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Notes

Insect Antifeedant Sesquiterpene Acetals from the Liverwort *Lepidolaena clavigera*. 2. Structures, Artifacts, and Activity

Nigel B. Perry,*[,†] Elaine J. Burgess,[†] Lysa M. Foster,[†] Philippa J. Gerard,[‡] Masao Toyota,[⊥] and Yoshinori Asakawa[⊥]

New Zealand Institute for Crop & Food Research Ltd, Dept of Chemistry, University of Otago, P.O. Box 56, Dunedin, New Zealand, AgResearch, Ruakura Research Centre, Private Bag 3123, Hamilton, New Zealand, and Faculty of Pharmaceutical Sciences, Tokushima Bunri University, Yamashiro-cho, Tokushima 770-8514, Japan

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Clavigerin A (**1**) was isolated from the New Zealand liverwort *Lepidolaena clavigera* and shown to be a polyoxygenated bergamotane sesquiterpene with an unusual ring system. *L. clavigera* shows infraspecific variation, since **1** was the only clavigerin detected in a North Island collection, whereas the previously reported clavigerins B (**2**) and C (**3**) were found in South Island collections with no sign of **1**. Three new clavigerins, **4**–**6**, were identified, but these are artifacts formed by alcoholysis of the acetoxy acetal group of the clavigerins **2** and **3**, with either the extraction solvent ethanol or the RP column eluent methanol. The insect antifeedant and cytotoxic activities of these compounds are reported, and it is proposed that they act as hidden 1,4-dicarbonyl compounds.

Liverworts are rich sources of new bioactive compounds.[1] Two approaches to discovering such compounds have been successful: examining NMR spectra of extracts for high levels of compounds with new structural features;[2] and screening extracts for biological activities relevant to the development of new pharmaceuticals or agrochemicals.[3] These two approaches independently focused interest on the liverwort *Lepidolaena clavigera* (Hook.f.) Trevis. (family Lepidolaenaceae),[4] which is endemic to New Zealand and grows on both main islands.[5]

The first report on the chemistry of *L. clavigera* was by the Tokushima group, who noted the presence of clavigerin A (**1**), plus some common sesquiterpenes.[2] However, no information was given in that report[2] on the isolation, structural characterization, or bioactivity of **1**, which also escaped the Chemical Abstracts Registry file. The Dunedin group reported clavigerins B (**2**) and C (**3**) as the main insect antifeedants in their collections of *L. clavigera*.[6] The clavigerins are polyoxygenated bergamotane sesquiterpenes with an unusual 7-oxatricyclo[4.3.0.03.9]nonane ring system. A few other naturally occurring bergamotanes have been reported with this ring system, but these have either an ether or ester oxygen linked to C-3, as in massarinolin C (**7**),[7] rather than the acetoxy acetal linked to C-1 of clavigerins **1**–**3**. The *L. clavigera* samples that contained clavigerins **2** and **3** also yielded two new polyoxygenated diterpenes, the first atisanes reported from any liverwort.[8] Other *Lepidolaena* species contain different polyoxygenated sesqui- and diterpenes: *L. taylorii* and *L. palpebrifolia* yielded cytotoxic kauranes and seco-kauranes,[9,10] and *L. hodgsoniae* gave a new class of sesquiterpene with insecticidal activity.[11–13]

We now report the full characterization of clavigerin A (**1**), plus three artifacts (**4**–**6**) formed by alcoholysis of the acetoxy acetal group. The insect antifeedant and cytotoxic activities of these clavigerins are reported, and a mode of action is proposed.

A North Island collection of *L. clavigera* was shown by GC-MS to contain the known sesquiterpenes bicyclogermacrene, germacrene D, and spathulenol.[2] The ^{1}H NMR spectrum of the extract showed the presence of a different major component. This compound (**1**) was purified and the NMR data showed signals for 24 protons and 17 or more carbons (Table 1). EIMS did not give a molecular ion consistent with this data, but positive ion ESIMS gave a base peak corresponding to $C_{19}H_{24}O_6Na$. The mass spectra showed facile losses of two acetic acid molecules, consistent with signals for two acetates in the NMR spectra. HMBC correlations (Table 1) showed that these acetates were each attached to CH groups giving sharp singlets, one at δ_C 96.4 ppm suggesting another oxygen substituent. Further HMBC correlations from these CH groups linked them into the rest of the structure, which contained two separate proton–proton spin systems shown by COSY correlations, giving the proposed connectivity shown in Figure 1 for **1**.

The relative configurations at C-1, C-4, C-6, and C-7 of **1** were fixed by the requirements of the tricyclic ring system. The proposed configuration at C-14 was based on weak, but definite, NOE interactions between H-14 and H-2, and H-14 and H-4. Molecular modeling (MM3 force field) with the proposed C-14 stereochemistry of **1** placed H-14 about 3.3–3.4 Å from those atoms, compared with separations of over 4 Å with the alternative configuration at C-14. We had hoped to determine the relative configuration at C-8 by conformational searching and molecular modeling on the two epimers. However, both epimers were predicted to give similar strong NOE interactions between H-8 and each of H-4, H-6, and one H-5, as found experimentally. The absolute configuration proposed for **1** is tentative, based on the absolute configuration of bergamotane derivative **8** found in the liverwort *Gackstroemia decipiens*,[14] in the same Lepidolaenaceae family as *L. clavigera*.

This structure of clavigerin A (**1**) was proposed in the preliminary communication by the Tokushima group[2] independent of the Dunedin group's proposed structure for the closely related clavigerin B (**2**).[6] The NMR data for **1** (Table 1 published here for the first time) are very similar to those for **2**. Clavigerin A (**1**) was isolated from one collection of *L. clavigera* from the North Island of New Zealand, which did not contain detectable levels of clavigerins B (**2**) and C (**3**). Conversely, compounds **2** and **3** were consistently present in several collections of *L. clavigera* from two sites about 30 km apart on the South Island,[6] which showed no sign of

* To whom inquiries should be addressed. Tel: +64-3-479-8354. Fax: +64-3-4798543. E-mail: perryn@crop.cri.nz.
† Crop & Food Research.
‡ AgResearch.
⊥ Tokushima Bunri University.

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Table 1. NMR Spectroscopic Data for Clavigerin A (**1**), Methoxy Clavigerin C (**4**), Methoxy Clavigerin B (**5**), and Ethoxy Clavigerin B (**6**)[a]

	clavigerin A (**1**)			methoxy clavigerin C (**4**)		methoxy clavigerin B (**5**)		ethoxy clavigerin B (**6**)	
position	δ_H (J in Hz)	δ_C	HMBC[d]	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C
1	4.88, dd (5,4)	78.6	2, 3, 7, 14	4.73, dd (5.0, 4.8)	77.5	4.74, t (5)	77.2	4.73, t (5)	77.3
2	5.50, br m	116.5		5.41, br m	115.9	5.42, br m	116.0	5.41, br m	115.9
3		147.3			148.3		148.4		148.4
4	2.40, td (6,2)	46.6	2, 3, 5, 7, 8, 15	2.39, td (5.7, 1.8)	47.3[b]	2.43, td (6, 2)	47.3	2.45, td (6, 2)	47.4
5	2.65, dt (9.5,6)	35.1	1, 3, 4, 6	2.32, m	34.5	2.35, ddd (10, 6, 5)	34.7	2.34, ddd (10, 6, 5)	34.6
5	1.46, d (9.5)		1, 3, 4, 6, 7	1.34, d (9.0)		1.33, d (9)		1.33, d (9)	
6	3.28, q (6)	42.2	2, 4, 7, 8	2.49, br q (5.5)	42.7[b]	2.50, br q (5)	42.8	2.51, br q (5)	42.9
7		62.6			58.5		58.7		58.9
8	5.58, s	77.3	4, 7, 9, 14, 8- O<u>C</u>=O	3.04, d (17.8)	44.1	3.07, d (18)	44.9	3.09, d (18)	45.0
8				2.69, d (17.8)		2.77, d (18)		2.76, d (18)	
9		194.3			209.6		199.4		199.7
10	6.12, br m	120.2	9, 12, 13	2.2–2.3, m	52.1	6.06, br m	124.2	6.08, br m	124.3
11		158.1		2.13, m	24.6		153.6		153.4
12	1.93, s	28.2	10, 11, 13	0.91, d (6.6)	22.5	1.87, d (1)	27.5	1.87, d (1)	27.5
13	2.17, s	20.8[b]	10, 11, 12	0.91, d (6.6)	22.5	2.10, d (1)	20.5	2.10, d (1)	20.5
14	5.86, s	96.4	4, 7, 14- O<u>C</u>=O	4.65, s	106.4	4.71, s	106.7	4.77, s	105.5
15	1.81, d (2)	20.8[b]	2, 3, 4	1.80, d (2.0)	20.9	1.81, d (2)	21.0	1.81, d (2)	21.0
14-OR′		169.1[c]		3.30, s	57.3	3.32, s	57.5	3.71, dq (9.5, 7) + 3.37, dq (9.5, 7)	66.1
14-OR′	1.91, s	21.2[b]						1.05, t (7)	15.1
8-OAc		170.5[c]							
8-OAc	2.18, s	20.8[b]							

[a] In $CDCl_3$. [b] Assignments with same superscript interchangeable within columns. [c] Assignments with same superscript interchangeable within columns [d] Carbon atoms correlated to this proton signal.

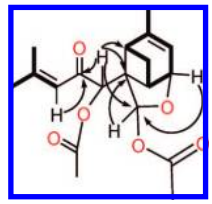


Figure 1. Key HMBC correlations for clavigerin A (**1**), with proton–proton spin systems bolded.

compound **1**. Therefore this is another example of infraspecific variation in liverwort chemistry, to add to the 15 cases highlighted in a review of the chemosystematics of liverworts.[15]

We did isolate some different clavigerins from South Island collections of *L. clavigera*. In our first fractionation we used reversed-phase (RP) flash chromatography with an H_2O-CH_3OH gradient, rather than the H_2O-CH_3CN gradient later used to isolate clavigerins B (**2**) and C (**3**).[6] One purified compound, **4**, had a similar ^1H NMR spectrum (Table 1) to clavigerin C (**3**), and another compound, **5**, was spectroscopically similar to clavigerin B (**2**). However, **4** and **5** showed methoxy signals (δ 3.3) rather than the acetoxy signals in **3** and **2**, and the acetoxy carbonyl signals were also missing from the IR spectra of **4** and **5**. The other main differences in the ^1H NMR spectra of **4** and **5** were the replacement of the singlets due to the acetoxy acetal H-14 (δ 5.9) in **3** and **2** by singlets at δ 4.7 (Table 1). Therefore these compounds were assigned as methoxy clavigerin C (**4**) and methoxy clavigerin B (**5**), confirmed by 2D NMR studies on **5** (data not shown). The relative configuration at C-14 was shown by the 2D NOESY spectrum of methoxy clavigerin B (**5**), focusing on the NOE interactions of H-14. We could not distinguish a possible NOE interaction between H-14 and H-2, expected for the C-14 configuration shown in structure **5** (see above), because of the close chemical shifts of H-14 and H-1 (Table 1). However, there was no detectable NOE interaction between H-14 and either of the C-8 protons, whereas molecular modeling of the structure with the alternative configuration at C-14 predicted a close approach (2.5–2.8 Å) of H-14 and one H-8. Compounds **4** and **5** are artifacts produced during RP chromatography with H_2O-CH_3OH, since their characteristic methoxy acetal ^1H NMR signals were not detected in crude extracts, nor in fractions from RP chromatography with H_2O-CH_3CN.

Another clavigerin derivative was purified during one isolation of clavigerins B (**2**) and C (**3**). This compound (**6**) had NMR signals mostly similar to methoxy clavigerin B (**5**), with an H-14 signal at δ_H 4.77 (the corresponding signal in **4** is at δ 4.71, Table 1) but no methoxy signal. Instead, there were signals at δ_H 3.71 and 3.37, both doublets of quartets (J = 9 and 7 Hz) coupled to one another and to a methyl triplet at δ 1.05. These observations led to the proposal of the ethoxy acetal structure **6**, which was confirmed by EIMS showing a very weak molecular ion at 276 Da, which readily lost ethanol. The ^{13}C NMR spectrum (Table 1) was also appropriate for structure **6**. We assume that **6** was formed from clavigerin B (**2**) by reaction with the ethanol used for this particular extraction. Nonhydroxylic solvents were preferred for later isolations to give higher yields of clavigerins B (**2**) and C (**3**).[6]

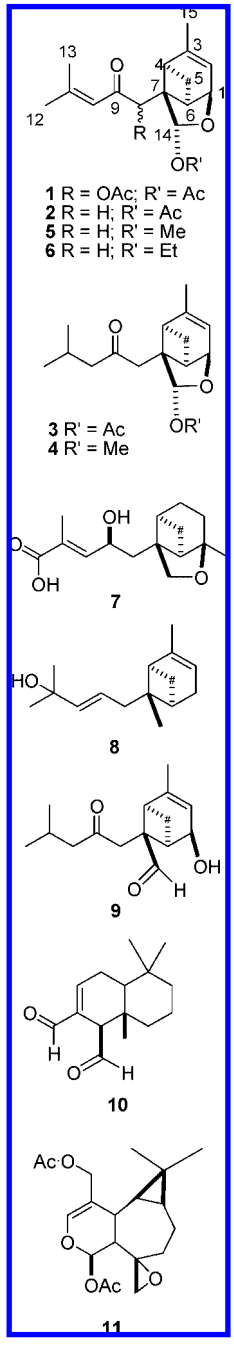
The instability of acetoxy acetal natural products has been noted by other groups, e.g., Williams et al., who observed facile hydrolysis to aldehydes.[16] When clavigerins B (**2**) and C (**3**) were recovered from CH_3CN-H_2O mixtures by rotary evaporation of solvents, ^1H NMR spectra showed singlets at ca. δ 9.8, which we attribute to partial hydrolysis to aldehydes, e.g., **9** from clavigerin C (**3**). We were not able to purify these aldehydes because of their instability, but we propose that they are the key to understanding the biological activities of the clavigerins.

Clavigerins B (**2**) and C (**3**) both showed insect antifeedant activities.[6] We now report that clavigerin A (**1**) and methoxy clavigerin C (**4**) showed significantly less antifeedant activity toward webbing clothes moth larvae, *Tineola bisselliella* (Lepidoptera), than clavigerins B (**2**) and C (**3**) (Table 2). Compounds **2** and **3** were also tested against Australian carpet beetle larvae, *Anthrenocerus australis* (Coleoptera), at three different doses. Clavigerins B (**2**) and C (**3**) both showed antifeedant activity toward the beetle larvae, with compound **3** significantly active at a lower dose than **2** (Table 2). Survival of the beetle larvae was not affected by the treatments, in contrast to the moth larvae (Table 2). We have previously shown that these beetle larvae survive starvation better than the moth larvae,[17] so reduced survival of the moth larvae (Table 2) may be due to starvation rather than to insecticidal activity of the clavigerins. The treated cloth squares from the beetle assays were stored at room temperature for 35 days, then tested again for antifeedant

Table 2. Insect Antifeedant Activity and Survival, and Mammalian Cell Cytotoxicity Data for Clavigerins A (**1**), B (**2**), and C (**3**) and Methoxy Clavigerin C (**4**)

	Tineola bisselliella larvae[a]			*Anthrenocerus australis* larvae[a]			BSC cells[b]	
compound	Dose (% w/w)	Feeding[c]	Survival[d]	Dose (% w/w)	Feeding[c]	Survival[d]	Dose (μg/disk)	Cytotoxicity[e]
clavigerin A (**1**)	0.1	+2 NS	95 ± 5 NS				30	+(2)
clavigerin B (**2**)	0.1	−74**	27 ± 6 **	0.1	−78**	95 ± 3 NS	30	+(2)
clavigerin B (**2**)				0.05	−79**	98 ± 2 NS		
clavigerin B (**2**)				0.025	−34 NS	98 ± 2 NS		
clavigerin C (**3**)	0.1	−95**	5 ± 3**	0.1	−86**	96 ± 3 NS	30	++(2)
clavigerin C (**3**)				0.05	−62**	97 ± 3 NS		
clavigerin C (**3**)				0.025	−50*	98 ± 2 NS		
methoxy clavigerin C (**4**)	0.1	−12 NS	86 ± 6 NS				60	+(2)
permethrin[f]	0.01	−100**	0 ± 0 **	0.01	−100**	96 ± 3 NS		

[a] *Tineola bisselliella* = clothes moth, *Anthrenocerus australis* = carpet beetle, on woolen test cloth.[17] [b] Monkey kidney cells.[24] [c] Mean % wool weight loss compared to untreated control, ** = statistically significant ($P < 0.01$), * = statistically significant ($P < 0.05$), NS = not significant. [d] Mean % larval survival compared with untreated control. [e] Mean size of cytotoxic effect: 0 = no zone; + = 1–4 mm zone; ++ = >4 mm zone (number of replicates). [f] Positive control in insect assays.



activity. After this time there were no significant antifeedant effects at any dose of clavigerin B (**2**) or C (**3**) (permethrin was still antifeedant), so the clavigerins are not good starting points for the development of stable, commercial insect antifeedants.

The insect antifeedant activity of acetoxy acetals **2** and **3** could be due to their proposed facile hydrolysis to corresponding aldehydes, e.g., **9** from **3** (see above). Aldehyde **9** is a 1,4-dicarbonyl compound, with some structural similarities to the well-known insect antifeedant polygodial (**10**).[17,18] The mode of action of polygodial (**10**) has been ascribed to reaction of the 1,4-dicarbonyl groups with biological nucleophiles, either amine or thiol groups.[18,19] We propose that **4** is less biologically active than **3** (Table 2) because hydrolysis of methoxy acetals, e.g., **4** to **8**, is slower than hydrolysis of acetoxy acetals.[20] However, this hypothesis does not explain the lower antifeedant and toxic activities of clavigerin A (**1**) (Table 2), which could also be hydrolyzed to a dicarbonyl compound. The insect antifeedant activity of another group of sesquiterpene acetoxy acetals from liverworts, including plagiochiline A (**11**),[21] may also be due to the fact that they can hydrolyze to dialdehydes.[1] The clavigerins **1**–**4** all showed some cytotoxicity against BSC cells (Table 2).

Experimental Section

General Experimental Procedures. The Tokushima procedures were as follows: optical rotation measured on a JASCO DIP-1000 digital polarimeter; EI-MS at 70 eV on a JEOL AX-500 spectrometer; IR spectra on a JASCO FT/IR 5300 spectrometer; UV spectra on a Hitachi U-3000; ^{1}H and ^{13}C NMR spectra on a JEOL GX-400 at 400 MHz for ^{1}H and 100 MHz for ^{13}C; normal-phase column chromatography on silica gel 60 (40–63 μm); CH_2Cl_2−MeOH (1:1) used for CC on Sephadex LH-20; and HPLC carried out with a JASCO TRI ROTAR-V and UVIDEC-100-V. The Dunedin group used previously described experimental procedures[22] and conformational searching and molecular modeling methods.[23]

Isolation of Clavigerin A (1). *L. clavigera* (voucher specimen No. NZ121117 retained in Tokushima) was collected in 1994 at Auckland in New Zealand. *L. clavigera* was gently washed with water, impurities were removed, and the plant material (9 g dry weight) was ground mechanically, then extracted with diethyl ether for 2 weeks. The crude extract (0.82 g) was subjected to silica gel CC using an *n*-hexane−EtOAc gradient to give nine fractions. Further purification of a midpolarity fraction (60 mg) by Sephadex LH-20 chromatography, followed by preparative HPLC on a 250 × 10 mm silica gel column using *n*-hexane−EtOAc (4:1 v/v) gave clavigerin A (**1**) (10.9 mg).

Clavigerin A (1): colorless oil; $[\alpha]_D$ 0 (*c* 0.55, $CHCl_3$); UV (EtOH) λ_{max} nm (log ϵ) 242 (4.24), 204 (4.04) nm; IR (film) ν_{max} 1750, 1694, 1618, 1443, 1375, 1217, 1003, 901 cm^{-1}; ^{1}H and ^{13}C NMR, see Table 1; EIMS *m*/*z* 228 [M − HOAc × 2]$^+$ (3), 200 (2), 181 (4), 163 (33), 146 (31), 131 (5), 118 (14), 105 (5), 91 (7), 83 (100), 71 (3), 55 (21), 43 (34); ESIMS *m*/*z* [M_2 + Na]$^+$ 719 (30), 371.1468 [M + Na]$^+$ (100, calcd for $C_{19}H_{24}O_6Na$ 371.1471), 289 [M − OAc]$^+$ (20), 229 [M − OAc − HOAc]$^+$ (60).

Isolation of Methoxy Clavigerins C (4) and B (5). *L. clavigera* was collected from coastal rain forest, near Haast, on the West Coast of the South Island of New Zealand in June 1993 (collection code

930609-02, voucher specimen in the Otago University Herbarium OTA046629). Dried liverwort (21 g) was extracted with EtOH (1 × 300 mL, then 4 × 100 mL), in a Waring blender. The solvent was removed by rotary evaporation of the combined, filtered extracts to give a green gum (0.34 g). A portion of this crude extract (0.33 g) was coated onto C18 silica (0.66 g) and packed onto a C18 column (5 g), which was developed in steps with H_2O, MeOH, and $CHCl_3$. Fractions eluted with H_2O−MeOH (1:3 and 1:9) were combined (44 mg). This material was fractionated on silica gel (3 g), developed in steps with 9:1, 1:1, and 0:1 hexane−EtOAc. Fractions eluted with 9:1 hexane−EtOAc gave a pure sample of methoxy clavigerin C (**4**) (3 mg), and a slightly more polar fraction contained methoxy clavigerin B (**5**) (4 mg).

Methoxy clavigerin C (4): colorless oil; $[\alpha]_D$ +6 (*c* 0.3, $CHCl_3$); IR ν_{max} ($CDCl_3$) 2954, 1709, 1600, 1216 cm^{-1}; ^1H and ^{13}C NMR, see Table 1; EIMS *m/z* 264 $[M]^+$ (1), 246 (1), 232.1448 $[M - MeOH]^+$ (10, calcd for $C_{15}H_{20}O_2$ 232.1463), 204 (35), 164 (28) 147 (50) 132 (40), 119 (85), 105 (60), 91 (70), 87 (90), 85 (95), 77 (55), 70 (35), 57 (100), 43 (45).

Methoxy clavigerin B (5): colorless oil; $[\alpha]_D$ −16 (*c* 0.2, $CHCl_3$); IR ν_{max} (film) 2957, 1687, 1623, 979 cm^{-1}; ^1H and ^{13}C NMR, see Table 1; ESIMS *m/z* 285.1483 $[M + Na]^+$ (100, calcd for $C_{16}H_{22}O_3Na$ 285.1461).

Isolation of Ethoxy Clavigerin B (6). *L. clavigera* was collected from Cascade River Flats, south of Haast, in November 1995 (collection 951102-01, voucher OTA046818). Dried liverwort (58 g) was extracted with EtOH (1 × 800 mL, then 3 × 150 mL), in a Waring blender. The solvent was removed by rotary evaporation of the combined, filtered extracts to give a green gum (1.04 g). A portion of this crude extract (0.86 g) was coated onto C18 silica (2 g) and packed onto a C18 column (10 g). This column was developed in steps with H_2O, then CH_3CN−H_2O mixtures, then CH_3CN and $CHCl_3$. Fractions eluted with 1:3 H_2O−CH_3CN (144 mg) contained mostly clavigerins B (**2**) and C (**3**).[6] A subsample (40 mg) was placed onto a silica gel column (170 mg) and developed in stages from 9:1 cyclohexane−EtOAc to EtOAc. The second fraction eluted with 9:1 cyclohexane−EtOAc (13 mg) was fractionated by HPLC on a C18 LichroCART 250–10 column (10 μm) (Merck). The mobile phase of 60:40 MeCN−H_2O was used with a flow rate of 5 mL/min, 100 μL injection, and detection at 206 nm. This gave a pure sample of ethoxy clavigerin B (**6**) (2 mg, retention time 14.1 min).

Ethoxy clavigerin B (6): colorless gum; silica gel TLC R_f 0.75 (2:1 cyclohexane−EtOAc, UV active spot, purple/pink spot with vanillin dip); UV (MeOH) λ max (log ϵ) 235 (3.71) nm; IR (film) ν_{max} 2925, 1687, 1442, 1376, 1115, 978 cm^{-1}; ^1H and ^{13}C NMR, see Table 1; EIMS *m/z* 276 $[M]^+$ (<1), 247 (<1), 230.1307 $[M - EtOH]^+$ (2, calcd for $C_{15}H_{18}O_2$ 230.1307), 215 (3), 202 (18), 178 (40), 147 (65), 119 (60), 101 (70), 83 (100), 73 (60), 55 (90).

Biological Assays. The insect assays[17] and the cytotoxicity assay[24] have both been described in detail elsewhere. Results from the insect assays were log transformed and statistically analyzed using ANOVA.

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