Feeding and Toxic Effects of Floral Sesquiterpene Lactones, Diterpenes, and Phenolics from Sunflower (*Helianthus annuus* L.) on Western Corn Rootworm

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Feeding deterrents for adult western corn rootworm, *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae), some of which were neurotoxic, were isolated from inflorescences of cultivated sunflower by chromatography of ethyl acetate solubles on Toyopearl TSK HW-40F and silica gel. Antifeedants, as measured through a consumption bioassay with treated squash flower disks containing cucurbitacin feeding stimulants for rootworm, were characterized by UV, ^{1}H and ^{13}C NMR, and EIMS. Fractionation by these methods gave 15 active principles of which argophyllin A and 3-*O*-methylniveusin A, both sesquiterpene lactone angelates, were the most potent. Feeding deterrency decreased in the order sesquiterpenes ≫ diterpenes > flavonoids > dicaffeoylquinic acids. The diterpenoic acid grandifloric acid and its 15-angelate and the flavonoids nevadensin and quercetin β-7-*O*-glucoside were much poorer antifeedants, although more abundant components of sunflower. Synergistic or antagonistic interactions for combinations of deterrents within or between the sesquiterpene, diterpene, and flavonoid classes were not found, indicating sunflower antifeedants act jointly in an additive fashion. The highly active antifeedant germacranolide angelates exhibit structural features and injected symptoms in adult rootworm similar to picrotoxinin, a γ-aminobutyric acid gated chloride channel antagonist, suggesting a link between sesquiterpene neurotoxicity and GABA.

INTRODUCTION

Floral chemicals are increasingly recognized for their antiherbivore action on insects in addition to their attraction and rewarding of essential pollinators. In the Asteraceae, both monoterpene derivatives such as the insecticidal pyrethrins from *Chrysanthemum* spp. (Casida, 1980) and toxic sesquiterpene lactones and diterpenoic acids concentrated in the floret achenes of wild *Helianthus* spp. (Gershenzon et al., 1985; Rossiter et al., 1986; Rogers et al., 1987) clearly protect flowers from excessive herbivory. Both niveusin A from *H. niveus* and 8β-sarracinoyloxycumambranolide from *H. maximiliani* deter feeding of the sunflower moth larvae, *Homoeosoma electellum* (Rossiter et al., 1986). Thus, these sesquiterpene lactones found in glandular trichomes of the anther are thought to prevent pollen feeding by this sunflower pest. Foliar sesquiterpenes including argophyllin A (1, Figure 1) from *H. argophyllus* (Rogers et al., 1987) and diterpenes such as kaurenoic acid (7; Elliger et al., 1976) may also explain antibiosis in *Helianthus* for this and other pests including the chrysomelid *Zygogramma exclamationis* (F.). Documentation and identification of anti-insect factors in wild sunflower species have been largely limited to leaf tissues and may not fully represent the defensive chemistry directed against pollen thieves.

Plant sesquiterpenes and diterpenes are major determinants of insect–plant interactions (Stipanovic et al., 1977; Mabry and Gill, 1979; Kubo and Nakanishi, 1979; van Beek and de Groot, 1986). Many insecticidal and antifeedant sesquiterpenoids (Mabry and Gill, 1979; Ivie and Witzel, 1982; Rodriguez, 1985; Picman, 1986; Isman et al., 1989) and diterpenes (Kubo and Nakanishi, 1979; Cooper-Driver and Le Quesne, 1987) are epoxides. Occasionally, the same compound, while normally inhibitory to herbivores, may for adapted insect species or at low concentrations have a stimulatory effect (Brattsten, 1983). Cyclic sesquiterpenes and diterpenes inhibitory to insect herbivores have been identified from at least 28 genera of the terpenoid-rich Asteraceae [references cited in Mullin et al. (1991)]. These studies were largely confined to extrafloral tissues. Among the most noted of chrysomelid–terpenoid investigations have been *Diabrotica* spp. feeding associations with squash cucurbitacins, triterpenoidal-derived electrophiles that serve as potent feeding stimulants for corn rootworms (Metcalf et al., 1980; Andersen and Metcalf, 1987).

Many polyhydroxylated flavonoids and related phenolics are also deterrent to insect herbivores (Harborne, 1988). Inhibitory actions by phenolics often require both the high concentrations naturally present in plants and chemical structures bearing adjacent (ortho) hydroxyl groups. As in the case of terpenoids, stimulatory rather than inhibitory effects on feeding may result at lower dosages or with phenolic specialists. Ultraviolet (UV)-absorbing flavonoids (Hedin et al., 1968) with pro- or anti-insect activities are increasingly being found within floral tissues, suggesting that their adaptive roles extend beyond the visual orientation of pollinators (Thompson et al., 1972).

Adult western corn rootworms, *Diabrotica virgifera virgifera* LeConte (WCR; Coleoptera: Chrysomelidae), avoid flowers of the Asteraceae that are readily acceptable to northern corn rootworm, *D. barberi* Smith & Lawrence (NCR). Rearing adult WCRs continuously on inflorescences of cultivated sunflower *H. annuus* L. var. Giant Gray Stripe, or Canadian goldenrod *Solidago canadensis* L. var. *canadensis*, reduces its longevity by 40% and 70%, respectively, to that on corn ears. By contrast, NCR's longevity is not significantly affected by host shifts from

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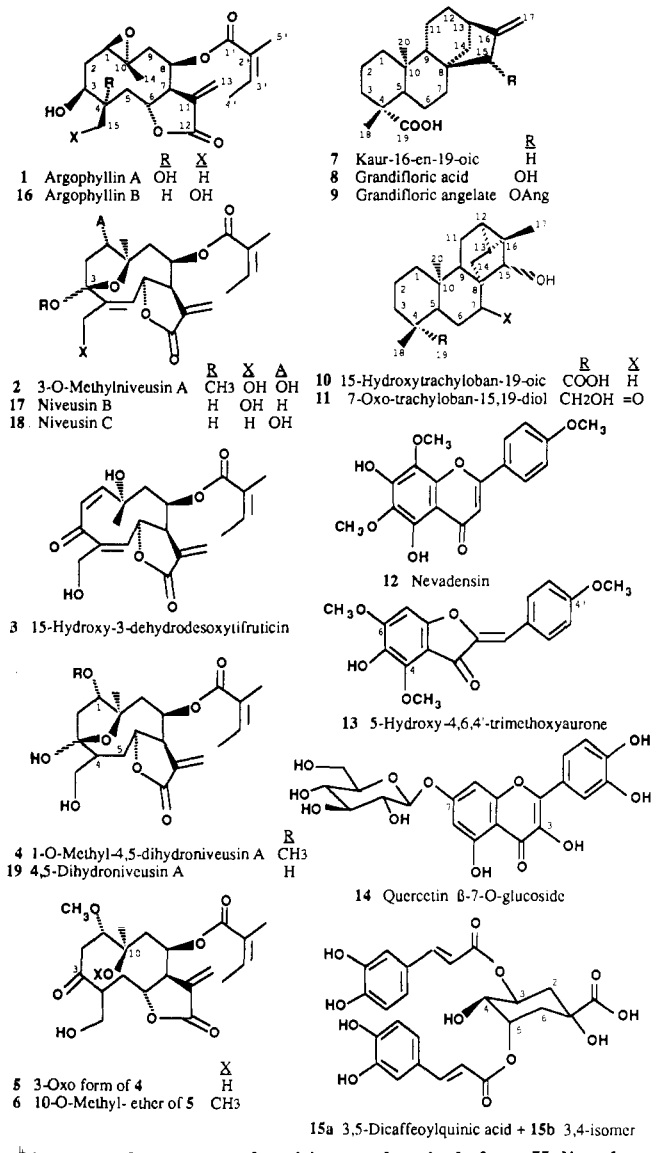

Figure 1. Structures of anti-insect chemicals from *Helianthus*.

corn to Asteraceae (Siegfried and Mullin, 1990). In the short term (<24 h), an antifeedant action of this food was observed for WCR. Reported herein are the isolation of antirootworm sesquiterpene lactones, diterpenoic acids, and phenolics from floral tissues of the cultivated sunflower.

MATERIALS AND METHODS

Insect and Plant Materials. A multicrop field plot (0.2–0.4 ha) has been established for 6 consecutive years at the Pennsylvania State University Field Research Lab at Rock Springs in Centre County, PA, as a source for fresh plant materials and adult corn rootworms. These plots contain 3–9-m strips of winter squash, *Cucurbita maxima* Duchesne var. Blue Hubbard, sweet corn, *Zea mays* L. var. Pennfresh ADX, and giant gray stripe sunflower grown on a silt loam soil. Squash are started in the greenhouse and then replanted into the field so that insecticide treatments for striped cucumber beetle control are not necessary. Herbicides are used to establish the corn and sunflower prior to rootworm hatch, after which time all weed control is by cultivation. Adult WCR forages on corn and squash, whereas the endemic NCR feeds equally well on all of these host plants. Fresh squash flowers for consumption bioassays are grown under controlled temperature and halide lamps (16 h of light and 8 h of darkness) in the greenhouse.

Reagents. TSK Toyopearl HW40-F (formerly EM Fractogel) was obtained from Supelco, and silica gel chromatography

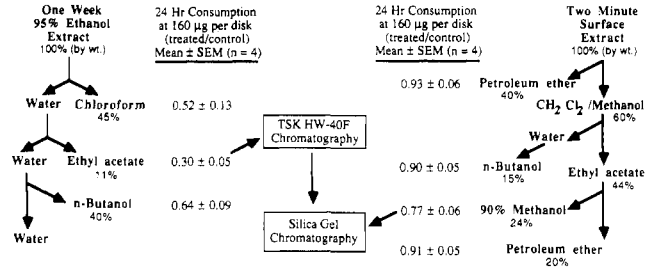

Figure 2. Isolation scheme for western corn rootworm antifeedants from floral tissues of sunflower.

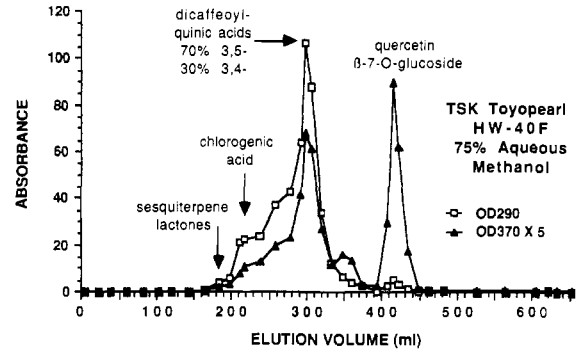

Figure 3. Chromatography of the floral ethyl acetate extract from sunflower on Toyopearl HW-40F.

supplies were from E. Merck. All other chemicals or solvents were of reagent grade or better.

General Identification Methods. UV spectra were obtained with a Perkin-Elmer Lambda 3B spectrophotometer. Unless otherwise noted, a Bruker WM-360 was used for ^1H (including NOE and homonuclear spin-decoupling experiments) and ^{13}C (90.6 MHz) nuclear magnetic resonance (NMR) determinations. This and ^{13}C-gated spin echo (GASPE) experiments on a Bruker WP-200 at 50.3 MHz and electron impact mass spectroscopy (EIMS) on a Kratos 950 were performed at the Pennsylvania State University NMR and Mass Spectrometry Facilities.

Phytochemical Isolation and Characterization. In 1988, residues from 1-week ice-cold 95% ethanol extracts of petals and florets from 21 sunflower heads were dispersed in water and repeatedly extracted sequentially with chloroform, ethyl acetate, and 1-butanol. Most of the original toxic and feeding deterrent activities to rootworm (see below) were associated with the ethyl acetate fraction (Figure 2). Further fractionation of the ethyl acetate residue on Toyopearl TSK HW-40 (F) using methanol–water (75:25) gave three major phenolic components (Figure 3), two with UV spectra resembling caffeoyl and/or other hydroxycinnamate esters and a flavonol with UV characteristics resembling a glycoside of quercetin with a free 3-hydroxy group (MeOH, λ_{max} 255 and 371 nm with similar absorbance and a shoulder at 268 nm; addition of NaOMe gives λ_{max} 247, 265, and 425 nm). We have identified these phenolics as a mixture of 3,5-dicaffeoyl- and 3,4-dicaffeoylquinic acids and the flavonol as quercetin 7-glucoside. Further analysis of the unbound compounds eluting from the Toyopearl gel filtration column for sunflower gave after silica gel chromatography additional terpenoids including angelate esters of polar sesquiterpene lactones.

Subsequently, sequential 2-min surface extraction of 84 whole sunflower heads by petroleum ether, methylene chloride–methanol (3:1 v/v), and methanol followed by partitioning of the residues (22 g) from the combined methylene chloride–methanol extracts between water and ethyl acetate and the ethyl acetate residue (16 g) between 90% aqueous methanol and petroleum ether yielded methanolic solubles (8 g) rich in antifeedant activity (Figure 2). Normal-phase column, flash, and thin-layer (TLC) silica gel chromatography of these solubles using solvent systems containing chloroform, ethyl acetate, and methanol gave the following WCR-active compounds: **7, 9, 13, 8, 11, 10, 17, 2, 3, 12, 6, 5, 1, 4, 19, 14,** and **15a,b** in approximate order of increasing polarity. Purity was assessed by TLC and for some sesquiterpenes by reversed-phase high-performance liquid chromatog-

raphy (HPLC) on C_8 and C_{18} columns using acetonitrile–water gradients. Only spectral data that supplement literature values will be included below. Data for the new compounds **2**, **6**, **11**, and **13** will be presented elsewhere (Alfatafta and Mullin, unpublished data).

Argophyllin A (1). Our spectral data including extensive ^1H NMR ($CDCl_3$) homonuclear spin-decoupling experiments for this angelate were consistent with the 90-MHz data of Watanabe et al. (1982) for **1** from leaves of *H. argophyllus* except H-7 and H-1 were resolved at δ 3.33 (m) and 3.25 (dd), respectively, instead of a combined multiplet centered at 2.86 and H-8 resonated at δ 5.47 instead of 5.2. Additional ^1H NMR assignments to that previously published include a combined multiplet at δ 3.70 for H-2a and H-5a with H-2b and H-5b being located at δ 2.30. Although only M − 18 was available by EIMS, a M + H^+ ion of 381 was obtained by fast atom bombardment in glycerol at room temperature (J.-C. Chou, unpublished data). This compound also has been isolated from leaves of a wild variety of *H. annuus* (Melek et al., 1985).

15-Hydroxy-3-dehydrodeoxytifruticin (3). This major floral conjugated dienone gave the following: UV λ_{max} 228, 255 nm in acetonitrile; EIMS *m/z* (% intensity) 376 (M^+, 0.2); ^1H NMR ($CDCl_3$) δ 7.03 (d, J = 17 Hz, H-1), 6.28 (d, J = 17 Hz, H-2), 6.09 (br d, J = 9.3 Hz, H-5), 4.45, 4.30 (dd each, J = 13, 1.1 Hz, H-15α,β), 1.55 (s, Me-14) diagnostic for **3** (Spring et al., 1982).

1-*O*-Methyl-4,5-dihydroniveusin A (4) and Its 3-Oxo Form (5). EIMS [*m/z* (% intensity) 410 (M^+, 0.63)] and ^1H NMR homonulear spin-decoupling experiments [($CDCl_3$) δ 6.18, 5.41 (d each, H-13α,β), 4.62 (ddd, H-6), 4.55 (ddd, H-7), 3.96 (dd, H-1), 3.67 (m, H-15α,β), 3.39 (s, OMe), 2.39, 2.13 (dd each, H-2α,β), 2.28 (m, H-4), 1.79, 1.75 (H-9α,β)] established **4** as the 1-methylated derivative of 4,5-dihydroniveusin A known from *H. annuus* (Spring et al., 1989). Its 3-oxo form **5**, a minor component (Spring et al., 1989), was distinguished from **4** primarily by its higher mobility on silica gel and slight shifts for H-7 (δ 4.46), H-1 (3.84), and H-15α,β (3.62, 3.56) resonances in the ^1H NMR.

Kaur-16-en-19-oic Acid (7). Crystals from methanol were confirmed to be *ent*-kaur-16-en-19-oic acid by EIMS [*m/z* (% intensity) 302 (M^+, 49)], ^1H NMR [($CDCl_3$) δ 4.80 and 4.74 (two br s for H-17α,β), 2.65 (br m, H-13), 1.25 (s, Me-18), 0.95 (s, Me-20)], and ^{13}C NMR [($CDCl_3$) δ 184.6 (C-19), 155.9 (C-16), 103.0 (C-17)] as expected on the basis of data for ester derivatives of this dominant diterpenoic acid of sunflower inflorescences (St. Pyrek, 1970, 1984).

Grandifloric Acid (8). This solid was identified as the 15-hydroxylated derivative of **7** by EIMS [*m/z* (% intensity) 318 (M^+, 22)] and ^1H NMR [($CDCl_3$) δ 5.22 and 5.08 (two s for H-17α,β), 3.81 (br s, H-15), 2.75 (br m, H-13), 1.26 (s, Me-18), 0.96 (s, Me-20)] as found previously from *H. annuus* (Panizo and Rodriguez, 1979).

Grandifloric Acid Angelate (9). This oily solid gave spectra consistent with the 15-angeloyl derivative of **8**: EIMS *m/z* (% intensity) 400 (M^+, 5.2), 300 (M^+ − angelic acid, 28), 83 (C_5H_7O, 100); ^1H NMR ($CDCl_3$) δ 6.03 (qq, J = 7.2, 1.4 Hz, H-3′), 5.35 (s, H-15), 1.98 (dq, J = 7.2, 1.4 Hz, Me-4′), 1.88 (q, 1.4, Me-5′); ^{13}C gated spin echo (GASPE) NMR ($CDCl_3$) δ 166.2 (absorption, C-1′), 137.2 (dispersion, C-3′), 128.2 (abs, C-2′), 82.5 (disp, C-15), 20.7 (disp, C-5′; not C-11 as assigned in Ohno et al. (1979); this is an absorption peak at 18.5), 15.9 (disp, C-4′). **9** has been reported previously from *H. annuus* (Panizo and Rodriguez, 1979).

15-Hydroxytrachyloban-19-oic Acid (10). The EIMS [*m/z* (% intensity) 318 (M^+, 40)] and ^1H NMR [($CDCl_3$) δ 3.29 (s, H-15), 1.23 (s, Me-18), 1.21 (s, Me-17), 0.90 (s, Me-20)] are consistent with the diterpenoid *ent*-15β-hydroxytrachylobanoic acid formerly found in cultivated sunflower heads (Ferguson et al., 1982).

Nevadensin (12). This flavone gave a ^1H NMR identical to that reported (Farkas et al., 1966) and has been found previously in leaf tissues of *H. annuus* (Rieseberg et al., 1987).

Quercetin β-7-*O*-Glucoside (14). The identity of **14** was confirmed by UV spectroscopy with shift reagents, ^1H and ^{13}C NMR in DMSO-d_6, and cochromatography by TLC with quercetin and glucose after its hydrolysis in 0.5 N trifluoroacetic acid at 100 °C (Markham, 1982). The resonance of the glucose H-1 doublet at δ 5.06 (J = 7.3 Hz) indicated a β-7-O linkage later confirmed by ^{13}C NMR. Spectra were identical with the literature except that our higher field ^{13}C NMR resolved C-2 and C-4′ at δ 147.6 and 148.0 in contrast to a single δ 147.9 peak reported by K. R. Markham in Harborne and Mabry (1982).

3,5- (15a) and 3,4-Dicaffeoylquinic Acids (15b). The Toyopearl TSK HW-40F residues eluting between 260 and 320 mL (Figure 3) were identified as a mixture of 3,5- and 3,4-dicaffeoylquinic acids by UV spectroscopy with basic shift reagents and extensive homonuclear spin-decoupling experiments with ^1H NMR in DMSO-d_6. The overall 70% **15a** and 30% **15b** composition of the mixture was determined primarily by the relative NMR intensities of proton 3 (quinic acid moiety) at δ 5.30 (ddd, $J_{3,4}$ = 10.4 Hz, $J_{2a,3}$ = 10.5, $J_{2e,3}$ = 4.7) for **15a** and δ 5.48 (ddd, $J_{3,4}$ = 10, $J_{2a,3}$ = 10, $J_{2e,3}$ = 5.1) for **15b**. ^1H NMR data were essentially as described (Corse et al., 1966; Morishita et al., 1984).

Niveusin B (17) and 4,5-Dihydroniveusin A (19). The EIMS and ^1H NMR ($CDCl_3$) data for the sunflower germacranolide angelates **17** (Ohno and Mabry, 1980; Spring et al., 1981, 1982) and **19** (Melek et al., 1985) were as reported.

Insect Bioassays. For consumption bioassays, chemicals were applied to the surface of 1.5-cm disks of a Blue Hubbard squash blossom in 8 μL of a carrier solvent. The flowers of this winter squash variety contain moderate amounts of cucurbitacins, potent, nonfitness reducing feeding stimulants for the *Diabrotica* (Andersen and Metcalf, 1987). This size of disk is totally consumed by two WCR adults within 24 h. Two solvent control and two treated disks are placed on a moist filter paper in a 20 × 100 mm Petri dish with four WCR beetles per dish. Relative consumption after 5, 24, and 48 h is estimated visually using a 0–10 scale (rank 0, no consumption; rank 5, half consumption; rank 10, total consumption). Consumption ratios of treated to solvent control per dish are arcsin transformed, and the significance of differences between mean ratios for compound/dose is determined by Wilcoxon's signed rank test if necessary.

An Alchemy II molecular modeling program (Tripos Associates) was used to test goodness of antifeedant fit to a picrotoxinin-like binding site on a γ-aminobutyric acid (GABA) receptor.

DMSO solutions for two of the more active sesquiterpene antifeedants were injected (up to 10 μg/insect in 200 nL) lateroventrally through the metepimeron suture into the thorax of WCR adults. Parallel injections were made with picrotoxinin (Sigma) and with up to 1 μg of avermectin (gift from Merck, Sharpe & Dohme). Toxicity symptoms were recorded over a 2-day period.

RESULTS AND DISCUSSION

Isolation and Characterization of Rootworm Feeding Deterrents. Rootworm longevity reducing factors were initially explored by two-dimensional TLC (Mullin et al., 1991) whereby a lavender fluorescing compound (366 nm) that accumulates in WCR after long-term feeding (2+ weeks) on sunflower petals was identified within both the plant and insect after isolation on silica gel and acid hydrolysis as *trans*-caffeic acid: UV λ_{max} (ethanol, nm) 291, 322; EIMS *m/z* (% intensity) 180 (M^+, 100), 163 (32), 136 (34), 134 (50), 89 (36); ^1H NMR (DMSO-d_6) δ 6.15 (1 H, d, J = 15.9 Hz), 6.74 (1 H, d), 6.96 (2 H, m), 7.40 (1 H, d, J = 15.9 Hz). This and a gold fluorescing flavonoid on the TLC plate cochromatographing with quercetin indicated that considerable phenolics were being consumed and sequestered from sunflower petals by WCR.

A more systematic fractionation of anti-rootworm factors from floral tissues of sunflower (Figure 2) was then conducted, guided by a squash disk bioassay in which relative consumption by adult rootworm of solvent- or compound-treated flower disks is measured. This bioassay was designed to detect only highly active antifeedants that counteract the potent feeding stimulatory effect of cucurbitacins. Residues from 95% ethanolic extracts of sunflower petals and florets were partitioned between water and, in order, chloroform, ethyl acetate, and 1-butanol. Most of the original antifeedant activity concen-

Table I. Floral Feeding Deterrents for Adult Western Corn Rootworm among Ethyl Acetate Soluble Chemicals from the Sunflower[a]

chemical		rel consumption (treated/control)[b]			
		40 μg/disk		80 μg/disk	
no.	compd by class	5 h	24 h	5 h	24 h
	sesquiterpenes				
1	argophyllin A (+ some 4)	0.32 ± 0.06	0.23 ± 0.04		
2	3-*O*-methylniveusin A	0.62 ± 0.10	0.75 ± 0.08	0.43 ± 0.09	0.30 ± 0.09
3	15-hydroxy-3-dehydrodeoxytifruticin	0.55 ± 0.10	0.97 ± 0.16	0.68 ± 0.36	0.75 ± 0.08
4	1-*O*-methyl-4,5-dihydroniveusin A	0.87 ± 0.09	0.97 ± 0.09	0.63 ± 0.08	0.71 ± 0.02
5	3-oxo-10-hydroxy derivative of 4			0.37 ± 0.07	0.72 ± 0.08
6	10-*O*-methyl ether of 5	0.47 ± 0.08	0.80 ± 0.05	0.49 ± 0.08	0.64 ± 0.02
	diterpenes				
7	kaur-16-en-18-oic acid	1.00 ± 0.21	1.05 ± 0.21	0.77 ± 0.20	0.85 ± 0.13
8	grandifloric acid	1.09 ± 0.17	0.91 ± 0.05	0.66 ± 0.15	0.61 ± 0.14
9	grandifloric acid angelate[c]	1.44 ± 0.27	0.92 ± 0.04	0.23 ± 0.06	0.70 ± 0.13
10	15-hydroxytrachyloban-19-oic			0.49 ± 0.07	0.96 ± 0.03
11	7-oxotrachyloban-19-ol	1.22 ± 0.30	1.00 ± 0.01	0.41 ± 0.04	0.98 ± 0.01
	flavonoids and phenolics				
12	nevadensin			0.55 ± 0.16	0.93 ± 0.06
13	5-hydroxy-4,6,4′-trimethoxyaurone			0.67 ± 0.16	1.01 ± 0.01
14	quercetin β-7-*O*-glucoside[c]		0.77 ± 0.08	0.81 ± 0.09	0.86 ± 0.05
15a,b	3,5- and 3,4-dicaffeoylquinic acid[c]	1.25 ± 0.25	1.65 ± 0.30		0.95 ± 0.16

[a] Dual-choice tests with the squash disk bioassay using 8 μL of solvent or compound solution/1.5 cm flower disk (average weight of 37.9 ± 2.3 mg for 24 disks). [b] Mean ± SEM for four replicates per dose; shown are floral compounds with a significant feeding effect (except 7) on WCR relative to a methanol control ($p < 0.05$ for at least one dose–time combination). [c] Dosages were 50 and 100 μg/disk, respectively, for compound 9, 29 and 114 μg/disk for 14, and 32 and 129 μg/disk for 15.

trated in the ethyl acetate fraction, with the chloroform extract containing much of the remaining activity. Toyopearl TSK HW-40F chromatography of ethyl acetate residues gave three major phenolic components and a number of unbound polar terpenoids (Figure 3). The bulk of the eluted phenolics (20% of ethyl acetate residue) were the faster mobile 3,5-dicaffeoylquinic acid (**15a**) together with the not fully resolved isomer, **15b**. Quercetin β-7-*O*-glucoside (**14**, 7% of ethyl acetate fraction) and monocaffeoyl esters cochromatographing with chlorogenic acid were the next major components of this residue. Only the flavonol acted as an antifeedant for WCR in a squash disk bioassay (Table I). Interestingly, the mixture of 3,5- and 3,4-dicaffeoylquinic acids was stimulatory to rootworm feeding at a low dose (32 μg/disk) but not at a higher dose (129 μg/disk) more representative of the intact flower. The flavonol **14** has been reported in flowers (Sando, 1925; Harborne and Smith, 1978) and pollen (Ohmoto et al., 1986) of *H. annuus*, and **15a** has been identified in sunflower seeds (Mikolajczak et al., 1970).

The unbound terpenoids from Toyopearl (Figure 3) were further purified by silica gel chromatography and identified as the sesquiterpene lactones argophyllin A (**1**) and 3-*O*-methylniveusin A (**2**), the former contaminated with the lesser antifeedant 1-*O*-methyl-4,5-dihydroniveusin A (**4**). These sesquiterpene lactone angelates (SQLA) are greater than 5 times more potent than quercetin 7-β-*O*-glucoside as antifeedants for WCR (Table I). Since the majority of these terpenoids are probably located in trichomes or the epicuticle of sunflowers (Spring et al., 1989), subsequent bulk isolation of these compounds was by surface extraction of sunflower heads by a 2-min solvent immersion. Most of the polar flavonol glucosides and caffeoylquinic acids were not extracted by this method, substantiating their expected intracellular localization, and negated the need for Toyopearl chromatography. Column, flash, and thin-layer silica gel chromatography of ethyl acetate solubles gave more than 65 compounds, which in decreasing order of abundance were primarily diterpenoic acids, SQLA, and methoxylated flavonoids. Indeed, the flower of this annual species of *Helianthus* was quite chemically complex. Thirty-four of these compounds were bioassayed for deterrency to WCR as above. The order of feeding deterrency and persistence of activity (out to 48 h) was sesquiterpenes (seven compounds) ≫ diterpenes (six) > methoxylated flavonoids (four). Bioassay data (Table I) and structures are included here for the more potent antifeedants. Included among these are novel sesquiterpenes **2** and **6**, a diterpene **11**, and a new aurone **13**.

The most potent feeding deterrents included SQLA of the germacranolide type with epoxidation (e.g., **1**) or 4,5-unsaturation (e.g., **2**). For the diterpenoic acids, the 16-kaurene system [e.g., grandifloric acid and its 15-angelate (**8**, **9**)] appeared more active than the trachylobane types (**10**, **11**), while 15-hydroxylation improved antifeedant activity. Grandifloric acid angelate **9**, like the dicaffeoylquinic acids, was stimulatory to WCR after 5 h of feeding at the low dose, but this effect was lost after 1 day. In general, sunflower diterpenoic acids and flavonoids had less residual activity (i.e., good bioactivity up to or beyond 24 h) than the sesquiterpenes.

Identification of 10 germacranolides in extrafloral aerial tissue (primarily leaves) of *H. annuus* has recently been reported by two other laboratories. Melek et al. (1985) have identified **1** as the major component and another epoxide, argophyllin B (**16**), along with niveusin B (**17**) and 4,5-dihydroniveusin A (**19**) as minor components, whereas Spring et al. (1989) have identified **3** and its hemiketal as the major components, and **16**, niveusin C (**18**), **4**, its oxo form **5**, and its anhydro analogue as the minor components. Part of the discrepancy between these studies might be due to cultivar differences since a wild variety was used in the former and var. Giganteus was used in the latter study. In addition to **1**–**6** and **17**, we have also isolated small amounts of **16** and **19** (J.-C. Chou and Mullin, unpublished data). However, cyclic sesquiterpene epoxides similar to the argophyllins (Geissman, 1973; Fischer, 1990) are sensitive to both acid- and base-catalyzed rearrangements that form tetrahydrofurans (e.g., niveusins) and ultimately conjugated systems such as a deoxytifruticin (**3**). Thus, **1** might be dehydrated and the epoxide undergo hydration-rearrangement to form the tetrahydrofuran **18**. Similarly, **16** would result in **19**. Lability of these terpenoids is evident from comparison

Table II. Joint Antifeedant Actions of Sunflower Sesquiterpenes, Diterpenes, and Flavonoids on Adult Western Corn Rootworm[a]

	rel consumption (treated/control)[b]			rel consumption (treated/control)[b]	
compd	5 h	24 h	compd	5 h	24 h
2	0.16 ± 0.05	0.36 ± 0.07	2	0.38 ± 0.08	0.86 ± 0.04
3	0.29 ± 0.11	0.34 ± 0.01	8	0.79 ± 0.05	1.00 ± 0.01
2 + 3	0.24 ± 0.05	0.45 ± 0.06	2 + 8	0.34 ± 0.07	0.72 ± 0.04
2	0.40 ± 0.11	0.86 ± 0.05	2	0.60 ± 0.14	0.79 ± 0.07
4	0.67 ± 0.13	1.00 ± 0.01	9	1.67 ± 0.60	1.07 ± 0.09
2 + 4	0.40 ± 0.06	0.77 ± 0.03	2 + 9	0.83 ± 0.35	0.67 ± 0.12
2	0.57 ± 0.38	0.38 ± 0.05	4	0.53 ± 0.14	0.82 ± 0.05
6	1.03 ± 0.55	0.57 ± 0.13	9	0.94 ± 0.16	0.96 ± 0.05
2 + 6	0.92 ± 0.55	0.32 ± 0.06	4 + 9	0.45 ± 0.08	0.78 ± 0.04
3	0.28 ± 0.09	0.44 ± 0.11	2	0.43 ± 0.16	0.84 ± 0.03
6	0.54 ± 0.18	0.73 ± 0.07	12	0.68 ± 0.18	1.00 ± 0.01
3 + 6	0.27 ± 0.04	0.37 ± 0.08	2 + 12	0.40 ± 0.12	0.78 ± 0.06

[a] Four beetles per dish presented a choice between one disk each of the methanol control, the first chemical, the second chemical, and the combination of the two. Each compound was applied at a 40-μg dose. [b] Mean ± SEM for five replicates.

of freeze-dried leaf to fresh leaf samples where the profile of the predominant six SQLA markedly shifts upon freeze-drying from argophyllin–niveusin types to the conjugated compound 3 (Chou and Mullin, unpublished data). We also found that EIMS by probe of 1 in wet methanol gives an EIMS identical to 18. In addition, niveusin A in acetic anhydride reportedly gives the corresponding diacetate of 3 (Ohno and Mabry, 1980). Sufficient acidity for these reactions may result from co-occurring diterpenoic acids on the plant surface. Thus, the argophyllins or more labile epoxides may be the actual or, at least, predominant forms in which these C-6 lactonized germacranolide angelates are present in sunflower.

Many of the sesquiterpenes were isolated as methoxylated derivatives that may have resulted from the interaction of methanol with precursor epoxides under acid conditions. Since the more abundant diterpenoic acids, probably present with the sesquiterpenes in the same trichomes (Melek et al., 1985; Rogers et al., 1987; Spring et al., 1989), could provide the requisite low pH for these additions–rearrangements, we hypothesized that isolating the terpenoids under buffered conditions in the absence of alcoholic solvents may result in chemistry more representative of the intact plant. A 30-s bulk extraction of 139 freshly cut sunflower inflorescences with a heterogeneous solvent (75% ethyl acetate–25% 50 mM potassium phosphate, pH 8, buffer, 4 by 4 L) gave after silica gel chromatography no 1-methoxy-substituted sesquiterpene lactone angelates. These latter germacranolides, some previously reported from sunflower (Spring et al., 1989), are thus probably artifacts from methanol in the isolation method. Nevertheless, some 2 is present and appears to be synthesized de novo in the plant.

Joint Actions of Sunflower Terpenoids and Phenolics on WCR. Binary combinations between a more potent rootworm feeding deterrent at an active dose with a less antifeedant compound were explored using the squash disk bioassay. Overt synergistic or antagonistic interactions for eight combinations of deterrents within or between the sesquiterpene (2–4, 6), diterpene (8, 9), and flavonoid (12) classes were not noted (Table II). This indicates that the suite of antifeedants present in sunflower inflorescences act in an additive fashion. Nevertheless, a small yet unexplained antagonism seems to occur between sesquiterpenes 2 and 3, suggesting more complex antagonistic effects at SQLA chemoreceptors in WCR.

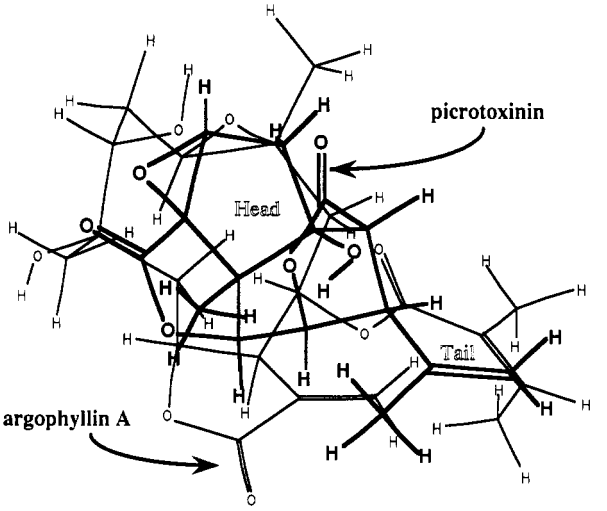

Figure 4. Molecular fit between picrotoxinin and argophyllin A.

Picrotoxinin-like Neurotoxicities of Sesquiterpene Antifeedants in WCR. DMSO solutions of the more active antifeedant sesquiterpenes 2 and 3, when injected into WCR adults at dosages >2.5 μg, gave neurotoxic symptoms at 24 h (excitability, hyperextension of ovipositor, egg expulsion, tarsal tetany) similar to that of picrotoxinin but not like that of avermectin (sluggish movements, paralysis), a GABA agonist. Picrotoxinin, a sesquiterpene epoxide lactone from *Anamirta* spp. and a type A GABA-gated chloride channel antagonist (Klunk et al., 1983; Ozoe et al., 1990), has structural similarities to these *Helianthus* germacranolides (Figure 4) and to another $GABA_A$ convulsant, dieldrin. All have bulky head groups with multiple electronegative atoms and a narrow hydrophobic tail containing an electrophilic site. Hence, all may similarly orientate and bind to the picrotoxinin site at the GABA-dependent chloride channel.

CONCLUSIONS

Diabrotica leaf beetles are major Pan-American pests on corn, of which WCR is the most pestiferous. Rootworm control presently depends largely on organophosphate and carbamate soil insecticides, all of which act on the nerve acetylcholinesterase. Development of broad insecticide insensitivity at this site would impair rootworm control and severely reduce corn production. Moreover, these insecticides have high mammalian toxicity, and applicator exposures and residue toxicities are problems increasingly associated with their use. New safer avenues of chemical control of WCR are needed.

The *Helianthus* genera are remarkable for the breadth of chemicals synthesized, many of which serve as allelochemicals for protection against insect herbivory (Mullin et al., 1991). From cultivated sunflowers, we have identified germacranolide antifeedants and neurotoxicants for adult rootworm, among which the most potent are epoxylactone angelates. These compounds may have direct cross-reactivities with GABA nerve receptors. Populations of WCR, in contrast to the mostly nonsusceptible NCR, are uniformly resistant to aldrin, dieldrin, and other cyclodiene insecticides, all of which are antagonists of $GABA_A$-gated chloride channels. GABA-antagonizing antifeedants may explain both the decreased longevity of WCR and its 7-fold decreased tolerance to aldrin when fed on floral parts of cultivated sunflower in comparison to corn (Siegfried and Mullin, 1989, 1990). Selective

chemical control strategies for rootworm may evolve from their vulnerability to phytochemical antagonism of GABA action.

ABBREVIATIONS USED

EIMS, electron impact mass spectroscopy; GABA, γ-aminobutyric acid; GASPE, gated spin echo; NCR, northern corn rootworm; SQLA, sesquiterpene lactone angelates; WCR, western corn rootworm.

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