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Structure–activity relationship of chemical defenses from the freshwater plant *Micranthemum umbrosum*

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Abstract

Vascular plants produce a variety of molecules of phenylpropanoid biosynthetic origin, including lignoids. Recent investigations indicated that in freshwater plants, some of these natural products function as chemical defenses against generalist consumers such as crayfish. Certain structural features are shared among several of these anti-herbivore compounds, including phenolic, methoxy, methylenedioxy, and lactone functional groups. To test the relative importance of various functional groups in contributing to the feeding deterrence of phenylpropanoid-based natural products, we compared the feeding behavior of crayfish offered artificial diets containing analogs of elemicin (**1**) and β-apopicropodophyllin (**2**), chemical defenses of the freshwater macrophyte *Micranthemum umbrosum*. Both allyl and methoxy moieties of **1** contributed to feeding deterrence. Disruption of the lactone moiety of **2** reduced its deterrence. Finally, feeding assays testing effects of **1** and **2** at multiple concentrations established that these two natural products interact additively in deterring crayfish feeding.

Keywords: *Micranthemum umbrosum*; Scrophulariaceae; Antifeedant; Structure–activity relationship; Chemical defense; Plant–herbivore interaction; Lignan; Elemicin; β-Apopicropodophyllin; *Procambarus acutus*

1. Introduction

Phenylpropanoid-derived natural products, biosynthesized via the shikimate pathway, are widespread among vascular plants (Lewis and Davin, 1999) and have been shown to exhibit antimitotic, antiviral, insect antifeedant, and root growth inhibition properties (Loike and Horwitz, 1976; Elakovich and Stevens, 1985; Gnabre et al., 1995; Harmatha and Nawrot, 2002). Most biological studies related to phenylpropanoid-based natural products have focused on their potential application in medicine and agriculture. A smaller number of ecological studies have indicated that some of these metabolites function as chemical defenses against co-occurring herbivores. Among freshwater macrophytes, 11 shikimate-derived natural products (10 lignoids and one monomeric phenylpropanoid) and one potential shikimate metabolite (a *p*-hydroxybenzyl ester) have been shown to deter herbivory by generalist crayfish (Bolser et al., 1998; Wilson et al., 1999; Kubanek et al., 2000, 2001; Parker et al., 2006), whose feeding behavior can dramatically affect macrophyte distribution and abundance (Lodge, 1991) (Fig. 1a). In these studies, isolation of deterrent natural products was guided by feeding assays such that structurally-related but non-deterrent compounds were not identified. A comparison of structural features for deterrent and non-deterrent metabolites of the same biosynthetic class has therefore not been possible, preventing rigorous analysis of the structural basis for chemical defense.

It is expected that there exists a definable relationship between the structure of phenylpropanoid-based plant natural products and feeding deterrence, when considering a

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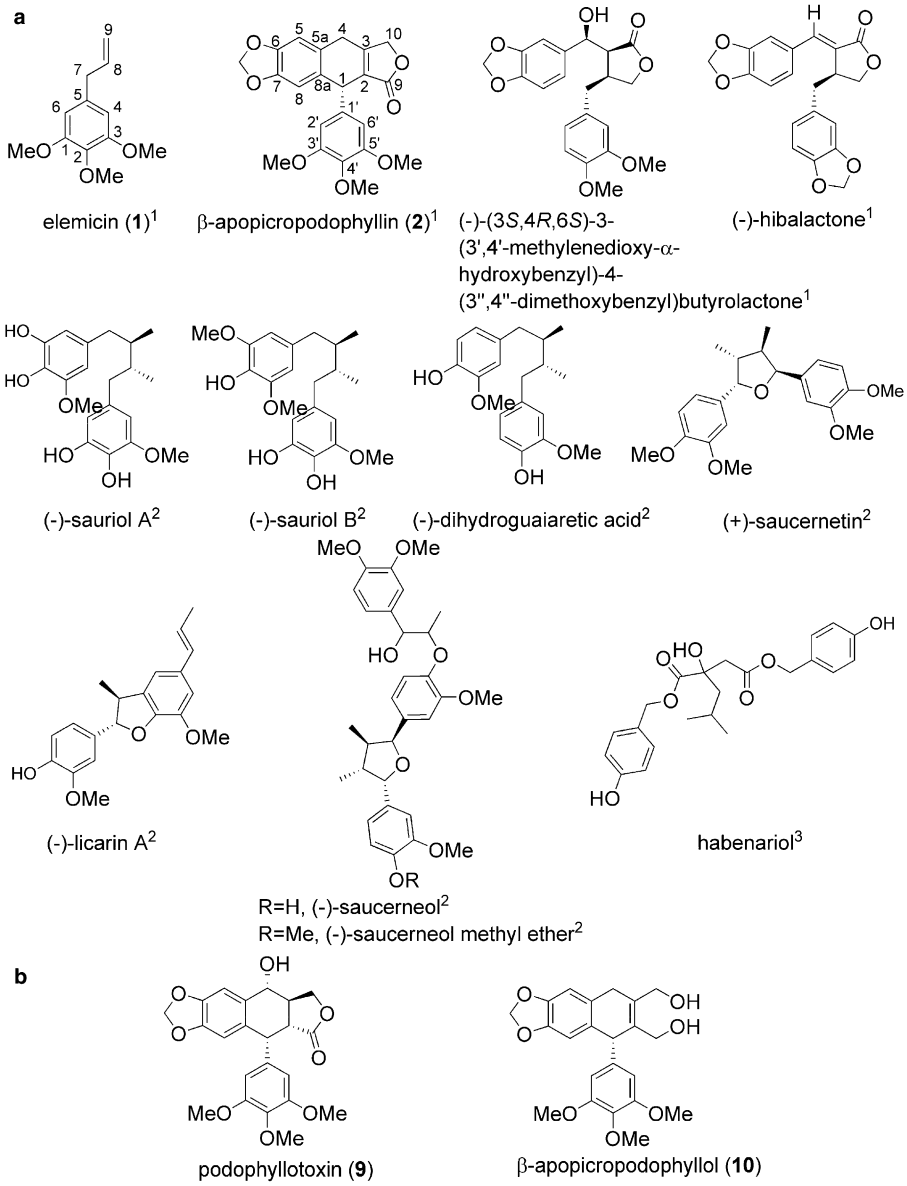

Fig. 1. (a) Freshwater plant shikimate-derived metabolites previously demonstrated to deter crayfish feeding. [1] Parker et al. (2006); [2] Kubanek et al. (2000, 2001); [3] Bolser et al. (1998), Wilson et al. (1999). (b) Analogs of β-apopicropodophyllin (**2**) for which crayfish feeding deterrence was assessed in the current study.

population or species of herbivores. From assessment of a group of deterrent lignoids possessing a common carbon skeleton, Kubanek et al. (2000) suggested that increased aryl hydroxylation may be associated with increased deterrence. By strategic manipulation of the molecular structures of natural products, quantification of biological activities, and statistical analyses to test for differences, insights may be gained into the precise nature of this structure–activity relationship. Such studies have commonly been used to explore the pharmacological (e.g., Lee et al., 1999; Zhang et al., 2004) and agricultural (e.g., Kim and Mullin, 2003; Morimoto et al., 2003) activities of natural products. However, few studies have investigated such relationships in an ecological context (but see Assmann et al., 2000; Lindel et al., 2000; Silva and Trigo, 2002), and no previous studies have evaluated this for phenylpropanoid-derived molecules. Such studies could lead to testable hypotheses regarding mechanisms of chemoreception and other physiological responses. Structure–activity relationship studies may also provide insights into the evolution of chemical defenses: if herbivore pressure is intense and chemical defenses carry significant costs, one might predict that plants evolved pathways to produce the most deterrent compounds, suggesting that natural products should have greater deterrent potency than unnatural, but structurally-related compounds, and that concentrations of chemical defenses within plants are likely to be adequate, but not excessive, for deterring herbivores.

In the current study, we selected elemicin (**1**) and β-apopicropodophyllin (**2**) (Fig. 1a), phenylpropanoid-based

chemical defenses of the freshwater plant *Micranthemum umbrosum* (Parker et al., 2006), as model structures for the evaluation of structure–activity relationships among freshwater plant chemical defenses. We obtained eight analogs of **1** and **2** by semi-synthesis or from commercial sources, and from feeding assay data using the crayfish *Procambarus acutus* we generated dose–response curves to quantify deterrent potency and to predict structural requirements for deterrence. Additionally, we compared the deterrent potencies of **1** and **2**, and evaluated the interaction (additive, antagonistic, or synergistic) between **1** and **2** in the chemical defense of the freshwater plant *M. umbrosum*.

2. Results and discussion

2.1. Feeding deterrence of natural products elemicin (*1*) vs. β-apopicropodophyllin (*2*)

Comparison of $\log EC_{50}$ values for the feeding deterrence of the phenylpropanoid elemicin (**1**) and the lignoid β-apopicropodophyllin (**2**) against the crayfish *Procambarus acutus* indicated that **2** is approximately 750 times more deterrent than **1** ($p < 0.0001$; Fig. 2). Consistent with this finding, in an investigation of the insect feeding deterrence activity of compounds belonging to these two structural groups, Harmatha and Nawrot (2002) reported that lignoids were generally more bioactive than phenylpropanoid monomers.

According to the optimal defense theory (Rhoades and Cates, 1976), if natural products evolved to fulfill a specific ecological function such as chemical defense, one would expect natural concentrations of these defenses to approximately match the sensitivity of potential consumers, in order to minimize costs associated with chemical defense. The experimental EC_{50} for elemicin (**1**), 8.3 mM (Fig. 2), was found to be similar to its natural concentration of 3.2 mM (Parker et al., 2006). In contrast, the natural concentration of β-apopicropodophyllin (**2**), 0.96 mM (Parker et al., 2006), was nearly 100 times greater than its experimental EC_{50} value, 0.011 mM (Fig. 2). These data suggest that **2** is more important than **1** in deterring crayfish from feeding on *M. umbrosum*, and may indicate that both metabolites serve multiple ecological functions, as natural concentrations of the combined compounds are greater than that required for feeding deterrence, or that metabolite concentrations are not optimally tuned to herbivore sensitivity.

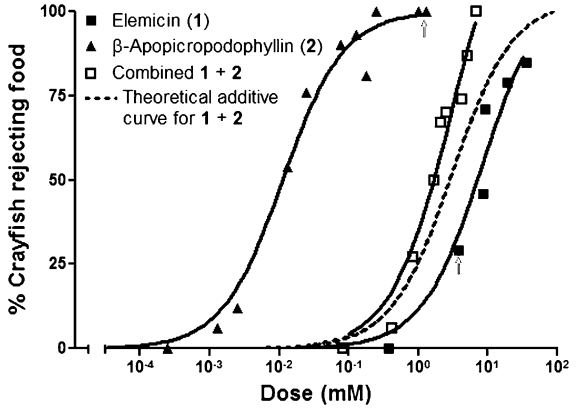

Fig. 2. Effect of compound concentration on crayfish feeding behavior for *M. umbrosum* natural products elemicin (**1**) and β-apopicropodophyllin (**2**), and effect of combined doses of **1** and **2**; $n = 13$–24 crayfish for each data point. Grey arrows denote natural concentrations of **1** and **2** in *M. umbrosum* from Parker et al. (2006). In determining the effect of combined doses, **1** and **2** were added to artificial diets in 1:1 molar ratios of the EC_{50} values for each compound. The theoretical additive curve was calculated from best fit dose–response curves developed individually for **1** and **2**.

2.2. Interaction between elemicin (*1*) and β-apopicropodophyllin (*2*) in the chemical defense of Micranthemum umbrosum

Previous studies have indicated that some phenylpropanoids and lignoids interact synergistically in the deterrence of agricultural pests (Yamashita and Matsui, 1961). Alternatively, antagonistic or additive effects could occur. When **1** and **2** were investigated for combined effects in deterring crayfish feeding, the experimental $\log EC_{50}$ value for the combined compounds was not significantly different from the value obtained from a theoretical additive curve (F test, $p = 0.73$; Fig. 2), leading us to reject the hypothesis that these two natural products behave synergistically or antagonistically in deterring crayfish feeding.

2.3. Structure–activity relationship of elemicin (*1*) analogs

At 12× the natural molar concentration of elemicin (**1**), allylbenzene (**3**) was not significantly deterrent to crayfish (Fisher's exact test, $p > 0.99$; Fig. 3), indicating that substituents on the allylbenzene scaffold are necessary for feeding deterrence. In contrast, dimethoxy-substituted methyl eugenol (**4**) was the most effective deterrent of compounds tested within this group, with an EC_{50} value 87% less than that of **1** (F test, $p < 0.001$; Fig. 3). The greater potency of **4** relative to trimethoxy-substituted **1** indicates that the third methoxy substituent on the phenyl ring of **1** undermines deterrence, which may indicate that steric hindrance of the bulkier **1** alters the interaction with a *P. acutus* chemoreceptor. Although crustacean receptors for detecting plant chemical defenses have not yet been identified, it seems likely that taste or odorant chemoreceptors are involved, given the quick response time (seconds) and lack of apparent injury to crayfish rejecting chemically-defended foods which might be expected if a deterrent caused a non-receptor-mediated effect such as burning (Lane and Kubanek, pers. observ.). The enhanced activity (relative to **1**) of dimethoxy-substituted **4**, a metabolite previously isolated from other vascular plants (e.g., Mata et al., 2004), also indicates that *M. umbrosum* has not evolved to produce the metabolite most deterrent to this particular herbivore.

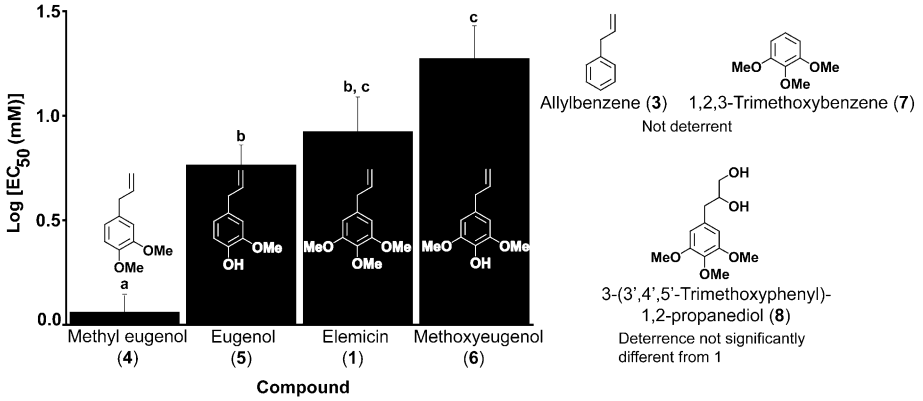

Fig. 3. Comparison of crayfish feeding deterrence of elemicin (**1**) and analogs. Different letters indicate treatments differing significantly from each other (F test, $p \leqslant 0.05$); bars represent standard error. Replacement of aryl methoxy groups with hydroxy groups resulted in decreased potency, as seen in **4** vs. **5** and **1** vs. **6**. Increased aryl substitution was also associated with decreased activity (**4** vs. **1**; **5** vs. **6**), except that **3**, with only an allyl substituent, was completely inactive. Dose–response curves were used to calculate EC_{50} values for **1** (Fig. 2) and **4**–**6** (data not shown). For **3** and **7**, EC_{50} values could not be calculated because these compounds were not deterrent at any concentration tested (see text). For **8**, an EC_{50} value could not be calculated because low synthetic yield prohibited testing at sufficient concentrations (see text).

In addition to the number of aryl methoxy groups affecting deterrence, the identity of substituents also appeared to play an important role. The presence of a hydroxy group *para* to the allyl substituent as in eugenol (**5**) and methoxyeugenol (**6**), instead of a methoxy group as in elemicin (**1**) and methyl eugenol (**4**), was associated with substantially reduced deterrent potency for trisubstituted benzenes **5** vs. **4** (F test, $p < 0.001$; Fig. 3), and marginally reduced deterrent potency for tetrasubstituted benzenes **6** vs. **1** ($p = 0.17$; Fig. 3). Both hydroxy and methoxy substituents may function as hydrogen bond acceptors in interactions with crayfish chemoreceptors, whereas a hydroxy substituent can also act as a hydrogen bond donor, which could potentially affect deterrent potency by altering the orientation in which a ligand interacts with a chemoreceptor. Alternatively, the bulkier, more hydrophobic methoxy substituent in the *para* position of **4** may enhance deterrence (relative to **5**) via a better steric fit and/or stronger van der Waals attractive forces within the chemoreceptor binding site. The current finding, that aryl hydroxy groups are associated with weaker crayfish deterrence than are aryl methoxy groups among monomeric phenylpropanoids, is contrary to the suggestion of Kubanek et al. (2000) regarding the crayfish deterrence among a group of lignans.

Eugenol (**5**) was previously demonstrated to cause paralysis in crayfish placed in an aqueous solution of this compound (Ozeki, 1975). Given the structural similarity of elemicin (**1**) and **5**, it is possible that **1** may also be toxic at certain concentrations; however, we did not observe incapacitation or mortality of *P. acutus* during or after feeding assays. This may indicate that toxicity is diminished when consumed as part of a diet rather than absorbed from surrounding water. If **1** or **5** is indeed toxic at high doses or following repeated consumption of plants containing these metabolites, this may have favored evolution of chemoreception in consumers such as *P. acutus*, in order to identify and avoid consuming toxic foods.

Supporting the hypothesis that the allyl group at C-5 of elemicin (**1**) is important in feeding deterrence, 1,2,3-trimethoxybenzene (**7**) was palatable at 38 mM, 12× the natural molar concentration of **1** (Fisher's exact test $p = 0.23$). 3-(3′,4′,5′-Trimethoxyphenyl)-1,2-propanediol (**8**), synthesized from **1**, was significantly deterrent at the three concentrations at which it was tested, spanning 2.9–8.4 mM (Fisher's exact test, $p = 0.0006$–0.05). Due to limited yield of synthetic product, a full dose–response curve could not be established for **8**. However, comparison of the deterrence of **1** and **8** at 3.8 mM indicated that potency did not appear to differ significantly between these two compounds (Fisher's exact test, $p > 0.99$). Thus, conversion of the allyl substituent to a diol did not appear to reduce bioactivity, despite the enhanced bulkiness, polarity, and hydrogen bonding capacity of this group relative to the allyl substituent of **1**. Although the lack of deterrence of trimethoxybenzene (**7**) indicated that a non-hydrogen substituent was essential at C-5 for deterrence, it appears that the *P. acutus* chemoreceptor for **1** is not highly specific for the C-5 substituent.

2.4. Structure–activity relationship of β-apopicropodophyllin (2) analogs

A number of structural features may be expected to influence the bioactivity of β-apopicropodophyllin (**2**), including the unsaturated lactone, trimethoxyphenyl group, methylenedioxy moiety, and stereochemistry at C-1. We focused on the role of the lactone moiety in crayfish feeding behavior, as this group was most amenable to synthetic modification and has been demonstrated to affect bioactivity in pharmacological studies of analogous lignoids including podophyllotoxin (**9**) (Loike and Horwitz, 1976; Brewer et al., 1979).

Podophyllotoxin (**9**) was marginally less deterrent than β-apopicropodophyllin (**2**) (F test, $p = 0.07$; Table 1),

Table 1
Comparison of crayfish feeding deterrence for β-apopicropodophyllin (**2**) and analogs

Analog	log[EC_{50} (mM)] ± SE	EC_{50} (mM)
β-Apopicropodophyllin (**2**)	-1.95 ± 0.08[a]	0.011
Podophyllotoxin (**9**)	-1.70 ± 0.07[a]	0.020
β-Apopicropodophyllol (**10**)	(Non-deterrent at all concentrations tested)	

Dose–response curves were used to calculate EC_{50} values for **2** (Fig. 2) and **9** (data not shown).
[a] logEC_{50} values for **2** and **9** differ marginally (F test, $p = 0.07$).

which may have resulted from different lactone conformations, from the lack of the C-2–C-3 unsaturation in **9**, or from the presence of a hydroxy at C-4 in **9**. Whereas **2** was significantly deterrent at 1% of its natural molar concentration (Fisher's exact test, $p = 0.001$) and nearly 100% deterrent at its natural concentration ($p < 0.0001$; Fig. 2), disruption of the lactone by reduction to the diol β-apopicropodophyllol (**10**) resulted in a complete lack of deterrence for **10** at either of these concentrations ($p > 0.99$ for both concentrations); **10** was significantly less potent than **2** (Fisher's exact test, $p < 0.001$ for comparison of deterrence of **2** and **10** at both concentrations). Feeding assays at higher concentrations were not feasible for **10**, due to insufficient synthetic product yields. This conversion of the lactone ring in **2** to a diol moiety in **10**, while retaining the C2–C3 unsaturation, may have resulted in loss of activity by a diminished capacity of the more polar diol to bind with hydrophobic regions of the chemoreceptor, or by increased conformational flexibility which could disrupt orientation of hydrogen bonding or dipole–dipole interacting groups between a receptor and ligand.

The role of the methylenedioxy moiety of β-apopicropodophyllin (**2**) in crayfish feeding deterrence remains unclear, as attempts at selective synthetic modification of this group were unsuccessful (data not shown). Kubanek et al. (2000) reported a series of seven antifeedant lignoids that did not possess methylenedioxy groups, indicating that these groups are not essential for crayfish feeding deterrence among lignoids (although different, albeit congeneric, species of crayfish were used in Kubanek et al. (2000) vs. the current study). In contrast, methylenedioxy moieties have been suggested to be important in the effectiveness of insect feeding deterrents (Harmatha and Nawrot, 2002).

2.5. Conclusions

Previous investigations aimed at elucidating the molecular structural requirements for ecological function have made assessments on the basis of one or a few feeding assay data points (e.g., Assmann et al., 2000; Kubanek et al., 2000; Lindel et al., 2000), making it difficult to quantitatively compare the sensitivity of consumers to chemical cues in their food. To our knowledge, this study marks the first aquatic chemical ecology investigation in which structure–activity relationships were evaluated through the use of dose–response curves and represents the first investigation of the structure–activity relationship of plant feeding deterrents against a freshwater herbivore.

Comparison of dose–response curves for two *M. umbrosum* natural products, elemicin (**1**) and β-apopicropodophyllin (**2**), and eight analogs (**3**–**10**) of these natural products indicated that the allyl and methoxy moieties of **1** influenced crayfish feeding behavior, as did the lactone moiety of **2**. The 12-member collection of natural products previously reported to deter crayfish feeding includes some molecules with none of these functionalities (Wilson et al., 1999; Kubanek et al., 2000), suggesting that several different chemoreceptive mechanisms are likely involved in crayfish feeding deterrence and/or that crayfish species or populations differ in their responses. Studies of interactions between these molecules and individual herbivore chemoreceptors and manipulation of chemoreception physiology will be necessary to develop further understanding regarding receptor–ligand interactions involving chemical defense.

3. Experimental

3.1. General experimental procedures

Fisher Scientific ACS grade solvents were used for extractions, flash CC, and chemical transformations; Fisher Scientific HPLC grade solvents were used for HPLC. Compounds **3**–**7**, **9**, and chemicals for synthetic modifications were purchased from Sigma–Aldrich (St. Louis, MO, USA). HPLC analyses were conducted with Zorbax RX-SIL (Agilent Technologies) semi-preparative normal phase columns using a Waters HPLC system (Waters 515 pump; Waters 2487 dual wavelength absorbance detector) with UV absorbance monitored at 220 and 254 nm. ^{1}H, ^{13}C, and two-dimensional inverse-detected NMR spectroscopic data (COSY, HMQC, HMBC) were acquired with a Bruker DRX-500 MHz spectrometer. All NMR experiments were conducted using $CDCl_3$ and referenced to residual $CHCl_3$ (7.24 and 77.0 ppm, for ^{1}H and ^{13}C, respectively). High and low resolution electron impact (EI) mass spectra were collected with a VG Instruments 70SE spectrometer.

3.2. Isolation of plant chemical defenses (*1*–*2*)

Whole *M. umbrosum* plants were collected in June, 2003, from ponds at the Owens and Williams Fish Hatchery (Hawkinsville, GA, USA) and were frozen until extraction. A voucher specimen GIT-MICR-001 is stored at the Georgia Institute of Technology. Plant material was shredded in MeOH and extracted successively with MeOH (2×), Me_2CO (2×), and CH_2Cl_2 (2×). Extracts were combined, filtered, and concentrated in vacuo. This crude extract was partitioned between petroleum ether and MeOH/H_2O (9:1); the MeOH/H_2O (9:1) portion was further partitioned

between MeOH/H_2O (3:2) and $CHCl_3$. TLC R_f values of authentic samples of **1** and **2** were compared with values for compounds in liquid–liquid partition fractions, in order to identify fractions containing these natural products.

β-Apopicropodophyllin (**2**) was found exclusively in the $CHCl_3$ fraction, and so this fraction was further separated by flash CC on silica with gradient elution of hexanes/EtOAc (9:1) to EtOAc. Finally, **2** (23 mg) was purified by normal phase silica HPLC with CH_2Cl_2/Me_2CO (49:1) as the mobile phase. Elemicin (**1**) was found in both the $CHCl_3$ and hexanes fractions following liquid–liquid partitioning. Both fractions were separated by flash CC as described above for **2**, except that the hexane-soluble portion was subjected to a gradient mobile phase of hexanes to EtOAc. Finally, **1** (48 mg) was purified by normal phase silica HPLC using a hexanes/EtOAc (49:1) mobile phase. The structures of **1** and **2** were determined by spectroscopic analysis and verified by comparison with lit. values (Gensler et al., 1971; Achenbach and Frey, 1992). Elemicin (**1**): ^{1}H NMR (500 MHz, $CDCl_3$): δ 6.41 (2H, *s*, H-4 and H-6), 5.95 (1H, *m*, H-8), 5.12 (1H, *m*, H-9), 5.08 (1H, *m*, H-9), 3.85 (6H, *s*, OMe-1 and -3), 3.82 (3H, *s*, OMe-2), 3.32 (2H, *d*, $J = 6.5$ Hz, H-7). ^{13}C NMR (125 MHz, $CDCl_3$): δ 153.1 (C-1 and C-3), 137.2 (C-8), 136.2 (C-5), 135.8 (C-2), 116.0 (C-9), 105.3 (C-4 and C-6), 60.8 (OCH_3-2), 56.1 (OCH_3-1,3), 40.5 (C-7). EI (*m*/*z*): [M^+] calculated for $C_{12}H_{16}O_3$, 208.10994; found 208.10856. β-Apopicropodophyllin (**2**): ^{1}H-NMR (500 MHz, $CDCl_3$): δ 6.70 (1H, *s*, H-5), 6.62 (1H, *s*, H-8), 6.39 (2H, *s*, H-2′ and H-6′), 5.93 (1H, *d*, $J = 4.5$ Hz, OCH_2O), 5.90 (1H, *d*, $J = 4.5$ Hz, OCH_2O), 4.87 (1H, *m*, H-10,), 4.82 (1H, *m*, H-1), 4.80 (1H, *m*, H-10), 3.82 (1H, *dd*, $J = 2.5$, 27.9 Hz, H-4), 3.79 (3H, *s*, OMe-4′), 3.78 (6H, *s*, OMe-3′,5′), 3.64 (1H, *dd*, $J = 3.5$, 27.9 Hz, H-4). ^{13}C NMR (125 MHz, $CDCl_3$): δ 172.2 (C-9), 157.3 (C-3), 153.2 (C-3′ and C-5′), 147.2 (C-4′), 147.0 (C-6), 138.6 (C-7), 136.9 (C-1′), 129.6 (C-5a), 128.1 (C-2), 123.7 (C-8a), 109.5 (C-8), 107.7 (C-5), 105.5 (C-2′,6′), 101.3 (OCH_2O), 71.0 (C-10), 60.7 (OCH_3-4′), 56.1 (OCH_3-3′,5′), 42.7 (C-1), 29.2 (C-4). EI (*m*/*z*): [M^+] calculated for $C_{22}H_{20}O_7$, 396.12090; found 396.12071.

3.3. Oxidation of elemicin (*1*) to 3-(3′,4′,5′-trimethoxyphenyl)-1,2-propanediol (*8*)

A solution of $KMnO_4$ (0.050 mmol) in deionized H_2O (780 μl) was added to an ice-bath cooled solution of **1** (0.070 mmol) in EtOH/H_2O (2:1) and stirred for 3 min. The reaction mixture was filtered and then partitioned into Et_2O and aqueous layers; TLC indicated presence of the diol product in the aqueous layer. The aqueous layer was extracted with *n*-BuOH (2 × 15 ml). The *n*-BuOH extract was concentrated in vacuo and **8** was purified by normal phase silica HPLC with a mobile phase of hexanes/EtOAc (3:7). The structure of **8** (0.011 mmol; 20% yield) was determined by spectroscopic analysis and verified by comparison with previous data (Dong et al., 1989; Gonzalez et al., 1991). ^{1}H-NMR (500 MHz, $CDCl_3$): δ 6.43 (2H, *s*, H-2′ and H-6′), 3.94 (1H, *m*, H-2), 3.84 (6H, *s*, OMe-3′ and -5′), 3.81 (3H, *s*, OMe-4′), 3.70 (1H, *dd*, $J = 11.5$, 2.5 Hz, H-1), 3.49 (1H, *dd*, $J = 11.5$, 6.5 Hz, H-1), 2.74 (1H, *dd*, $J = 13.5$, 4.3 Hz, H-3); 2.67 (1H, *dd*, $J = 13.5$, 8.7 Hz, H-3). ^{13}C-NMR (125 MHz, $CDCl_3$): δ 53.6 (C-3′ and C-5′), 137.0 (C-1′), 133.6 (C-4′), 106.4 (C-2′ and C-6′), 73.2 (C-2), 66.4 (C-1), 61.1 (OMe-4′), 56.4 (OMe-3′ and -5′), 40.4 (C-3). EI (*m*/*z*): [M^+] calculated for $C_{12}H_{18}O_5$, 242.11542; found 242.11564.

3.4. Reduction of β-apopicropodophyllin (*2*) to β-apopicropodophyllol (*10*)

A solution of **2** (0.010 mmol) in CH_2Cl_2 (100 μl) was added to dry Et_2O (5 ml) suspension of $LiAlH_4$ (0.025 mmol) and stirred 15 h at room temperature (Anjanamurthy and Rai, 1987). Aqueous HCl (2 M, 15 ml) were then added, and the mixture was stirred for 30 min. The Et_2O layer was washed with deionized H_2O (2 × 15 ml) and dried over dry Na_2SO_4. Et_2O was removed in vacuo, and **10** was purified as a white powder by normal phase silica HPLC with CH_2Cl_2/EtOAc (7:3) as mobile phase. Compound **10** (0.0047 mmol, 47% yield) was identified by spectroscopic analysis and comparison with published data (Anjanamurthy and Rai, 1987). ^{1}H-NMR (500 MHz, $CDCl_3$): δ 6.63 (1H, *s*, H-5), 6.51 (1H, *s*, H-8), 6.38 (2H, *s*, H-2′ and H-6′), 5.88 (1H, *d*, $J = 1.5$ Hz, OCH_2O), 5.84 (1H, *d*, $J = 1.5$ Hz, OCH_2O), 4.53 (1H, *m*, H-1), 4.33 (2H, *m*, H-10), 4.26 (1H, *dd*, $J = 12.0$, 2.5 Hz, H-9), 4.10 (1H, *dd*, $J = 12.0$, 7.8 Hz, H-9), 3.78 (6H, *s*, OMe-3′ and -5′), 3.76 (3H, *s*, OMe-4′), 3.72 (1H, *dd*, $J = 21.5$, 4.2 Hz, H-4), 3.53 (1H, *dd*, $J = 21.5$, 3.6 Hz, H-4). ^{13}C-NMR (125 MHz, $CDCl_3$): δ 53.2 (C-3′ and C-5′), 146.1–146.0 (C-4′ and C-6), 140.5 (C-7), 136.4 (C-1′), 135.3 (C-2), 134.1 (C-3), 130.2 (C-5a), 125.4 (C-8a), 107.8 (C-8), 107.1 (C-5), 104.7 (C-2′ and C-6′), 100.5 (OCH_2O), 62.4 (C-10), 61.0 (C-9), 60.6 (OMe-4′), 55.8 (OMe-3′ and -5′), 50.0 (C-1), 33.6 (C-4). EI (*m*/*z*): [M^+] calculated for $C_{22}H_{24}O_7$, 400.15220; found 400.15173.

3.5. Bioassays and statistical methods

Feeding assays were conducted using the omnivorous crayfish, *P. acutus*, collected from the wetlands in the Chattahoochee National Recreation Area (Atlanta, GA, USA) in 2003 and 2004, and identified by comparison of morphological traits (Hobbs, 1981). Crayfish were housed individually in 12.5 × 12.5 cm chambers of a recirculating water table at 23 °C and maintained on a diet of commercial trout food pellets.

Assays were completed following procedures described in Parker et al. (2006). Artificial food for each feeding assay was prepared by suspending 100 mg of a 1:1 mixture of freeze-dried, ground broccoli and lettuce in Me_2CO, to which was added the test compound (natural product or synthetic analog) dissolved in Me_2CO. The mixture was shaken and the Me_2CO removed by rotary evaporation.

This test food powder was then mixed with sodium alginate (30 mg) in 1 ml of deionized H_2O, and dispensed through a syringe into a 0.10 M aqueous solution of $CaCl_2$. Test food was allowed to solidify in this solution for 1 min, then rinsed with deionized H_2O, and cut into test food pellets ca. 3 mm in length. Test compound concentrations were recorded as millimoles of compound per ml of food mixture. Control food pellets were prepared in the same way, including the use of Me_2CO as solvent, but without the addition of test compounds. Feeding assays were conducted by first feeding a crayfish a control food pellet to confirm that the crayfish was not already satiated, and if that control pellet was consumed, then offering the crayfish a test food pellet. If the crayfish consumed the test food pellet, it was considered accepted. Test food pellets were considered rejected if a crayfish took the pellet into its mouth cavity twice and rejected it each time, in which case, a second control pellet was offered to verify the crayfish did not reject the test food pellet due to satiation. Feeding deterrence was recorded as the frequency of 14–23 crayfish rejecting a test food pellet, but accepting both control pellets; crayfish that refused control pellets were not included in the analysis. A Fisher's exact test was applied to test for significance of feeding deterrence data for each compound at individual concentrations. A Fisher's exact test was also used to compare deterrence of two different compounds at equal molar concentrations (Zar, 1998).

Dose–response curves were constructed using data from 5–11 feeding assays for each test compound, by plotting the frequency of crayfish rejecting a test food pellet against the log of the concentration of the test compound in food pellets. These data were fit to a sigmoidal dose–response curve with a Hill slope of 1; this Hill slope provided the largest R^2 goodness-of-fit value for all data sets. Differences among dose–response curves for different test compounds were analyzed by an F test of the $\log EC_{50}$ values for each compound using GraphPad Prism version 4 (Motulsky, 1995). For compounds which were not significantly deterrent at any tested concentration, the $\log EC_{50}$ could not be calculated. The highest concentration tested was 38 mM (12× the natural concentration of **1**) for analogs of **1**, and 1.3 mM (1.4× the natural concentration of **2**) for analogs of **2**. One compound, 3-(3′,4′,5′-trimethoxyphenyl)-1,2-propanediol (**8**) was synthesized in limited yield and so was tested only up to the EC_{50} of **1** (8.3 mM).

The interaction of natural products **1** and **2** in affecting crayfish feeding behavior was assessed using feeding assays incorporating these compounds at nine different concentrations representing 1:1 ratios of experimentally determined EC_{50} values for the two compounds (Luszczki and Czuczwar, 2003). The resulting feeding response curve was compared to a theoretical additive curve, developed on the basis of best fit dose response curves developed individually for **1** and **2** (Tallarida et al., 1997). An F test was applied to test for significant difference between $\log EC_{50}$ values associated with the theoretical additive curve vs. the observed plot (using GraphPad Prism version 4).

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References

Achenbach, H., Frey, D., 1992. Cycloartanes and other terpenoids and phenylpropanoids from *Monocylanthus vignei*. Phytochemistry 31, 4263–4274.

Anjanamurthy, C., Rai, K.M.L., 1987. Synthesis of podophyllotoxin and related analogs. Part 3. Synthesis of β-apopicropodophyllin analogues with modified lactone ring. Indian J. Chem. 26B, 131–135.

Assmann, M., Lichte, E., Pawlik, J.R., Kock, M., 2000. Chemical defenses of the Caribbean sponges *Agelas wiedenmayeri* and *Agelas conifera*. Mar. Ecol. Prog. Ser. 207, 255–262.

Bolser, R.C., Hay, M.E., Lindquist, N., Fenical, W., Wilson, D., 1998. Chemical defenses of freshwater macrophytes against crayfish herbivory. J. Chem. Ecol. 24, 1639–1658.

Brewer, C.F., Loike, J.D., Horwitz, S.B., Sternlicht, H., Gensler, W.J., 1979. Conformational analysis of podophyllotoxin and its congeners. Structure–activity relationship in microtubule assembly. J. Med. Chem. 22, 215–221.

Dong, X., Mondranondra, I.O., Che, C.T., Fong, H.H.S., Farnsworth, N.R., 1989. Kmeriol and other aromatic constituents of *Kmeria duperreana*. Pharm. Res. 6, 637–640.

Elakovich, S.D., Stevens, K.L., 1985. Phytotoxic properties of nordihydroguaiaretic acid, a lignan from *Larrea tridentata* (Creosote bush). J. Chem. Ecol. 11, 27–33.

Gensler, W.J., Ahmed, Q.A., Muljiani, Z., Gatsonis, C.D., 1971. Ultraviolet irradiation of α-apopicropodophyllin. J. Am. Chem. Soc. 93, 2515–2522.

Gnabre, J.N., Brady, J.N., Clanton, D.J., Ito, Y., Dittmer, J., Bates, R.B., Huang, R.C.C., 1995. Inhibition of human immunodeficiency virus type 1 transcription and replication by DNA sequence-selective plant lignans. Proc. Natl. Acad. Sci. USA 92, 11239–11243.

Gonzalez, A.G., Barrera, J.B., Arancibia, L., Diaz, J.G., De Paz, P.P., 1991. Two phenylpropanoids from *Todaroa aurea* subsp. *suaveolens*. Phytochemistry 30, 4189–4190.

Harmatha, J., Nawrot, J., 2002. Insect feeding deterrent activity of lignans and related phenylpropanoids with a methylenedioxyphenyl (piperonyl) structure moiety. Entomol. Exp. Appl. 104, 51–60.

Hobbs, H.H., 1981. The Crayfishes of Georgia. Smithsonian Institution Press, Washington, DC.

Kim, J.H., Mullin, C.A., 2003. Antifeedant effects of proteinase inhibitors on feeding behaviors of adult western corn rootworm (*Diabrotica virgifera virgifera*). J. Chem. Ecol. 29, 795–810.

Kubanek, J., Fenical, W., Hay, M.E., Brown, P.J., Lindquist, N., 2000. Two antifeedant lignans from the freshwater macrophyte *Saururus cernuus*. Phytochemistry 54, 281–287.

Kubanek, J., Hay, M.E., Brown, P.J., Lindquist, N., Fenical, W., 2001. Lignoid chemical defenses in the freshwater macrophyte *Saururus cernuus*. Chemoecology 11, 1–8.

Lee, I.S., Jung, K.Y., Oh, S.R., Park, S.H., Ahn, K.S., Lee, H.K., 1999. Structure–activity relationships of lignans from *Schisandra*

chinensis as platelet activating factor antagonists. Biol. Pharm. Bull. 22, 265–267.

Lewis, N.G., Davin, L.B., 1999. Lignans: biosynthesis and function. In: Barton, D.H.R., Sir, Nakanishi, K., Meth-Cohn, O. (Eds.), Comprehensive Natural Products Chemistry, vol. 1. Elsevier, Amsterdam, pp. 639–712.

Lindel, T., Hoffmann, H., Hochgurtel, M., Pawlik, J.R., 2000. Structure–activity relationship of inhibition of fish feeding by sponge-derived and synthetic pyrrole-imidazole alkaloids. J. Chem. Ecol. 26, 1477–1496.

Lodge, D.M., 1991. Herbivory on fresh water macrophytes. Aquatic Bot. 41, 195–224.

Loike, J.D., Horwitz, S.B., 1976. Effects of podophyllotoxin and VP-16-213 on microtubule assembly in vitro and nucleoside transport in HeLa-cells. Biochemistry 15, 5435–5443.

Luszczki, J.J., Czuczwar, S.J., 2003. Isobolographic and subthreshold methods in the detection of interactions between oxcarbazepine and conventional antiepileptics – a comparative study. Epilepsy Res. 56, 27–42.

Mata, R., Morales, I., Perez, O., Rivero-Cruz, I., Acevedo, L., Enriquez-Mendoza, I., Bye, R., Franzblau, S., Timmermann, B., 2004. Antimycobacterial compounds from *Piper sanctum*. J. Nat. Prod. 67, 1961–1968.

Morimoto, M., Tanimoto, K., Nakano, S., Ozaki, T., Nakano, A., Komai, K., 2003. Insect antifeedant activity of flavones and chromones against *Spodoptera litura*. J. Agric. Food Chem. 51, 389–393.

Motulsky, H., 1995. Intuitive Biostatistics. Oxford University Press, Oxford.

Ozeki, M., 1975. Effects of eugenol on nerve and muscle in crayfish. Comp. Biochem. Physiol. 50C, 183–191.

Parker, J.D., Collins, D.O., Kubanek, J., Sullards, M.C., Bostwick, D., Hay, M.E., 2006. Chemical defenses promote persistence of the aquatic plant *Micranthemum umbrosum*. J. Chem. Ecol. 32, 815–833.

Rhoades, D.F., Cates, R.G., 1976. Toward a general theory of plant antiherbivore chemistry. In: Wallace, J.W., Nansel, R.L. (Eds.), Recent Advances in Phytochemistry, vol. 10. Plenum Press, NY, pp. 168–213.

Silva, K.L., Trigo, J.R., 2002. Structure–activity relationships of pyrrolizidine alkaloids in insect chemical defense against the orb-weaving spider *Nephila clavipes*. J. Chem. Ecol. 28, 657–668.

Tallarida, R.J., Kimmel, H.L., Holtzman, S.G., 1997. Theory and statistics of detecting synergism between two active drugs: cocaine and buprenorphine. Psychopharmacology 133, 378–382.

Wilson, D.M., Fenical, W., Hay, M., Lindquist, N., Bolser, R., 1999. Habenariol, a freshwater feeding deterrent from the aquatic orchid *Habenaria repens* (Orchidaceae). Phytochemistry 50, 1333–1336.

Yamashita, K., Matsui, M., 1961. Studies on phenolic lactones. Synergistic activities of phenolic lactones. Agric. Biol. Chem. 25, 141–143.

Zar, J.H., 1998. Biostatistical Analysis. Prentice Hall, Englewood Cliffs, NJ.

Zhang, H.Z., Kasibhatla, S., Wang, Y., Herich, J., Guastella, J., Tseng, B., Drewe, J., Cai, S.X., 2004. Discovery, characterization, and SAR of gambogic acid as a potent apoptosis inducer by a HTS assay. Bioorg. Med. Chem. 12, 309–317.