Antifeedant Effects of Marine Halogenated Monoterpenes

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In this work the antifeedant effects of the halogenated monoterpenes **1**−**13** were tested against several divergent insect species. These compounds have been isolated from *Plocamium cartilagineum* (**6** was isolated as an acetyl derivative), except for **4**, which was isolated from *Pantoneura plocamioides*. We have also included the semisynthetic derivatives **1a**, **2a**, and **7**. Compounds **1**, **1a**, **2**, **4**, **5**, **7**, **8**−**10**, and **13** were antifeedants against *Leptinotarsa decemlineata* with varying potencies. The aphids *Myzus persicae* and *Ropalosiphum padi* were strongly deterred in the presence of compounds **2**, **12**, and **13**. This effect was correlated with the electronic recording of their probing behavior. Compounds **2** and **12** were toxic to *L. decemlineata* and had selective cytotoxicity to insect-derived Sf9 cells. None of the tested compounds showed phytotoxic effects. The antifeedant and cytotoxic effects of these compounds were compared with those of the polyhalogenated insecticide γ-hexachlorocyclohexane (lindane).

KEYWORDS: Halogenated monoterpenes; insect antifeedants

INTRODUCTION

Polychlorocycloalkane insecticides are noncompetitive γ-aminobutyric acid$_A$ (GABA$_A$) receptor antagonists which bind to the picrotoxinin binding site within the GABA-gated chloride channel and inhibit GABA-induced chloride flux (*1*). These compounds have been widely used for insect control, causing environmental, health, and resistance problems that led to severe restrictions of their use (*2, 3*). However, theses types of compounds continue to be explored as possible new insecticides and receptor probes.

The polychlorocycloalkanes are similar in structure to a number of natural polyhalogenated monoterpenes found in red algae of the Plocamiaceae and Rhyzophyllidaceae families (*4*). These compounds have diverse biological activities, including antialgal, cytotoxic, antimicrobial, and insecticidal effects (*5*−*11*), and could represent a new source of selective GABA receptor probes.

Molecular modeling and pharmacological approaches indicate that GABA-dependent ionophores may mediate sensillar transduction of gustatory behavior in insects (*12, 13*). Therefore, according to this hypothesis, putative insect GABA antagonists are good antifeedant candidates, and vice versa. However, little

is known about the effect of natural halogenated terpenes on the feeding behavior of insects.

Here we studied the antifeedant effects of 13 polyhalogenated monoterpenes against several divergent insect species, including the lepidopteran *Spodoptera littoralis*, the chrysomelid *Leptinotarsa decemlineata* (Colorado potato beetle), and two aphid species with diverse host adaptations (*Myzus persicae* and *Ropalosiphum padi*). The molecules tested included furoplocamioids A (**1**) and C (**2**), their acetates **1a** and **2a**, prefuroplocamioid **3**, pantoneurine AB (**4**), pirene **5**, the new linear terpene **6**, and cyclohexanes **7**−**13**, including mertensene (**11**) and violacene (**12**) (**Chart 1**). Additionally, we tested their insect toxicity on *S. littoralis* and *L. decemlineata*, their cytotoxicity on Sf9 and mammalian CHO cells, and their phytotoxic effects on tomato and barley leaves. Given the potential of this class of molecules as GABA modulators, their antifeedant effects have been compared with those of the GABA-gated chloride channel antagonists lindane (*2*), picrotoxinin (*14*), and thymol, an allosteric modulator for insect GABA receptors (*15*). The sesquiterpene farnesol was included as a positive control for aphid antifeedants (*16*).

MATERIALS AND METHODS

General Experimental Procedures. IR spectra were taken on a Perkin-Elmer 1600 FT spectrometer. NMR spectra were measured on a Bruker AMX2 500 MHz spectrometer with pulsed-field gradient, using the solvent as internal standard (CDCl$_3$ at δ_H 7.26 and δ_C 77.0).
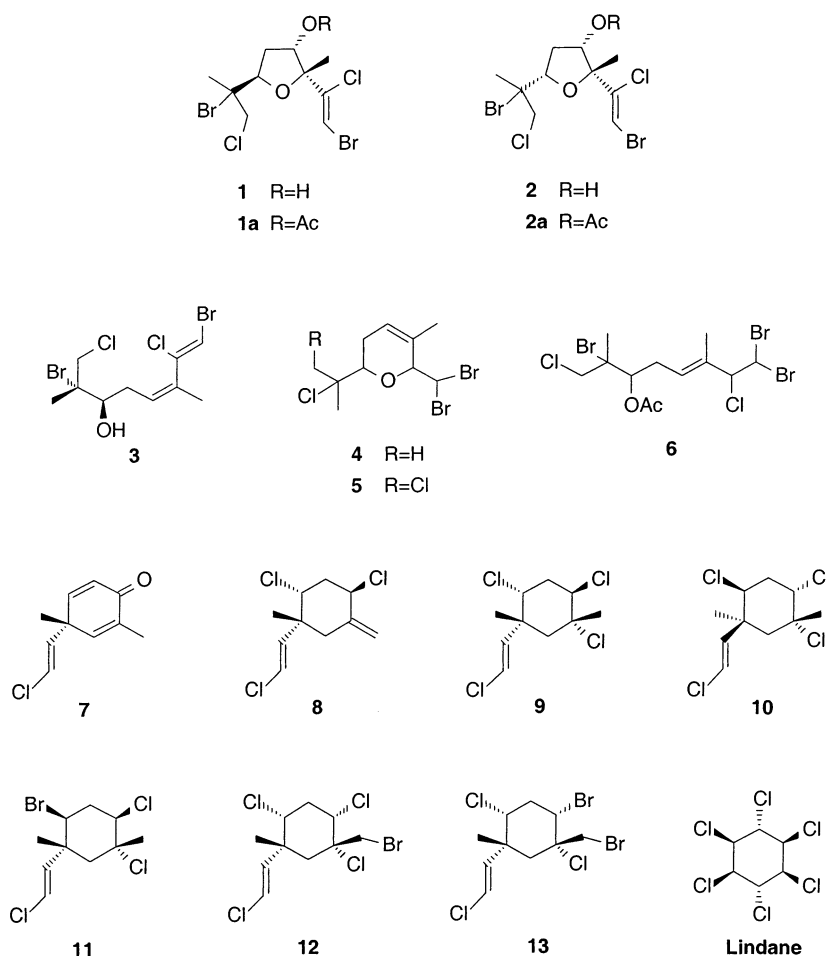
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Chart 1

Exact mass measurements and EIMS were recorded on an Autospect instrument at 70 eV. Sephadex LH-20 (Pharmacia) and silica gel from Merck (15111, 7741, 5554, and 5715) were used for column chromatography. Compounds were visualized on TLC with a 5% H_2SO_4 solution.

Compounds. Compounds **1**−**3**, **5**, and **8**−**13** have been previously isolated from *Plocamium cartilagineum* L. (Dixon), a marine alga with a wide geographic distribution; **4** was isolated from the antarctic endemism *Pantoneura plocamioides* Kylin (Delesseriaceae); and derivatives **1a** and **2a** were generated by acetylation of **1** and **2**, respectively (*5, 17*−*21*). The new compound **6** was isolated as its acetyl derivative from *P. cartilagineum*, and **7** was prepared from mertensene (**11**).

Insect Bioassays. *S. littoralis*, *L. decemlineata*, and the aphids *M. persicae* and *R. padi* were reared on artificial diet (*22*) and their respective host plants (*Solanum tuberosum*, *Capsicum annuum*, and *Hordeum vulgare*) and maintained at 22 ± 1 °C, >70% relative humidity, with a photoperiod of 16:8 h (light:dark) in a growth chamber.

Choice Feeding Assays. These experiments were conducted with *S. littoralis* L6 larvae, adult *L. decemlineata*, and apterous aphid adults. Percent feeding inhibition (%FI) and percent settling inhibition (%SI) were calculated as described by Reina et al. (*23*). Compounds with an FI/SI > 50% were tested in a dose−response experiment to calculate their relative potency (EC$_{50}$ values, the effective dose for 50% feeding reduction), which was determined from linear regression analysis (%FI or %SI on log dose).

Hemolymph Injection. Dimethyl sulfoxide (DMSO) solutions of the test compounds (10 μg/insect) were injected in 20 adult *L. decemlineata* beetles as described by Reina et al. (*23*). Beetle mortality was recorded up to 3 days after injection. Percent mortality was analyzed with contingency tables and corrected according to Abbott (*24*).

Oral Cannulation. This experiment was performed with preweighed, newly emerged *S. littoralis* L6 larvae under the same environmental conditions as above. Each experiment consisted of 20 larvae orally dosed with 20 μg of the test compound in 2 μL of DMSO (treatment) or solvent alone (control) as described previously (*23*). An analysis of covariance (ANCOVA) was performed on biomass gains and food consumption, with initial biomass as covariate.

Electronic Recording of the Probing Behavior. Aphid probing behavior was recorded using a DC system (*25*). *R. padi* starved for 1 h were tethered to an electrode of gold wire. This electrode was fixed to the dorsum of the thorax with silver paint and then connected to the amplifier before being placed on a barley leaf. The amplifier used was a four-channel DC system (with an input resistance of 1 GΩ and a gain of 50×). Leaves, aphids, and amplifier were placed in a Faraday cage. Aphid probing behavior on treated and control plants (with acetone) was recorded simultaneously for 2 h. A total of three paired waveforms (treatment−control) per treatment were analyzed using the EPG patterns described by Janssen et al. (*26*), and the following probing behavior variables were calculated: total noningestion time, total ingestion time, and salivation time. The data were analyzed by one-way ANOVA.

Cytotoxicity. Sf9 cells, derived from *Spodoptera frugiperda* pupal ovarian tissue (European Collection of Cell Cultures, ECCC), were maintained in TC-100 insect cell medium supplemented with 10% fetal bovine serum, 1% L-glutamine, and 1% penicillin/streptomycin at 26 °C. Mammalian Chinese hamster ovary cells (CHO) were grown in RPMI 1640 medium supplemented as above at 37 °C under a humidified atmosphere of 5% CO_2/95% air.

Cells seeded in 96-well flat-bottom microplates with 100 μL of medium per well (initial densities of 5×10^4 and 10^4 cells per well for the insect and mammalian cultures, respectively) were exposed for 48 h to serial dilutions of the test compounds in DMSO (<1% final concentration). Cell viability was analyzed by the MTT colorimetric assay method (*27*). The purple-colored formazan precipitate was dissolved with 100 μL of DMSO. Cell viability was calculated as the

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percent absorbance of the control (untreated cells). The active compounds were tested in a dose–response experiment to calculate their relative potency (ED_{50} values, the effective dose to give 50% cell viability), which was determined from linear regression analysis (% cell viability on log dose).

Phytotoxic Effects. Fully developed leaves of barley and tomato plants, irrigated with Hoagland solution and maintained in a greenhouse with 70% relative humidity, 25 °C/17 °C day/night, a photoperiod of 12 h, and a light intensity of 350 μmol quanta m^{-2} s^{-1}, were used to measure the phytotoxicity of the test compounds. In a first assay, the test compound (1% in acetone) was applied on the abaxial surface of the leaf. In a second assay, two applications were done within an interval of 80 h. In a third assay, a surfactant (0.5% Tween 20) was added to improve the contact of the compound with the leaf surface. Treated leaves (with water or compound) were dark-adapted for 30 min and then exposed to the excitation light at 1500 μmol quanta m^{-2} s^{-1} for 30 s with a Hansatech Plant Efficiency Analyzer. Initial (F_0), maximal (F_m), and variable ($F_v = F_m - F_0$) fluorescence values were determined directly after dark acclimation. The relative quantum efficiency of photochemistry by open PSII traps was calculated from the ratio of variable to maximal fluorescence yield (F_v/F_m) (*28*).

Compound 6. Air-dried *P. cartilagineum* (1500 g dry wt) was extracted with a mixture of hexane:EtOAc:CH$_2$Cl$_2$ (1:1:1) at room temperature and was concentrated to give a dark residue. The extract was chromatographed by flash chromatography on silica gel. The fraction eluted with hexane:EtOAc (95:5) was chromatographed on an LH-20 column to give a complex mixture that was subjected to acetylation with Ac$_2$O/Py and further separated on silica gel to give compound **6** (38 mg): colorless oil; IR ν_{max} 1744, 1223 cm^{-1}; ^{1}H NMR (500 MHz, CDCl$_3$) δ 1.73 (3H, s), 1.81 (3H, s), 2.06 (3H, s), 2.59 (2H, t, $J = 6.8$ Hz), 3.77 (1H, d, $J = 11.4$ Hz), 3.92 (1H, d, $J = 11.4$ Hz), 4.73 (1H, d, $J = 9.1$ Hz), 5.14 (1H, t, $J = 6.3$ Hz), 5.64 (1H, d, $J = 9.1$ Hz), 5.67 (1H, t, $J = 6.5$ Hz); ^{13}C NMR (125 MHz, CDCl$_3$) δ 44.9 (C1), 73.1 (C2), 134.2 (C3), 128.4 (C4), 30.8 (C5), 73.9 (C6), 67.7 (C7), 50.9 (C8), 26.4 (C10), 169.4 (C=O), 20.9 (*Me*C=O); EIMS *m/z* 440/442/444/446/448 [M − AcOH]$^+$ (<1, <1, <1, <1, < 1), 421/423/425/427 [M − Br]$^+$ (<1, <1, <1, <1), 361/363/365/367 [M − Br − AcOH]$^+$ (36, 100, 89, 32); HREIMS [M]$^+$ 499.8155 (calcd for C$_{12}$H$_{17}$O$_2$^{79}Br$_3$^{35}Cl$_2$, 499.8155).

Compound 7. A mixture of 50 mg of mertensene (**11**) and 300 mg of NaOH in DMSO (3 mL) was stirred at room temperature for 8 h, poured into water, and extracted with CHCl$_3$. The organic layer was dried over CaCl$_2$ and concentrated. The residue was chromatographed on silica gel and eluted with hexane: EtOAc (95:5) to give 22 mg of the new compound, **7**: colorless oil; ^{1}H NMR (500 MHz, CDCl$_3$) δ 1.35 (3H, s), 1.90 (3H, d, $J = 1.4$ Hz), 5.85 (1H, d, $J = 13.6$ Hz), 6.05 (1H, d $J = 13.6$ Hz), 6.25 (1H, d, $J = 9.9$ Hz), 6.54 (1H, m, $J = 1.5$ Hz), 6.74 (1H, dd, $J = 3.1$ and 9.9 Hz); ^{13}C NMR (125 MHz, CDCl$_3$) δ 119.3 (C1), 134.9 (C2), 43.1 (C3), 151.5 (C4), 127.9 (C5), 185.9 (C6), 134.9 (C7), 147.3 (C8), 15.9 (C9), 24.9 (C10); EIMS *m/z* 182/184 [M]$^+$ (5, 1), 167/169 [M − Me]$^+$ (3, 1), 147 [M − Cl]$^+$ (31); HREIMS [M]$^+$ 182.0497 (calcd for C$_{10}$H$_{11}$O^{35}Cl, 182.0498).

RESULTS AND DISCUSSION

Table 1 shows the antifeedant and repellent effects of the test compounds on the different insect species. The feeding activity of the polyphagous *S. littoralis* was not modified by any of the compounds tested (data not shown). Compounds **4** and **5** were the most active against *L. decemlineata*, with an antifeedant potency similar to that of thymol (overlapping 95% confidence limits), followed by furoplocamioids **1**, **1a**, and **2** and the halogenated cyclohexanes **8**, **10**, **7**, **9**, and **13** (in order of potency), with a moderate effect within the range of picrotoxinin. Compounds **2**, **12**, and **13** were strongly repellent to both aphid species (6−31 and 9−20 times more active than thymol against *M. persicae* and *R. padi*, respectively), followed by **10**, **8**, and **3**. In addition, *R. padi*, with a more restricted host-plant range than *M. persicae* (*29*), was selectively sensitive to **11** and **9**. Lindane had toxic (at 50 μg/cm^2) and antifeedant

Table 1. Effective Antifeedant Doses (EC$_{50}$) and 95% Confidence Limits (Lower, Upper) of the Test Compounds on Adult *L. decemlineata* and Apterous Adult *M. persicae* and *R. padi*

	EC$_{50}$ (nmol/cm^2)		
compd	*L. decemlineata*	*M. persicae*	*R. padi*
1	18.9 (8.0, 45.6)	na^a	>100
1a	17.1 (7.5, 40.2)	∼50	>100
2	19.1 (9.0, 40.6)	3.7 (2.0, 7.3)^b	1.6 (0.8, 3.2)^b
2a	na	na	>100
3	>100	25.3 (11.3, 57.2)	38.9 (24.8, 61.0)
4	2.4 (0.5, 12.5)	≈100	>100
5	8.3 (4.0, 16.9)	>100	>100
6	>70	na	>70
7	21.2 (8.3, 54.1)	>200	>200
8	12.9 (3.7, 42.6)	7.1 (3.8, 13.3)	44.2 (32.1, 60.5)
9	30.1 (12.3, 73.5)	>100	63.0 (28.3, 140.9)
10	13.0 (3.6, 46.0)	1.0 (0.5, 1.5)	15.9 (9.0, 27.5)
11	∼150	>150	16.6 (6.5, 43.0)
12	∼140	5.1 (1.1, 24.2)^b	2.5 (0.28, 4.2)^b
13	39.3 (14.3, 108.4)	1.0 (0.5, 1.5)^b	1.2 (0.7, 2.2)^b
lindane	0.1 (0.04, 0.3)		
picro-toxinin^c	27.5 (9.4, 80.1)	>100	>100
thymol^c	3.6 (1.1, 12.9)	30.9 (20.8, 45.9)	23.7 (15.4, 36.6)
farnesol^c	107.3 (59.9, 192.6)	67.1 (54.5, 82.4)	7.9 (5.2, 11.9)

^{a} na, insufficient compound available. ^{b} Total settling <50% at 50 μg/cm^2 (9%) and 10 μg/cm^2 (17–21%). ^{c} From González-Coloma et al (*30*).

Table 2. Effect of Compounds **11**–**13** on the Feeding Behavior of Aperous Adult *R. padi* ($n = 6$)^a

compd	concn (μg/cm^2)	ingestion time (min)	salivation time (min)	noningestion time (min)
control	0.0	9a	35a	16a
11	10.0	0	45b	15a
	2.0	1b	48b	15a
	1.0	3b	40b	17a
12	10.0	0	0	60b
	2.0	0	0.5c	59.5b
	1.0	0.2c	8d	51.8b
13	10.0	0	0	60b
	2.0	0	0	60b
	1.0	0.1c	12d	48b

^{a} Means followed by different letters within columns differ significantly ($P < 0.05$, LSD test).

effects to *S. littoralis* in choice tests (from a dose of 10 μg/cm^2), with an EC$_{50}$ of 16.8 nmol/cm^2 (6.7−42.6 nmol/cm^2, 95% confidence limits). *L. decemlineata* showed a stronger response to this chemical in choice tests (toxic down to 2 μg/cm^2, 170 times more antifeedant to this insect; **Table 1**), as expected from their different feeding adaptations. This insecticide had concentration-dependent lethal (100% mortality at 50 μg/cm^2) or toxic (>50% mortality at 10 and 2 μg/cm^2 against *M. persicae* and *R. padi*, respectively) effects against both aphid species, without being antifeedant at sublethal doses.

The monoterpenes tested showed species-related selective antifeedant effects. Furopolocamioids **1** and **1a**, pantoneurine AB (**4**), the related compound **5**, and the unsaturated cyclohexane **7** were selective antifeedants to *L. decemlineata*. Compounds **3** and **12** were selective antifeedants to both aphids, while **11** only affected *R. padi*.

Overall, *L. decemlineata* responded to a larger number of structures than did the aphids. However, the aphids showed higher molecular selectivity than *L. decemlineata*, as shown by comparison of the antifeedant effects of furoplocamioids A (**1**) and C (**2**) or cyclohexanes **9** and **10**. Additionally, the number

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Table 3. Injected Toxicity on Adult *L. decemlineata* [Mortality Data Corrected According to Abbott (*24*)], Consumption (*I*) and Biomass Gain (ΔB) of Orally Injected *S. littoralis* L6 Larvae, and Cytotoxic Effects on Sf9 and CHO Cells of Compounds **2**, **12**, and Lindane

	L. decemlineata	*S. littoralis*		ED$_{50}$ (μg/mL)^a	
compd	mortality, 72 h	ΔB^b	I^c	Sf9	CHO
2	na^d	89.8	91.8	0.03 (0.02, 0.05)	8.09 (6.53, 10.02)
12	80*	80.8	84.4	0.023 (0.005, 0.11)	29.84 (13.27, 67.09)
lindane	100*	nc^e	nc	0.0014 (0.0006, 0.003)	>100
p^f		>0.05	>0.05		

^{a} ED$_{50}$, concentration needed to produce 50% cell viability and 95% confidence limits (lower, upper). ^{b} ΔB, change in insect body weight (mg dry weight). ^{c} I, milligrams of food consumed (mg dry weight). ^{d} na, insufficient compound available. ^{e} nc, not calculated. ^{f} Treatment p-level, ANCOVA analysis with initial body weight as covariate. *Significantly different from the control, $p < 0.05$, contingency table analysis.

of halogenated substituents seemed to affect the aphid antifeedant action of the cyclohexanes (strong activity was observed when five halogen substituents were present, as in **12** and **13**). Furthermore, the substitution of a Cl by a Br atom enhanced the activity of the molecule (**13** versus **12** against *M. persicae*). However, the presence of six Cl atoms (lindane) conferred generalized toxicity and decreased the selective aphid antifeedant action of the molecule.

The structural similarities between cyclic halogenated monoterpenes and lindane, the antifeedant effects of lindane, thymol, and picrotoxinin, and the action of these compounds on insect GABA neuroreceptors suggest a GABA-mediated taste regulation by halogenated monoterpenes in insects. Similarly, tricyclic silphinenes with strong antifeedant effects against *L. decemlineata* and aphids are GABA antagonists at mammalian receptors (*1*, *30*, *31*), supporting the hypothesis of a GABA-mediated shared molecular mechanism for antifeedant taste chemoreception in divergent insect species (*13*).

To further investigate the aphid antifeedant mode of action of the active compounds, we carried out the electronic recording of the probing behavior of *R. padi* on barley leaves treated with compounds **11** (a moderate antifeedant in the choice test), **12**, and **13** (strong antifeedants in the choice test) (**Table 2**). Compound **11** reduced the ingestion time of *R. padi* at all the doses tested and increased the salivation time, with a total noningestion time similar to that of the control, indicating that this compound had a moderate antifeedant effect but did not decrease the insect's probing activity (**Table 2**). *R. padi* did not show any feeding activity (ingestion or salivation) in the presence of 50−10 μg/cm^2 of **12** and **13**. A dose of 2 μg/cm^2 of these compounds allowed significantly lower aphid ingestion/salivation activity than the control or compound **11** (**Table 2**). This effect correlated with the settling inhibition activity observed in the choice tests, indicating that these compounds are both repellent and antifeedant.

The probing and feeding behavior of aphids can result in the uptake of viruses from infected plants and subsequent transmission to healthy plants. It is possible to interfere with virus acquisition and transmission by influencing aphid host selection and feeding behavior with antifeedant chemicals (see *16*). Therefore, the halogenated monoterpenes **12** and **13** have the potential to interfere with aphid-mediated plant virus transmission.

Table 3 shows the toxic actions of **2**, **12**, and lindane. None of the natural compounds affected the performance of the polyphagous *S. littoralis*, but **12** was strongly toxic to *L. decemlineata* (a Solanaceae specialist). Lindane was lethal to both insect species (100% mortality).

Cyclic polyhalogenated monoterpenes from *P. cartilagineum*, including mertensene (**11**) and violacene (**10**), showed insecticidal effects after ingestion against the chrysomelid *Diabrotica*

virgifera and the aphids *Aphis fabae* and *Schizaphis graminum* (*5*, *32*). However, this is the first report on the antifeedant effects of **1**, **1a**, **2**, **3**, and **9−13**.

Antifeedants **2** and **12** were strongly cytotoxic to insect-derived Sf9 cells, suggesting metabolic detoxification by *S. littoralis*. Additionally, both compounds were less cytotoxic to mammalian CHO cells, with **12** being 4 times less active than **2**, indicating a selectivity of action of the cyclohexane **12** against insects probably related to membrane factors. Furthermore, lindane was cytotoxic to Sf9 but not to CHO. This cytotoxicity suggests a mode of action other than GABA-related neurotoxicity for these compounds, since these cell lines lack GABA receptors. Previous reports have shown that halogenated carbocyclic monoterpenes were cytotoxic to human tumor cells (*33*). Additionally, lindane alters the molecular dynamics of the sperm membrane bilayer (*34*). Similarly, these compounds could intercalate into membrane cells and cause selective cytotoxicity.

To investigate the phytotoxic effects of the test compounds, we measured the photosynthetic efficiency of treated barley leaves. The compounds assayed did not have any significant effect on plant photosynthetic efficiency (see Supporting Information). Similarly, a polyhalogenated monoterpene structurally related to **12** and **13** had a strong antialgal effect without inhibition of the photosynthesis (*10*).

In summary, we have demonstrated that furoplocamioid C (**2**) and cyclohexane polyhalogenated monoterpenes (**12** and **13**) are very efficient aphid repellents and antifeedants as well as selective insect cell toxicants. Additionally, these compounds had low mammalian toxicity and did not show phytotoxic effects. Given the structural similarities of some of the natural halogenated monoterpenes tested here to the insecticide lindane and their antifeedant effects, we hypothesize a GABA-mediated mode of antifeedant and action for these compounds.

Supporting Information Available: Table showing the phytotoxic effects of compounds **11−13** on barley and tomato leaves. This material is available free of charge via the Internet at http://pubs.acs.org.

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