmr ($CDCl_3$, D_2O exchanged) δ 1.06, 1.20, 1.35 and 1.43 (4s, 4 Me), 3.25 (dt, unassigned, *J* 16, 2), 3.66 (dd, H-3, *J* 2, 7), 4.09 (s, H-6 and H-7), 4.31 (s, H-14), 5.02 (2H), 5.09 and 5.14 (3s, H-17 and H-20); and mass spectrum *m/e* 390 (4%, M^+, $C_{23}H_{34}O_5$), 375 (10), 332 (13, M–Me_2CO), 314 (52), 253 (23), 225 (25), 199 (34), 157 (36), 145 (41), 129 (51), 105 (61), 91 (100), 79 (64), 69 (53) and 59 (72).

The residue from Fraction No. 81–90 crystallized from acetone as needles (3 mg) of dianhydrokalmitoxin-I acetonide-B (**15**): mp 165°; ir ($CHCl_3$) ν max 3530, 1655, 1640, 1383, 1370, 1205–1235, 1167, 1075, 995, 890 and 847 cm^{-1}; ^1H-nmr ($CDCl_3$, D_2O exchanged) 1.08, 1.22, 1.36 and 1.42 (4s, 4 Me), 1.79 (d, Me-17, *J* 1.3), 3.65 (dd, H-3, *J* 2, 7), 4.01 and 4.24 (ABq, H-6 and H-7, *J* 9.3), 4.51 (s, H-14), 4.99 and 5.06 (s, H-20), and 5.43 (m, $\omega_{1/2}$ 4 Hz, H-15); and mass spectrum *m/e* 390 (50%, M^-), 375 (21), 372 (8), 332 (15), 314 (100), 297 (17), 285 (31), 267 (13), 91 (31) and 59 (42). A 2 mg sample of the acetonide **15** was acetylated (acetic anhydride and pyridine) to a diacetate with R_f 0.66 on tlc and solvent F, and ^1H-nmr spectrum δ 1.03, 1.13, 1.34 and 1.40 (4s, 4 Me), 1.77 (d, H-17, *J* 1.3), 2.07 (s, 2 Ac), 3.78 and 4.06 (ABq, H-6 and H-7, *J* 9.4), 4.9 (m obscured, H-3), 4.93 and 5.00 (s, H-20), 5.56 (br s, H-15) and 5.66 (s, H-14).

KALMITOXIN-II (**5**).—The column fraction from separation of Fraction F-1A-2 on silica gel-silver nitrate was crystallized from toluene-methanol to give 95 mg (2 x 10^{-3}% yield) of kalmitoxin-II: mp 187–8°; $[\alpha]^{22}D-52°$ (*c* 0.43, MeOH); ir (KBr) ν max 3240–3460, 1628, 1445, 1408, 1045, 895 and 883 cm^{-1}; ^1H-nmr (pyr-d_5) δ 1.21, 1.55 and 1.59 (3s, 3 Me), 3.27 (br s, 1H), 3.29 (d, 1H, *J* 14), 3.87 (br t, H-1, *J* 8.6), 3.99 (dd, H-3, *J* 2, 7), 4.48 (s, H-6 and H-7, after D_2O exchange an ABq, 4.39 and 4.46, *J* 8), 4.62 (br d, H-14, *J* 4, becomes singlet on D_2O exchange), 5.17 and 5.24 (2s, H-20); ^{13}C-nmr (pyr-d_5) 19.0, 24.3 (2 carbons), 24.7, 25.6, 39.4, 45.1, 49.2, 51.1, 54.1, 55.2 (2 carbons), 73.8, 78.2, 80.7, 81.2, 82.4, 83.2, 112.3 and 153.2; and mass spectrum *m/e* 368 (1%, M^-, $C_{20}H_{32}O_6$), 350 (9), 332 (74), 314 (70), 296 (33), 285 (51), 268 (37), 267 (37), 245 (33), 205 (37), 187 (43), 159 (55), 145 (62), 135 (58), 119 (60), 105 (70), 91 (88), 69 (100) and 58 (100). The chemical ionization (*i*-butane) mass spectrum showed a quasimolecular ion at *m/e* 369 (0.7%, MH^+) and base peak at 315 (100, MH–$3H_2O$).

KALMITOXIN-II TETRAACETATE.—A 27 mg sample of kalmitoxin-II (**5**) was mixed with 1 ml each of acetic anhydride and pyridine, and kept at ambient temperature for 25 hrs. The residue remaining after evaporation of the reaction mixture was chromatographed on 5 g of silica gel with solvent system E. Crystallization from diethyl ether gave 28 mg of kalmitoxin-II tetraacetate: mp 167–8°; $[\alpha]^{24}D+7°$ (*c* 0.61, MeOH); ir ($CHCl_3$) ν max 3570, 1735, 1618, 1370, 1255–1205, 1028 and 955 cm^{-1}; ^1H-nmr ($CDCl_3$) δ 0.98, 1.03 and 1.37 (3s, 3 Me), 2.05 (s, 3 Ac), 2.20 (s, Ac), 3.26 (br s, 1H), 3.45 (br t, H-1, *J* 8), 4.86 (dd, H-3, *J* 3.5, 6.7), 5.12 and 5.20 (s, H-20), 5.22 (s, H-6 and H-7), and 5.60 (s, H-14); and mass spectrum *m/e* 536 (1%, M^+, $C_{28}H_{40}O_{10}$), 518 (2), 500 (1), 476 (11, M–AcOH), 458 (10), 442 (7), 416 (64, M–2 AcOH), 398 (39), 374 (38), 356 (98, M–3 AcOH), 338 (72), 314 (77), 296 (98, M–4 AcOH), 278 (84), 268 (64), 253 (64), 109 (65), 91 (72) and 69 (100).

KALMITOXIN-III (**9**).—The fraction from the silica gel-silver nitrate column separation of Fraction F-1B-2 which showed tlc R_f 0.35 was crystallized from acetone to give colorless prisms (157 mg, 4 x 10^{-3}%) of kalmitoxin-III (**9**): mp 252–4°; $[\alpha]^{23}D+3°$ (*c* 0.54, MeOH); ir (Nujol) ν max 3430, 1745, 1380, 1240, 1123, 1043 and 910 cm^{-1}; ^1H-nmr (pyr-d_5) δ 1.04, 1.58 (double inten-

sity), 1.85 (3s, 4 Me), 2.20 (s, Ac), 3.33 (dd, H-1, *J* 5, 10), 3.86 (d, *J* 14.7), 3.89 (br d, H-3, *J* 4), 4.27 (t, H-7, *J* 10, on D_2O exchange changes to doublet, *J* 10), 5.35 (d, H-14, *J* 6, with D_2O becomes singlet), and 5.93 (d, H-6, *J* 9.5); and mass spectrum *m/e* 410 (1%, M-H_2O, $C_{22}H_{36}O_8$ needs 428), 392 (5), 374 (17), 358 (15), 332 (52), 314 (100, M-AcOH-3 H_2O), 296 (35), 271 (40), 209 (42), 191 (65), 171 (62), 109 (63), 91 (79) and 60 (91). The chemical ionization (*i*-butane) mass spectrum showed *m/e* 429 (0.2%, MH) and base peak at 315 (100, MH-3 H_2O-AcOH).

HYDROLYSIS OF KALMITOXIN-III (**9**) TO KALMITOXIN-I (**10**).—A solution of 11 mg of kalmitoxin-III in 2 ml of 5% methanolic sodium hydroxide was allowed to react for 5 hours at ambient temperature. The residue remaining after evaporation of the solvent under vacuum was dissolved in 0.7 ml of methanol and diluted with 9.3 ml of chloroform. The solution was added to a column of 2 g of silica gel and eluted with chloroform-methanol (93:7). The eluted material (8.5 mg) crystallized from acetone as prisms, mp 245° $[\alpha]^{25}D$ −16° (*c* 0.5, MeOH); and gave ir and ^{1}H-nmr spectra superimposable with those of kalmitoxin-I (**10**).

SODIUM METAPERIODATE TEST ON KALMITOXIN-III (**9**) AND KALMITOXIN-I (**10**).—The test was based on the literature procedure of Wood *et al.*, (19). Kalmitoxin-III (1.5 mg) was dissolved in 0.3 ml 0.1M pH 4.7 sodium acetate-acetic acid buffer and treated with 0.3 ml of 0.1M sodium metaperiodate and left to stand for 1.5 hrs. Evaporation of solvent under vacuum and analysis of the residue by tlc with solvent A indicated no change in the starting material. A similar test with kalmitoxin-I (**10**) showed complete loss of starting material.

KALMITOXIN-IV (**6**).—The fraction with R_f 0.5 material from the silica gel-silver nitrate column separation of Fraction F-1A-3 was crystallized from methanol to give 400 mg (10^{-2}%) of large colorless needles of kalmitoxin-IV (**6**): mp 231-3°; $[\alpha]^{22}D$ −33° (*c* 0.76, MeOH); ir (KBr) ν max 3450, 1718, 1373, 1250, 1095, 1038, 897 and 855 cm^{-1}; ^{1}H-nmr[5] ($CDCl_3$, D_2O exchanged) δ 1.00, 1.27, 1.38 and 1.49 (4s, 4 Me), 2.14 (s, Ac), 3.25 and 3.83 (2d, H-3 and H-2, *J* 3), 3.59 and 5.24 (ABq, H-7 and H-6, *J* 9.7), and 4.46 (s, H-14); and mass spectrum *m/e* 408 (2%, M-H_2O, $C_{22}H_{34}O_8$ requires 426), 390 (2), 372 (2), 348 (2, M-H_2O-AcOH), 330 (8), 312 (7), 264 (10), 246 (16), 203 (14), 191 (26), 161 (26), 149 (26), 116 (36), 109 (36), 85 (42), 55 (35) and 43 (100). The chemical ionization (*i*-butane) mass spectrum contained a quasimolecular ion at *m/e* 427 (0.4%, MH) with the base peak at 85.

HYDROLYSIS OF KALMITOXIN-IV (**6**). A) TO DEACETYLKALMITOXIN-IV (**16**).—An 11 mg sample of kalmitoxin-IV was treated with 1 ml of 5% methanolic sodium hydroxide for 5 hrs, at ambient temperature. Ethyl acetate (7 ml) was added and the mixture washed with brine. The brine was back extracted with ethyl acetate (3 x 7 ml), and the combined ethyl acetate solutions dried over anhydrous sodium sulfate. The residue left on evaporation of the solvent was crystallized from methanol to give glistening needles of deacetylkalmitoxin-IV (**16**): mp 240-3°; $[\alpha]^{22}D$ −64° (*c* 0.18, MeOH); ir (KBr) ν max 3440, 1373, 1150, 1035 and 855 cm^{-1}; ^{1}H-nmr (pyr-d_5, D_2O exchanged) δ 1.29, 1.55, 1.61 and 1.89 (4s, 4 Me), 2.94 (s, H-1), 3.28 and 4.22 (2d, H-3 and H-2, *J* 3), 3.70 (d, *J* 15), 4.04 and 4.24 (ABq, H-6 and H-7, *J* 9.5) and 5.13 (s, H-14); and mass spectrum *m/e* 366 (0.3, M-H_2O, $C_{20}H_{32}O_7$ requires 384), 348 (13), 330 (19), 312 (13), 301 (16), 245 (28), 209 (100), 191 (81), 163 (66), 140 (72), 116 (63), 85 (59) and 55 (63).

B) To *bis*-DEACETYLKALMITOXIN-VI (**17**).—A solution of 18 mg of kalmitoxin-IV (**6**) in 7 ml of 0.1M sodium acetate buffer at pH 4.7 was stirred with 2.5 g of neutral alumina for 1 hr. Then 0.5 g of sodium carbonate was added, the mixture was filtered, and the filtrate evaporated at reduced pressure. The residue was dissolved in 5 ml of 5% methanolic sodium hydroxide and allowed to react overnight. Ethyl acetate (7 ml) was added and the mixture extracted with brine. The aqueous phase was reextracted with ethyl acetate (7 ml, 3 times), and the combined ethyl acetate extract was evaporated to dryness after drying with sodium sulfate. The residue showed two spots (R_f 0.22 and 0.41) or tlc with solvent system A corresponding to *bis*-deacetylkalmitoxin-VI (**17**) and deacetylkalmitoxin-IV, respectively, when cochromatographed with authentic samples. The *bis*-deacetylkalmitoxin-VI (**17**) zone showed the same mobility as an authentic sample with the following tlc solvent systems: methylene chloride-isopropyl alcohol-water-acetic acid (80:20:2:0.1) R_f 0.38; benzene-isopropyl alcohol-water (40:15:2) R_f 0.28; and carbon tetrachloride-isopropyl alcohol-water-acetic acid (80:20:20.1) R_f 0.25.

REDUCTION OF KALMITOXIN-IV (**6**) TO KALMITOXIN-I (**10**).—A 15 mg sample of kalmitoxin-IV in 1 ml of dry tetrahydrofuran was treated with 15 mg of lithium aluminum hydride suspended in 1 ml of tetrahydrofuran. After refluxing 2 hrs, the reaction was quenched with 1 ml of ethyl acetate, then 1 ml of ethanol was added and the mixture was refluxed for 15 min. Filtration of the reaction mixture and evaporation of the filtrate left a residue that was dissolved in 0.85 ml of methanol and diluted with 9.15 ml of chloroform. The solution was passed into a

[5]In pyridine-d_5 kalmitoxin-IV shows, with time, the appearance of a second acetate singlet which suggests that an acyl migration is occurring, but this observation was not investigated further.

2 g column of silica gel. Elution with solvent system B gave a material that crystallized (5 mg) from acetone. Its tlc mobility, melting point, specific rotation and spectral properties (ir and ^{1}H-nmr) were identical with those of kalmitoxin-I (**10**).

SODIUM METAPERIODATE TEST ON KALMITOXIN-IV (**6**).—The test was performed on 2 mg of material as described for kalmitoxin-III (**9**) with kalmitoxin-I (**10**) serving as the positive control. No reaction was observed with kalmitoxin-IV (**6**).

KALMITOXIN-V (**2**).—The silica gel-silver nitrate separated Fraction F-1A-1 gave a residue that crystallized from methanol as tiny colorless needles (17 mg, 4 x 10^{-4}%) of kalmitoxin-V (**2**): mp 234–5°; $[\alpha]^{24}$D −9° (*c* 0.065, MeOH); ir (KBr) ν max 3490, 3430, 1745, 1720, 1375, 1255, 1230, 1095, 897 and 855 cm^{-1}; ^{1}H-nmr (CDCl$_3$, D$_2$O exchanged) δ 0.94, 1.25, 1.31 and 1.51 (4s, 4 Me), 2.07 and 2.09 (2s, 2 Ac), 2.56 (s, H-1), 3.25 and 3.85 (2d, H-3 and H-2, *J* 3), 4.41 (s, H-14), 5.25 and 5.44 (ABq, H-6 and H-7, *J* 10); and mass spectrum *m/e* 450 (0.1%, M-H$_2$O, $C_{24}H_{36}O_9$ requires 468), 432 (0.2), 408 (0.1), 390 (0.4), 374 (3), 348 (0.6), 330 (3), 312 (3), 246 (15), 204 (18), 161 (33), 135 (38), 109 (66), 83 (95) and 55 (100). The chemical ionization (*i*-butane) mass spectrum showed a quasimolecular ion peak at *m/e* 469 (2%, MH) with base peak at 313 (100, MH–2 H$_2$O–2 AcOH).

HYDROLYSIS OF KALMITOXIN-V (**2**).—A 5 mg sample of kalmitoxin-V (**2**) was left at ambient temperature in 0.5 ml of 5% methanolic sodium hydroxide for 3 hrs. The reaction mixture was diluted with 1 ml of water and extracted with ethyl acetate (3 x 5 ml). The combined ethyl acetate solution was dried (sodium sulfate) and evaporated at reduced pressure to give a residue showing a single spot on tlc with solvent A. The residue crystallized from methanol to give glistening needles, mp 240–3°, of *bis*-deacetylkalmitoxin-V, which showed the same R_f on tlc, ^{1}H-nmr spectrum and mp as deacetylkalmitoxin-IV (**16**).

KALMITOXIN-VI (**8**).—The fraction from chromatography of Fraction F-1B-1 on a column of silica gel-silver nitrate was crystallized from 2-butanone to give 240 mg (6 x 10^{-3}%) of kalmitoxin-VI (**8**): mp 225–6°; $[\alpha]^{23}$D +1.0° (*c* 0.46, MeOH); ir (KBr) ν max 3500–3420, 1730, 1373, 1240, 1110, 1030, 895 and 805 cm^{-1}; ^{1}H-nmr (pyr-d$_5$, D$_2$O exchanged) δ 1.55 (6H), 1.58, 1.85 (3s, 4 Me), 2.15 and 2.34 (2s, 2 Ac), 3.27 (d, H-1, *J* 5.7), 3.80 (d, *J* 15), 4.11 (d, H-3, *J* 3), 4.21 (d, H-6, *J* 10), 5.18 (dd, H-2, *J* 5.7, 3), 5.73 (d, H-7, *J* 10) and 6.33 (s, H-14); ^{13}C-nmr (pyr-d$_5$) δ_c 20.7, 21.7 (2 carbons), 22.2, 24.1, 24.9, 27.2, 30.0, 50.2, 51.8, 55.1, 55.5, 55.9, 60.0, 75.3, 77.4, 78.4, 80.6 (2 carbons), 82.7, 83.3, 86.9, 170.4 and 171.3; and mass spectrum *m/e* 468 (0.9%, M-H$_2$O, $C_{24}H_{38}O_{10}$ requires 486), 450 (4), 432 (4), 414 (4), 408 (2), 390 (22), 372 (23), 348 (60), 330 (100), 312 (96), 294 (99), 228 (40), 200 (40), 91 (42) and 60 (40).

Anal. Calcd for $C_{24}H_{38}O_{10}$: C, 59.24; H, 7.87. Found: C, 59.19; H, 7.89%.

HYDROLYSIS OF KALMITOXIN-VI (**8**).—A 14 mg sample of kalmitoxin-VI (**8**) was left at ambient temperature for 3 hrs in 2 ml of 5% methanolic sodium hydroxide. The reaction work up was as described for the hydrolysis of kalmitoxin-IV. The product residue was dissolved in 0.15 of methanol, diluted with 0.85 ml of chloroform, and applied to a 3 gm silica gel column. Elution by chloroform-methanol (17:3) yielded 11 mg of *bis*-deacetylkalmitoxin-VI (**17**) which crystallized from acetone: mp 220–1°; $[\alpha]^{24}$D −30° (*c* 0.56, MeOH); ir (KBr) ν max 3400, 1370, 1110, 1040, 910, 805 and 770 cm^{-1}; ^{1}H-nmr (pyr-d$_5$, D$_2$O exchanged) δ 1.53, 1.66 (double intensity), 1.80 (3s, 4 Me), 2.96 (d, H-1, *J* 5.7), 3.57 (d, *J* 15), 3.95 and 4.41 (AB q, H-7 and H-6, *J* 9.4); 4.07 (d, H-3, *J* 3), 5.10 (m hidden, H-2) and 5.11 (s, H-14); and mass spectrum *m/e* 384 (0.4, M-H$_2$O, $C_{20}H_{34}O_8$ requires 402), 366 (1), 348 (18), 330 (30), 312 (14), 287 (18), 264 (21), 246 (28), 209 (100), 191 (98), 163 (77), 140 (67), 109 (67), 93 (67) and 55 (60).

SODIUM METAPERIODATE TEST ON KALMITOXIN-VI (**8**).—The test was quantitated according to Jackson (22). Kalmitoxin-VI (4.8 mg, 0.01 mmoles) in 25 ml of 0.1M acetate buffer pH 4.7 was mixed with 30 ml of 0.001M sodium metaperiodate solution and allowed to react for 2 hrs at ambient temperature. Potassium iodide (50 mg) was added and followed, after 15 min, by 70 ml of a standard sodium arsenite solution (9.81 x 10^{-4}N). Ten minutes later 0.2 g of sodium bicarbonate and 1 ml of 0.5% starch solution were added. Titration of the excess arsenite with 9.36 x 10^{-4}N iodine solution required 8.5 ml (less blank), while the standard grayanotoxin-III (**11**, 3.7 mg, 0.01 mmoles) needed 5.1 ml. Kalmitoxin-VI consumed 1.7 times as much periodate as grayanotoxin-III.

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