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Inter- and Intraspecific Comparisons of Antiherbivore Defenses in Three Species of Rainforest Understory Shrubs

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Abstract Plants defend themselves against herbivores and pathogens with a suite of morphological, phenological, biochemical, and biotic defenses, each of which is presumably costly. The best studied are allocation costs that involve trade-offs in investment of resources to defense versus other plant functions. Decreases in growth or reproductive effort are the costs most often associated with antiherbivore defenses, but trade-offs among different defenses may also occur within a single plant species. We examined trade-offs among defenses in closely related tropical rain forest shrubs (*Piper cenocladum*, *P. imperiale*, and *P. melanocladum*) that possess different combinations of three types of defense: ant mutualists, secondary compounds, and leaf toughness. We also examined the effectiveness of different defenses and suites of defenses against the most abundant generalist and specialist *Piper* herbivores. For all species examined, leaf toughness was the most effective defense, with the toughest species, *P. melanocladum*, receiving the lowest incidence of total herbivory, and the least tough species, *P. imperiale*, receiving the highest incidence. Although variation in toughness within each species was substantial, there were no intraspecific relationships between toughness and herbivory. In other *Piper* studies, chemical and biotic defenses had strong intraspecific negative correlations with herbivory. A wide variety of defensive mechanisms was quantified in the three *Piper* species studied, ranging from low concentrations of chemical defenses in *P. imperiale* to a complex suite of defenses in *P. cenocladum* that includes ant mutualists, secondary metabolites, and moderate toughness. Ecological costs were evident for the array of defensive mechanisms within these *Piper* species, and the differences in defensive strategies among species may represent evolutionary trade-offs between costly defenses.

Keywords *Piper cenocladum* · *Piper imperiale* · *Piper melanocladum* · Piperaceae · Amides · Secondary metabolites · Chemical defense · Plant-herbivore interactions · Costa Rica · Tropical rain forest

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Introduction

In response to strong selective forces exerted by a broad array of enemies, which include insect herbivores, vertebrate herbivores, and pathogens (Coley et al. 1985), plants employ a variety of morphological, biochemical, and biotic defenses (Maxwell et al. 1972; Johnson 1975; Coley 1983; Kursar and Coley 1992; Harborne 2001). Differences in plant palatability that result from these defenses contribute to substantial variation in the extent of herbivore damage to plants (Coley 1983). However, each kind of defense is potentially associated with allocation costs (Levin 1976; Herms and Mattson 1992; Gershenzon 1994; Bergelson and Purrington 1996; Koricheva 2002; Strauss et al. 2002).

Many studies have addressed intraspecific trade-offs between defense and other plant metabolic functions (Zangerl et al. 1997). Because most plants produce several

different defenses (reviewed in Romeo et al. 1996), intraspecific trade-offs may occur between defenses (e.g., between chemical and biotic defenses or between two different chemical defenses; Mattson et al. 1988; Steward and Keeler 1988; but see Koricheva et al. 2004). In this case, rather than reducing plant allocation of resources to reproduction or growth, plants may express the costs of heightened defense production by diverting resources from one defense to another. These trade-offs among different defenses are referred to as "ecological costs" (Heil 2002) since reducing a given defense only incurs costs under the appropriate ecological conditions.

Evolutionary trade-offs among defenses may also be evident between closely related species because sympatric plant species that differ in the type or quantity of their defenses may be at a selective advantage when defending themselves against specialist herbivores that have evolved resistance (Cates and Rhoades 1977). Producing multiple defenses may be selectively advantageous either because of synergistic action, where the effect of a combination of defenses exceeds the sum of the effect of all individual defenses (e.g., Berenbaum and Neal 1985; Berenbaum et al. 1991; Calcagno et al. 2002; Dyer et al. 2003), or because multiple defenses may protect a plant against a diverse suite of enemies (Hay et al. 1994; Lindroth and Hwang 1996; Nelson and Kursar 1999; Dyer et al. 2003; Koricheva et al. 2004).

Ant–plant associations ranging from obligate mutualisms to loose facultative relationships are common (Heil and McKey 2003) and have long been used as model systems in the study of plant defense (e.g., Janzen 1966; Rehr et al. 1973; Dyer et al. 2001). Allocation of resources to production of domatia, food bodies, nectar, or other ant rewards in these systems presumably incurs some costs (Folgarait and Davidson 1995; Dyer et al. 2001; Heil et al. 2002). Leaf toughness and production of secondary metabolites are two widely distributed defenses in tropical plants (Coley and Barone 1996). Several studies have shown that toughness is an effective defense (Feeny 1970; Coley 1983; Sagers and Coley 1995; Coley and Barone 1996), but relatively few studies include toughness in examinations of ecological costs. Plant secondary metabolites are often effective in reducing herbivory (reviewed by Harborne 2001) but can be metabolically costly to produce (Gershenzon 1994; Zangerl et al. 1997; Dyer et al. 2001; reviewed by Bergelson and Purrington 1996, and Strauss et al. 2002), potentially necessitating trade-offs in resource allocation. For example, allocation of limited plant resources may necessitate trade-offs between allocation of carbon to ant rewards, construction of structural carbohydrates to enhance toughness, and/or chemical defense. Similarly, nitrogen may be allocated to ant rewards, nitrogen-based chemical defenses, or the enzymes and physiological machinery necessary to produce tougher leaves or higher levels of chemical defense. Plant species or individuals that invest in ant mutualisms often show reduced chemical defense when compared to closely related species or individual plants within a species that lack ant defenders (Rehr et al. 1973; Dyer et al. 2001; Heil et al. 2002).

By using field experiments and surveys, we examined relative efficacies of and trade-offs among three ecologically important tropical plant defenses: ant mutualists, secondary metabolites, and leaf toughness. Three closely related species in the genus *Piper* [*P. imperiale* (Miq.) C. DC., *P. cenocladum* C. DC., and *P. melanocladum* C. DC.) served as a model system. The phytochemistry of the diverse tropical genus *Piper* is variable and well documented (reviewed by Parmar et al. 1997; Dyer et al. 2004a). All species investigated to date (more than 112) produce mixtures of secondary metabolites, and the compounds discovered include alkaloids/amides/imides, lignans, neolignans, terpenes, propenylphenols, steroids, kavapyrones, chalcones, flavones, flavanones, and piperolides (Dyer et al. 2004a). The three *Piper* species utilized are closely related (as part of the sect. *Macrostachys* clade, Tepe et al. 2004), and share many species of generalist and specialist herbivores (Marquis 1991; L. Dyer, personal observations). They display a unique combination of ant mutualism, chemical defense, and leaf toughness. *Piper cenocladum* is defended by ant mutualists and nitrogen-containing chemicals, *P. imperiale* has a facultative relationship with ants, but its chemistry has not previously been investigated, and *P. melanocladum* has no relationship with ants, and its chemistry was previously unknown. The relative leaf toughness of all three has not previously been measured.

We tested for inter- and intra-specific trade-offs between multiple putative plant defenses and their effects on herbivory in order to address these two general questions: How is intra- and interspecific variation in plant defense associated with herbivory by specialists and generalists? Are ecological costs of different types of defense evident within and among these three *Piper* species?

We predicted that generalist herbivores would respond more to chemical defenses, while small, specialists would be deterred by ant defenders or leaf toughness (Dyer et al. 2004b). We expected to find trade-offs in allocation of resources to toughness, ant associations, and chemical defense within and among the three *Piper* species.

Materials and Methods

Study System *Piper cenocladum* is a tall (to 5 m) understory plant with large, long-lived leaves (Letourneau and Dyer 1998) found in the lowland wet forest of Costa Rica (Burger 1971). This species has defenses in the form of

amide/imide secondary metabolites (Dodson et al. 2000) and resident obligate ant mutualists (Risch and Rickson 1981; Risch 1982). *Piper cenocladum* leaves contain two imides and one amide [Appendix, structures 1–3: piplartine (1), 4′-desmethylpiplartine (2), and cenocladamide (3); Dodson et al. 2000] in relatively high concentrations of up to 3.8% dry weight (Dyer et al. 2004b). Piplartine is cytotoxic in vitro (Duh et al. 1990), and all three compounds in combination act synergistically to directly and indirectly affect the fitness and feeding preferences of generalist and specialist herbivores from a wide range of taxa (Dyer et al. 2003). *Pheidole bicornis* Forel (Hymenoptera: Formicidae: Myrmicinae) ant colonies are housed inside of *P. cenocladum* sheathing leaf bases (petioles), and the plants produce amino acid and lipid rich opalescent food bodies on the adaxial sides of the petioles (Risch and Rickson 1981). These food bodies comprise the majority of the ants' diet (Fischer et al. 2002). In return, the ants remove insect eggs, vines, and small particles from the surface of the leaves, and kill small lepidopteran larvae and stem boring weevils (Risch et al. 1977; Letourneau 1983; Letourneau and Dyer 1998). When plants are not inhabited by ants, the production of these food bodies declines, and amide production increases threefold or greater (Dodson et al. 2000; Dyer et al. 2001).

Piper imperiale is a large shrub or small tree to 6 m tall found in moist, shaded forest areas (Burger 1971). This species is characterized by large leaves and tubercles on stems and leaves but in many ways is morphologically and ecologically similar to *P. cenocladum* (Burger 1971). Several species of ants facultatively inhabit the sheathing leaf bases, but the plants produce no food bodies inside of the domatia. Whether the ants benefit the plants in any way is unknown.

Piper melanocladum is a small understory shrub to 1.6 m tall, with thick, glabrous, shiny lanceolate leaves (Burger 1971). It has small, partially open leaf bases, and has no demonstrated relationship with ants. We describe the first characterization of the nitrogen-based secondary metabolites of *P. imperiale* and *P. melanocladum*.

Field Survey This study took place in two lowland tropical wet forests at Tirimbina Rainforest Center and Estacion Biologica La Selva, Heredia Province, Costa Rica. La Selva is located at 10°25′N 84°05′W at circa 100 m elevation on the Caribbean slope. Tirimbina is located nearby at circa 210 m elevation, 10°24′4″ N, 84°6′29″ W.

We sampled *P. imperiale* (N=81), *P. cenocladum* (N=84), and *P. melanocladum* (N=94) plants along trails at Tirimbina and La Selva. Because *P. imperiale* and *P. cenocladum* reproduce vegetatively through fragmentation (Dyer et al. 2004c), individuals that we collected were separated by at least 5 m. On each plant, we chose the first fully mature leaf from the top of the plant for our measurements to standardize leaf age. We measured leaf toughness by using a modified penetrometer described by Feeny (1970). This device measures leaf toughness as the force, measured in grams of sand, needed to punch a 5-mm steel rod through a taut leaf. Immediately after removing the leaf from the plant, we took three toughness measurements, two on one side of the midrib and one on the other side. These measurements were made near the leaf tip, thus avoiding all major veins. The mean of these three toughness values was used in all analyses.

We used a translucent grid to measure the percent area removed from each leaf by each of the following major *Piper* herbivores: *Atta cephalotes* (leaf-cutter ants; Hymenoptera: Formicidae: Myrmicinae), katydids (Orthoptera: Tettigoniidae), beetles (Coleoptera: Chrysomelidae and Curculionidae), *Quadrus cerealis* caterpillars (Lepidoptera: Hesperiidae), and at least two species of *Eois* caterpillars (Lepidoptera: Geometridae), which were grouped together. Each type of herbivore damage is easily discernible based upon the pattern and shape of damage. Total percent damage was calculated for each leaf as the sum of damage by all herbivore types.

Imide Isolation and Quantification Imides were isolated from *P. imperiale* and *P. melanocladum*, and their structures were determined with the methods for *P. cenocladum* described by Dodson et al. (2000). All imide structures were confirmed by synthesis (Appendix). A randomly selected subset of *P. imperiale* (N=30), *P. melanocladum* (N=44), and *P. cenocladum* (N=34) leaves were air-dried and analyzed to determine secondary chemical content. Each leaf was extracted ×2 with ethanol overnight. The resultant extract was resuspended in a 3:1 water/ethanol mixture and exhaustively extracted with chloroform in a separatory funnel. Combined extracts were dried, resuspended in chloroform, and analyzed by gas chromatography. Standards for analysis were synthesized at Mesa State College (Appendix). For a more detailed description of extraction and GC methods, see Dodson et al. (2000) and Dyer et al. (2001).

Atta cephalotes Bioassays To determine the effectiveness of the newly identified *P.* imides as defenses, we performed a feeding choice experiment with *Atta cephalotes* colonies. We modeled our experiment on those performed by Folgarait et al. (1996). We presented foraging *A. cephalotes* colonies with a selection of leaf fragments coated with different compounds. Treatments applied to experimental leaf fragments included piplaroxide (**5**), the newly discovered compounds (**6**) and (**4**), and a control that contained

only methanol (all imides were in methanol solution). Treatments were applied to *Hyeronima alchorneoides* Allemao (Euphorbiaceae) leaves (a species shown to be palatable to *A. cephalotes*; Folgarait et al. 1996) and allowed to dry. Randomly distributed *A. cephalotes* colonies were simultaneously presented with five fragments of each of the four treatments (for a total of 20 leaf fragments per trial) in a cafeteria-style display. These trials continued for 15 min or until all 5 fragments of one of the treatments had been removed by the ants. It was repeated with 22 different colonies (N=22).

We modified this *A. cephalotes* feeding trial to use *P. imperiale* leaf ethanol extracts, which would contain most secondary metabolites and other ethanol-soluble compounds produced by the plant, and to force the ants to cut fragments from a large leaf section, so that their mouthparts would be exposed to any secondary metabolites during the cutting process. We applied an extract of *P. imperiale* to leaves of *Hieronyma alchorneoides* and compared the amount of leaf material removed from leaves treated with extract to control leaves. Fresh and dry mass measurements from 144 strips of *H. alchorneoides* were used to create a conversion factor for estimation of the dry mass of fresh leaf strips used in feeding choice trials (dry mass=0.2512 (fresh mass) +0.0054, R^2=0.83). Leaves were cut into paired strips (two from each leaf) weighing 0.25 g fresh weight, avoiding all major veins, and the leaf area for each strip was measured with a LICOR leaf area meter (leaf area mean ± SE=12.27±0.15 cm^2). We cut a pair of treatment and control leaf strips from the same leaf, applied *P. imperiale* extract at high or low concentrations of 0.0095% leaf dry weight compound 4 (Appendix) and 0.0051% leaf dry weight compound 5 (Appendix) (N=42), or 0.0024% leaf dry weight compound 4 (Appendix) and 0.0013% leaf dry weight compound 5 (Appendix) (N=36; dissolved in 0.24 and 0.06 ml ethanol, respectively) to the treatment leaf strip and a corresponding amount of ethanol to the control leaf strip and allowed the extract to dry. These concentrations are an order of magnitude lower than those found in *P. imperiale* leaves, and so should measure the lower limits of ant abilities to detect and respond to secondary metabolites. Leaves were placed in pairs (one control leaf and one leaf treated with *P. imperiale* extract) in an actively foraging column of leaf-cutter ants (modeled after the methods of Folgarait et al. 1996). Each trial was allowed to continue for 2 hr or until the ants had removed all of one leaf strip. Leaf strips were large enough that ants cut small (approximately 1 cm^2) sections to carry away. After each trial, final leaf weight and leaf area were measured.

Statistical Analyses Because we were not able to transform the percent herbivory data to meet requirements of normality for parametric statistics due to the low frequency of occurrence of herbivory, we used two methods of analyses to test for differences among *Piper* species in herbivory by different herbivores. First, to examine qualitatively host choice by herbivores, we categorized leaves as experiencing herbivory or escaping from herbivory, and we performed chi-square tests for contingency tables to test for differences among plant species in the frequency of escape from herbivory. We then quantitatively examined the extent of feeding by herbivores once feeding was initiated (i.e., excluding all plants lacking herbivory) by performing an analysis of covariance (ANCOVA) with plant species as the independent variable, herbivore damage as the dependent variable, and toughness as a covariate, followed by Tukey's multiple range tests. For this analysis, we removed all zero herbivory values from the data set and log transformed damage and toughness values to meet assumptions of residual normality and homogeneity of variance. The covariate and the interactive term were insignificant in all tests. In combination, these two analyses compare both the frequency of herbivory and the amount of herbivore damage among plant species. Both of these analyses were repeated for all damage types (total herbivory, *A. cephalotes*, tettigoniid, beetle, *Eois*, and *Q. cerealis*). To test for interspecific differences in toughness and concentration of amides/imides, we performed an analysis of variance (ANOVA) followed by Tukey's multiple range test.

We used logistic regression to examine intraspecific relationships among chemical defense (with chemical defense content used as a predictor variable), toughness, and herbivory by different herbivores. One test was performed for each type of herbivore (with the exception of *Q. cerealis* and *A. cephalotes*, which were excluded because of rarity) and for total percent herbivory. Damage by each type of herbivore was classified as either present or absent. We tested for evidence of intraspecific trade-offs among different defenses by examining correlations between average leaf toughness and amide content for each *Piper* species.

Due to intrinsic difficulties in analyzing cafeteria-style feeding choice data (Lockwood 1998, and works cited therein) and nonnormality of the data, we analyzed the first *A. cephalotes* bioassay feeding preference data by using log-linear models (Floyd 2001). Each leaf fragment treatment was used as a variable in specified models. We used the maximum likelihood method for parameter estimation of linear models and Chi-square statistics for hypothesis testing (Folgarait et al. 1996). Because we were interested in testing specific hypotheses, we used nonhierarchical models to test the significance of leaf treatments as predictors of *A. cephalotes* preference. For the second *A. cephalotes* bioassay feeding preference experiment that

used leaf extracts, two paired *t* tests were used to test for differences in the leaf area and mass removed from control and *P. imperiale* leaf extract treated leaf strips, for each concentration of extract.

Results

Secondary Metabolites *Piper imperiale* contains two imides (Appendix, structures 4, 5). The first imide, compound 4, is an analog of piplartine, a compound we previously found in *P. cenocladum*. Compound 4 (5′-desmethoxydihydropiplartine) has not been isolated previously from a species of *Piper*, and is accompanied by its epoxide deviative, compound 5, which has been isolated previously from *P. tuberculatum* by Capron and Wiemer (1996) and is named piplaroxide. Total imide content ranged from 0.0044% to 0.029% dry weight. *P. imperiale* did not have alkaloids but contained at least five different sesquiterpenes (Appendix, Experimental).

We isolated compounds **4** and **5** (piplaroxide; Appendix) as well as the 4′-desmethyl analog of piplaroxide from *P. melanocladum*. The latter compound is also new to the genus *Piper* and has structure **6** (Appendix). These imides are present at high levels (ranging from 0.016% to 0.40% dry weight) in the leaves. No other defensive compounds were detected in *P. melanocladum* (Appendix, Experimental).

Piper cenocladum total amide/imide content ranged from 0.17% to 1.068% leaf dry weight. This range of concentrations is consistent with ranges reported in other studies with this species where synergy (Dyer et al. 2003) and trade-offs (Dodson et al. 2000) have been demonstrated. No other defensive compounds were detected in *P. cenocladum* (Appendix, Experimental).

Interspecific Differences The three *Piper* species were different in their leaf toughness ($F_{2,\ 256}$=168.2, P<0.001; Fig. 1a). *Piper melanocladum* is the toughest species with a penetrometer value of 559.3±13.9 g, nearly twice as tough as the least tough *P. imperiale* (296.6±6.7 g). The toughness of *P. cenocladum* was intermediate (389.9±13.9 g). The concentration of amides/imides in *P. cenocladum* was higher than that in *P. melanocladum*, which in turn was higher than that in *P. imperiale* ($F_{2,\ 100}$=107.18, P<0.001; Fig. 1b).

When leaves were categorized as either experiencing or escaping herbivory, *Piper* species had significant differences in frequency of leaves escaping damage from beetles, *Eois*, and all herbivores combined (Fig. 2a; beetle: X^2=17.3, df=2, P<0.001; *Eois*: X^2=13.1, df=2, P=0.001; total: X^2=21.4, df=2, P<0.001). The number of individuals escaping total herbivory and beetle herbivory was lowest for *P. imperiale*, while the number of individuals escaping *Eois* herbivory was lowest for *P. cenocladum*. *Piper melanocladum* individuals escaped all three types of herbivory more frequently than the other two *Piper* species. Damage by *Atta cephalotes* and *Q. cerealis* was rare (N=4 for each herbivore), indicating that these herbivores avoid all three *Piper* species. Tettigoniids showed no preference for one *Piper* species over another (X^2=4.0, df=2, P=0.1).

Once a plant is attacked by an herbivore, the extent of damage differs among *Piper* species and among herbivores. When individuals with no herbivore damage were omitted from the analysis, beetle and total herbivore damage were significantly different between *Piper* species (Table 1, Fig. 2b). Herbivores fed more on *Piper imperiale* than on *P. cenocladum* or *P. melanocladum* (Fig. 2b). Beetles

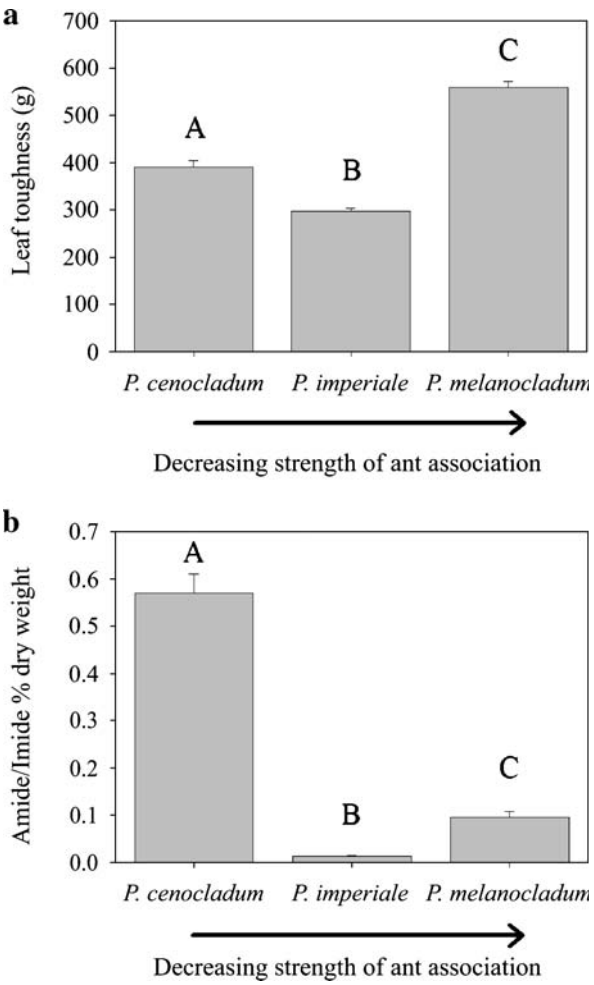

Fig. 1 Differences in: **a** mean leaf toughness (±1 SE) and **b** mean amide/imide content (±1 SE) among three species of *Piper* shrubs (*P. cenocladum*: N=84, *P. imperiale*: N=84, *P. melanocladum*: N=94). Data were analyzed using analysis of variance followed by Tukey's multiple range test; *different letters* above *Piper* species indicate significant differences

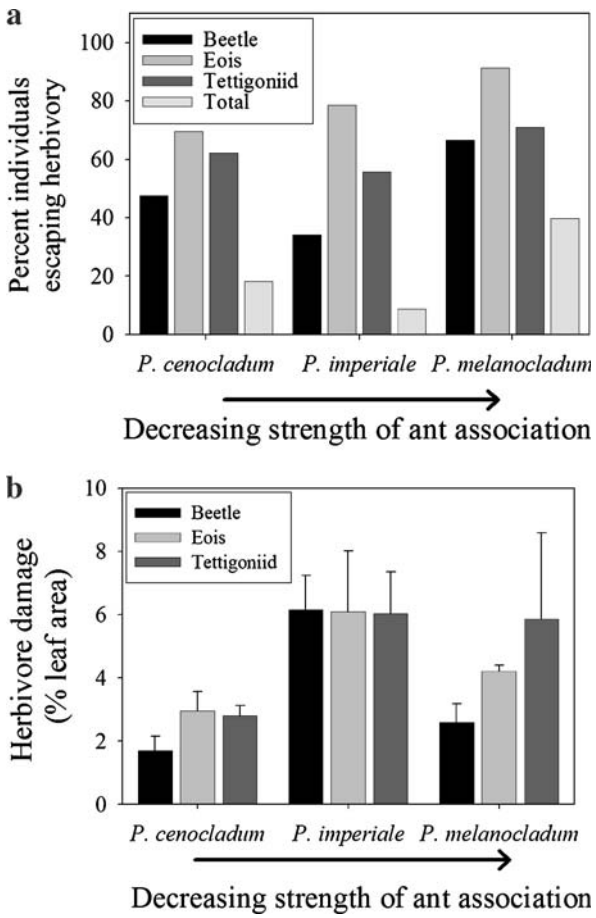

Fig. 2 Naturally occurring interspecific variation in herbivory for three closely related species of tropical shrubs, *Piper* spp. **a** Percentage of individuals completely escaping herbivory. Chi-square tests for contingency tables were utilized to test for differences between plant species in the frequency of escape from herbivory. Beetle, *Eois* and total herbivory differed significantly between *Piper* species (*P. cenocladum*: N=84, *P. imperiale*: N=84, *P. melanocladum*: N=94). A value of 100% indicates all individuals of that species escaped herbivory from a particular herbivore taxon. Total herbivory includes escape from all categories of herbivory, thus, it is always has a lower percentage than other categories. **b** Percent herbivory (±1 SE) on individual plants that did not escape herbivores. Analysis of Covariance (with toughness as a covariate) was utilized to test for differences in herbivory between species. *P. cenocladum*: N=72, *P. imperiale*: N=67, *P. melanocladum*: N=56)

showed a significant preference for *P. imperiale* over *P. cenocladum* but did not differentiate between *P. melanocladum* and the other two *Piper* species (Table 1). *Piper* damage by *Atta cephalotes* and *Q. cerealis* was excluded from the analysis due to small sample size. Levels of damage by *Eois* and tettigoniids were not significantly different among *Piper* species (Table 1).

Intraspecific Relationships Among Secondary Metabolites, Toughness, and Herbivory Secondary metabolite content in *P. melanocladum* was negatively correlated with levels of tettigoniid herbivory on leaves that experienced herbivory (R=−0.63, P=0.05). No other correlations between herbivory and secondary metabolites were found. We found no intraspecific relationships between toughness and herbivory (Table 2), but intraspecific variance was low compared to interspecific variance (Fig. 1a). We also found no evidence for trade-offs between toughness and secondary metabolites in the three species (*P. cenocladum* N=34, R=−0.22, P>0.05; *P. imperiale* N=30, R=0.033, P>0.05; *P. melanocladdum* N=44, R=0.10, P>0.05).

Atta cephalotes Feeding Trial In *P. imperiale* and *P. melanocladum*, compound **4**, piplaroxide (**5**), and compound **6** are clearly deterrent to *A. cephalotes* (Table 3, Fig. 3). The loglinear model revealed significant associations in removal of different leaf disks based on this deterrence. The (compound **4**×compound **6**) and (compound **4**×piplaroxide) interactions are significant since the frequencies of removal of both disk types are low in each case. In addition, associations between removal of control and treatment disks reflect the fact that the likelihood of the control disk being taken while the treatment disk was not taken was high, and the likelihood of the control disk being left while the treatment disk was taken was low. Whole leaf extract of *P. imperiale* was also deterrent to *A. cephalotes* (Fig. 4). Ants removed less leaf area and mass of leaves treated with leaf extracts in high (T=−2.38, df=41, P=0.02; T=−2.86, df=41, P=0.007) and low (T=−2.60, df=35, P=.01; T=−2.10, df=35, P=0.04) concentrations.

Discussion

We found evidence for ecological costs of the different defense mechanisms in the three *Piper* species studied. Secondary metabolites and their concentrations differed among species, along with toughness and the strength of the association of the plants with ants. *Piper melanocladum*, which has no ant association and low levels of chemical defense, has tough leaves. *Piper cenocladum* has the strongest association with ants and the highest levels of chemical defenses, but the leaves of this species are not likely to use toughness as a deterrent. *Piper imperiale* has tender leaves, low levels of defenses, and a loose association with ants. In addition to these contrasting suites of defenses, it is possible that there are trade-offs between the traits measured here and chemical defenses that are unknown or were not quantified, such as the sesquiterpenes that are present in *P. imperiale*. A comparison of toughness for the non-ant plant, *P. melanocladum*, with the ant-plants, *P. cenocladum* (obligate) and *P. imperiale* (facultative), provides support for the hypothesis that ant plants invest less in other defenses (Fig. 1a; Dyer et al. 2001; Heil et al.

Table 1 Qualitative differences in frequency of herbivory

Damage Type	Mean±SE (*N*)			*F* (*df*)
Plant Species (Total *N*)	*P. imperiale* (81)	*P. cenocladum* (84)	*P. melanocladum* (94)	
Total percent damage	9.6±1.21 (72)	4.5±1.1 (67)	5.0±1.4 (56)	9.6** (2)
Beetle percent damage	6.1±1.1% (52)	1.7±0.4% (43)	2.6±0.6% (31)	10.7** (2)
Tettigoniid percent damage	6.0±1.3 (35)	2.8±0.8 (31)	5.9±2.7 (27)	3.0* (2)
Eois percent damage	6.1±1.9 (17)	3.0 0.6 (25)	4.2 1.7 (8)	1.4 (2)
A. cephalotes percent damage	(0)	22.9±20.8 (3)	1.0 (1)	–
Q. cerealis percent damage	26.9±23.9 (2)	2.4 (1)	6.6 (1)	–

[a] Analysis of covariance
[b] Only herbivores experiencing herbivory were included in analyses, thus *N* varies by damage type and different types of herbivory do not add up to total percent damage.
*$P<.06$
**$P<.0001$

2002). On the other hand, the hypothesis that ant plants invest less than non-ant plants in chemical defenses, such as amides/imides, is only supported when comparing concentrations of these compounds in *P. imperiale* and *P. melanocladum* (Fig. 1b).

The effectiveness of different defensive mechanisms in these three species varies widely and differs according to the taxon of the attacking herbivore. The strong negative relationship between herbivory and toughness across the three species supports the findings of previous studies that demonstrate that toughness is an important defense (Coley 1983; Sagers and Coley 1995; Coley and Barone 1996). When choosing among multiple host species, generalist and oligophagous herbivores may avoid extremely tough species. In an examination of different types of anti-herbivore defenses and other plant characteristics across 46 tropical tree species, Coley (1983) found that leaf toughness was the plant characteristic most correlated with reduced herbivory. However, all patterns of herbivory cannot be accounted for with differences in leaf toughness. Specialist *Eois* caterpillar damage is higher on *P. cenocladum* vs. the other two species, although *P. cenocladum* is of intermediate toughness. *Eois* feeding may be deterred by both toughness (in *P. melanocladum*) and secondary metabolite content (in *P. imperiale*). Alternatively, as relatively specialized feeders, *Eois* species, some of which sequester imides/amides (Dyer et al. 2003), may be adapted to the defensive compounds in *P. cenocladum*. Tettigoniids, which are relatively generalized feeders, do not appear to have a host preference possibly because they feed on the young, expanding leaves of these species, which may have low toughness and secondary metabolite content (Kursar and Coley 1992).

Intraspecific variation in leaf toughness is not associated with changes in total herbivory, suggesting that feeding preferences within a single plant species may be motivated by other factors, such as nutrient content or secondary metabolism. Indeed, individual *Piper* species with lower levels of chemical defenses in their tissues may be preferred by insect enemies, such as leaf-cutter ants. We have shown here that piplaroxide (**5**) and compounds **4** and **6** (found in *P. imperiale* and *P. melanocladum*) are strongly deterrent to

Table 2 Logistic regression: toughness as predictor of herbivore damage

Species	Total Damage Likelihood Ratio χ^2, Standardized Estimate, Estimate χ^2	Beetle Damage Likelihood Ratio χ^2, Standardized Estimate, Estimate χ^2	Tettigoniid Damage Likelihood Ratio χ^2, Standardized Estimate, Estimate χ^2	*Eois* Damage Likelihood Ratio χ^2, Standardized Estimate, Estimate χ^2
Piper cenocladum (*N*=84)	0.40, −0.54, 0.31	0.78, −0.73, 0.63	1.86, −1.13, 1.33	0.19, 0.42, 0.18
Piper imperiale (*N*=81)	2.80, 1.67, 2.71	0.42, 0.65, 0.42	0.28, 0.53, 0.28	0.29, 0.53, 0.28
Piper melanocladum (N=94)	2.11, 1.44, 2.04	2.86, 1.72, 2.75	0.30, 0.56, 0.30	0.15, 0.38, 0.15

[a] Data analyzed with logistic regression; degrees of freedom=1.
[b] $P>0.05$ for all likelihood ratios, indicating that models are a good fit.
[c] $P>0.05$ for all standardized estimates, indicating no association between toughness and herbivore damage.

Table 3 Effects of *Piper* imides on *Atta cephalotes* feeding preference

Source	Degrees of Freedom	Chi-Square	*P* Value	Standardized Estimate
Compound 5×Compound 6	1	30.58	<0.0001	5.5303
Compound 5×Compound 4	1	23.29	<0.0001	4.8243
Compound 6	1	7.67	0.0281	2.7695
Control×Compound 6	1	4.41	0.0499	2.1002
Control×Compound 6	1	4.38	0.0250	2.0924
Control	1	3.79	0.0486	−1.9470
Control×Compound 5	1	0.49	0.5792	0.6967
Compound 6[a]	1	0.91	0.3396	0.9549
Compound 5[a]	1	0.06	0.8001	−0.2230
Likelihood ratio	4	5.13	0.2738	

Data analyzed utilizing logit models

[a] Nonsignificant variables included in former models.

Compound 4=1-[(2E)-3-(3′,4′-dimethoxyphenyl)prop-2-enoyl]-5,6-dihydropridin-2(1H)-one; Compound 5=piplaroxide; Compound 6=3-[(2*E*)-3-(4-hydroxy-3-methylphenyl)prop-2-enoyl]-7-oxa-3-azabicyclo[4.1.0]heptan-2-one

A. cephalotes (see also Capron and Wiemer 1996). In previous studies, we demonstrated also that the three imides/amides found in *P. cenocladum* deter feeding by *A. cephalotes* and act synergistically against a variety of herbivores (Dyer et al. 2003). Clearly, the imide/amide nitrogen-based defenses are effective deterrents to leaf-cutter ants, and the presence of these compounds is a likely explanation for why these *Piper* species are avoided by *A. cephalotes*.

The two sister species, *P. cenocladum* and *P. imperiale* exhibit different patterns of defense, tolerance, and trade-offs among defenses, despite the fact that these two species are morphologically similar, unresolved in molecular phylogenies (Tepe et al. 2004), and share almost identical herbivore fauna. In *P. imperiale*, herbivory is common, as the species appears to be investing few resources in defense and relying on tolerance. Dyer et al. (2004c) found that asexual reproductive success of *P. imperiale* was not affected by herbivory, which is consistent with a tolerance hypothesis. *Piper imperiale* fragments placed on the surface of lowland rainforest soils to simulate asexual reproduction through natural fragmentation appeared tolerant of high

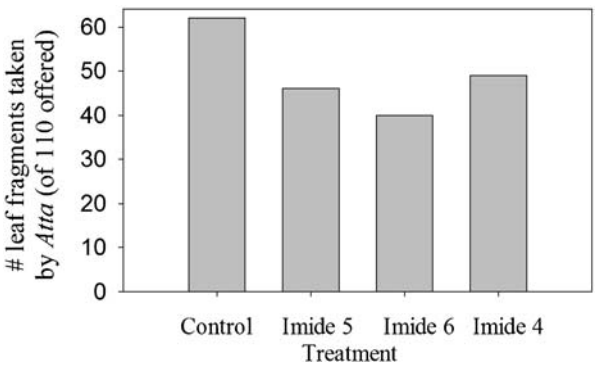

Fig. 3 The frequency of experimental leaf fragment removal by leafcutting ants, *Atta cephalotes* (Hymenoptera). Experimental fragments were treated with imides from two species of tropical shrubs, *P. imperiale* and *P. melanocladum*, and control fragments were treated only with solvent. A loglinear model uncovered significant associations between removal of pairs of leaf fragments with different solutions applied (e.g., when high numbers of control fragments were removed, low numbers of piplaroxide fragments were removed). Based on significant associations in this model, all defensive imides were deterrent to *A. cephalotes*. Imide 4=1-[(2E)-3-(3′,4′-dimethoxyphenyl)prop-2-enoyl]-5,6-dihydropridin-2(1H)-one; Imide 5=piplaroxide; Imide 6=3-[(2*E*)-3-(4-hydroxy-3-methylphenyl)prop-2-enoyl]-7-oxa-3-azabicyclo[4.1.0]heptan-2-one

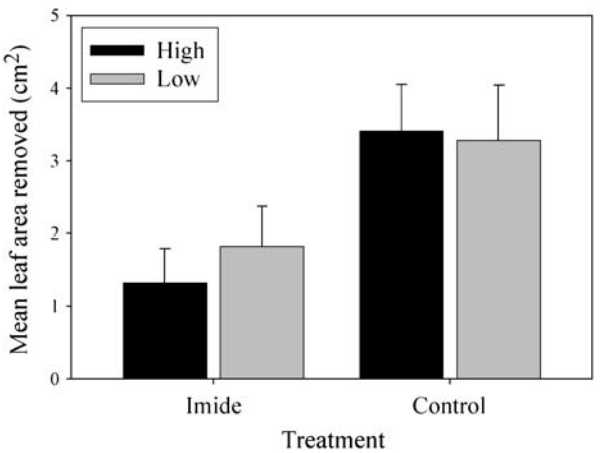

Fig. 4 Mean leaf area (±1 SE) removed from leaves of the tropical shrub, *P. imperiale*, by foraging leaf-cutter (*Atta cephalotes*; Hymenoptera) workers. Large leaf fragments were treated with *P. imperiale* extracts at high ($T=-2.38$, d.f.=41, $P=0.02$) and low ($T=-2.60$, d.f.=35, $P=0.01$) concentrations and ants were presented with a control leaf and a treatment leaf during a 2 h trial

levels of herbivory present on fragments before planting. In contrast, *P. cenocladum* exhibits high allocation of resources to defense, which may reflect low tolerance of herbivory in this species. *Piper cenocladum* relies primarily on vegetative fragmentation for reproduction (Greig 1993), and Dyer et al. (2004c) showed that *P. cenocladum* asexual reproductive success is reduced by natural herbivory on and artificial damage of the leaves of fragments. Prevention of herbivory by chemical and ant defense is associated with increased asexual reproductive fitness. One might predict that such high investment in defense would lead to trade-offs among different defenses. In *P. cenocladum*, a trade-off between secondary metabolite content and ant defense is indicated by a threefold increase in amide concentrations when ants are excluded from *P. cenocladum* plants (Dodson et al. 2000). In the current study, this increase in secondary metabolite content was not correlated with *Eois* herbivory, which is consistent with other studies that demonstrate that amides/imides do not protect *P. cenocladum* plants from feeding by specialized herbivores (Dyer et al. 2004b). There is evidence that the *Ph. bicornis* ant mutualists effectively protect plants against specialized herbivores, but they have little effect on large, generalist herbivores such as katydids or other caterpillars, which may be deterred by increased amide/imide concentrations (Dyer and Letourneau 1999; Dyer et al. 2001, 2004b). Although we found that *P. cenocladum* exhibits moderate investment in leaf toughness, we were unable to detect any correlations between toughness and herbivory in this species. Nevertheless, investment in leaf toughness could make it difficult for rare herbivores such as hesperiids (Lepidoptera) and *Atta* (Hymenoptera) from adding this plant species as a consistent food resource.

In summary, we have demonstrated a wide variety of defensive mechanisms among three closely related species of tropical shrubs. It is clear that redundancy in defense provides the plant with protection against a variety of herbivores. Previous work demonstrated intraspecific trade-offs between chemical and ant-mediated defense in *P. cenocladum*, but our interspecific results here were consistent with both the trade-off hypothesis as well as the alternative hypothesis that there are no trade-offs between indirect biotic and direct chemical defenses in plants (Heil et al. 2002). We were also unable to measure trade-offs between defense and other functions, but tough leaves may come at the expense of lower rates of growth or reproduction. Studies of chemical redundancy, ecological costs of antiherbivore defense, and interspecific variation in defensive attributes should consider the complex interaction between forces that select for optimum levels of defense against a variety of different herbivores and trade-offs between defenses, growth, and reproduction that allow plants to minimize the cost of defense.

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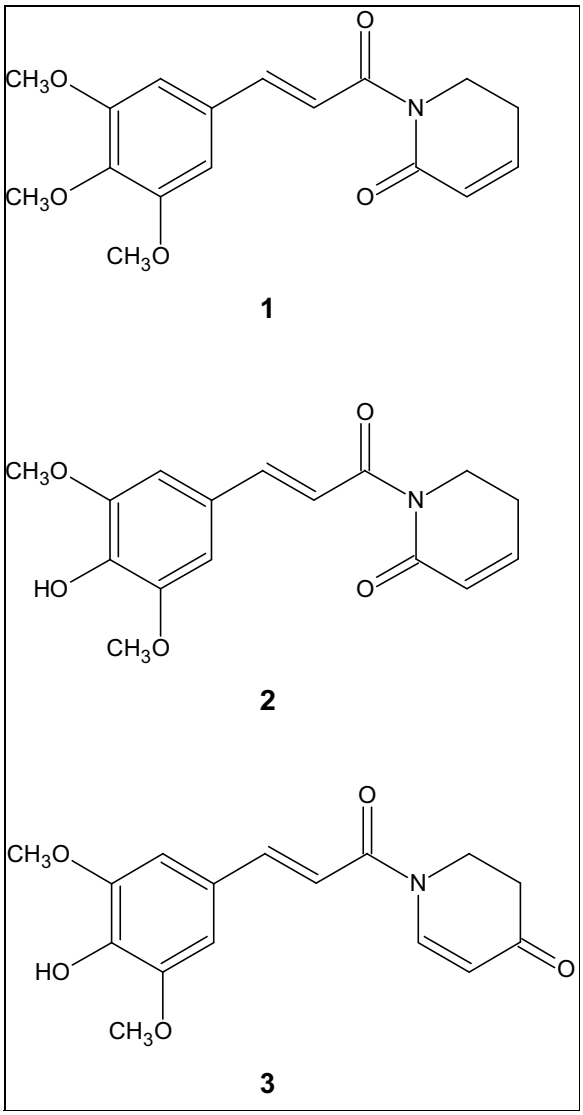
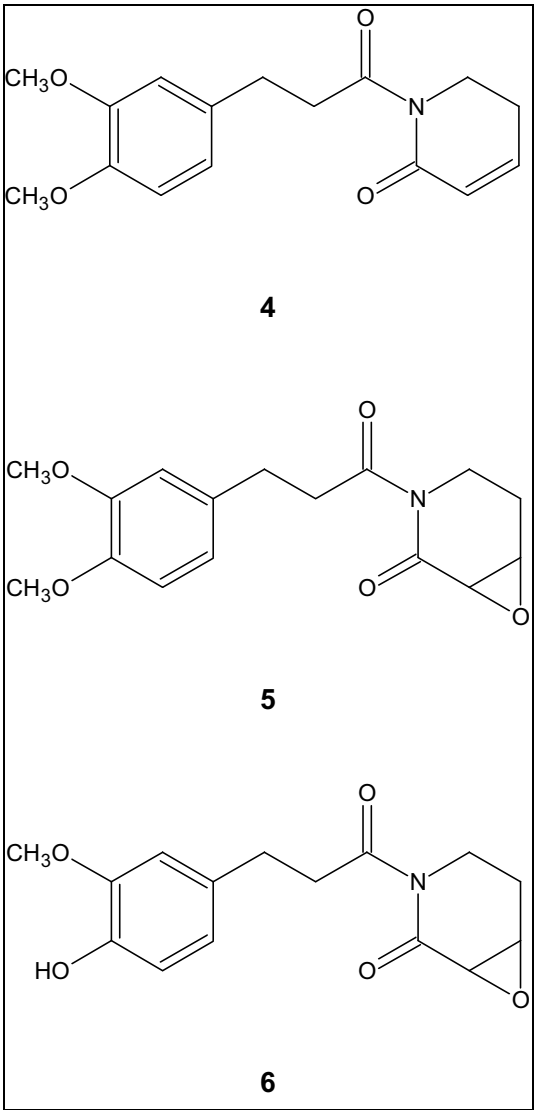
Appendix

Structures 1–3. Defensive metabolites isolated from *Piper cenocladum*



Structure 4–6. Defensive metabolites isolated from *Piper melanocladum*



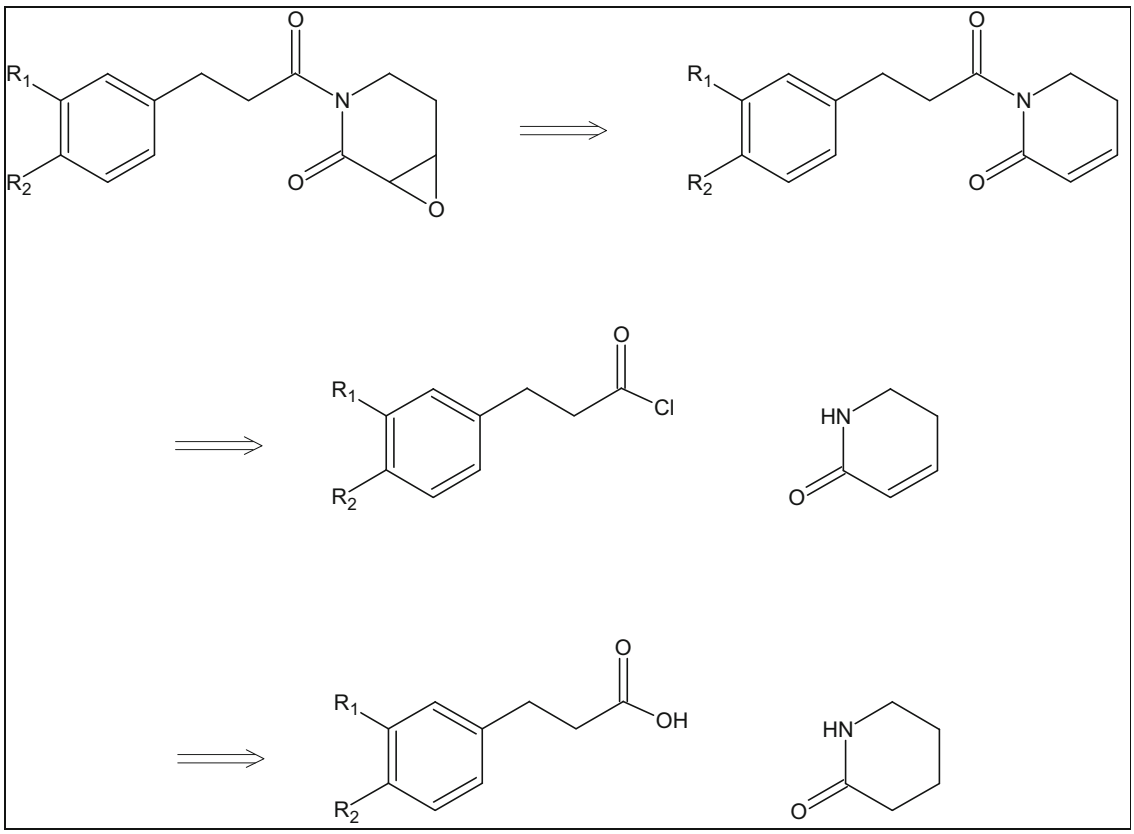
Synthesis of **4, 5, and 6** (Schemes 1, 2, 3, 4, 5, 6 and 7): The syntheses of 1-[(2E)-3-(3′′,4′′-dimethoxyphenyl)prop-2-enoyl]-5,6-dihydropridin-2(1H)-one (**4**), 3-[(2*E*)-3-(3,4-dimethylphenyl)prop-2-enoyl]-7-oxa-3-azabicyclo-[4.1.0] heptan-2-one (piplaroxide, **5**) and 3-[(2*E*)-3-(4-hydroxy-3-methylphenyl)prop-2-enoyl]-7-oxa-3-azabicyclo[4.1.0]-heptan-2-one (**6**) were accomplished via similar convergent approaches. These involved the synthesis of an appropriate functionalized pyridinone piece and its coupling with an appropriately activated acid derivative.

Experimental

General Information All ^{1}H NMR and ^{13}C NMR spectra were recorded at 300 MHz. All flash chromatography was performed on Aldrich silica gel (200–400 mesh, 60 Å). Tetrahydrofuran (THF) was redistilled from sodium/benzophenone. Dichloromethane (CH_2Cl_2) was redistilled from CaH_2. All other reagents and solvents were used as received unless otherwise noted.

1-(Methoxymethyl)piperidin-2-one (13) A 500-ml round bottom flask was flushed under N_2 while δ-valerolactam was melted on the oven. δ-Valerolactam (15.147 g, 153 mmol) and distilled THF (160 ml) were added to the flask, and the mixture was cooled on an ice bath for 10 min. After cooling, 2.25 M n-BuLi (50 ml) was added to the mixture slowly via syringe. CH_3OCH_2Cl (11.8 g, 157 mmol) was added to the flask, and the mixture stirred at room temperature for 2 h. The reaction mixture was transferred to a separatory funnel with hexane (50 ml), and washed with water (2×100 ml) and brine (1×50 ml). The combined aqueous layers were extracted with CH_2Cl_2 (5× 50 ml). The combined organic layers were washed with brine (1×60 ml), dried over anhydrous magnesium sulfate, and filtered. The solvent was removed under reduced pressure to yield a yellow liquid (15.2 g), which was purified via vacuum distillation (92–94°C @ 4 mm Hg) using a Vigeroux fractionating column to yield **13** as a colorless liquid [10.3 g, 49% yield; ^{1}H NMR ($CDCl_3$): δ 4.77 (2H, s), δ 3.32 (2H, t, *J*=5 Hz), δ 3.26 (3H, s), δ 2.39 (2H, t, *J*=5 Hz), δ 1.78 (4H, m). ^{13}C NMR ($CDCl_3$): δ 171.1, δ 77.1, δ 56.0, δ 46.2, δ 32.4, δ 23.1, δ 21.3].

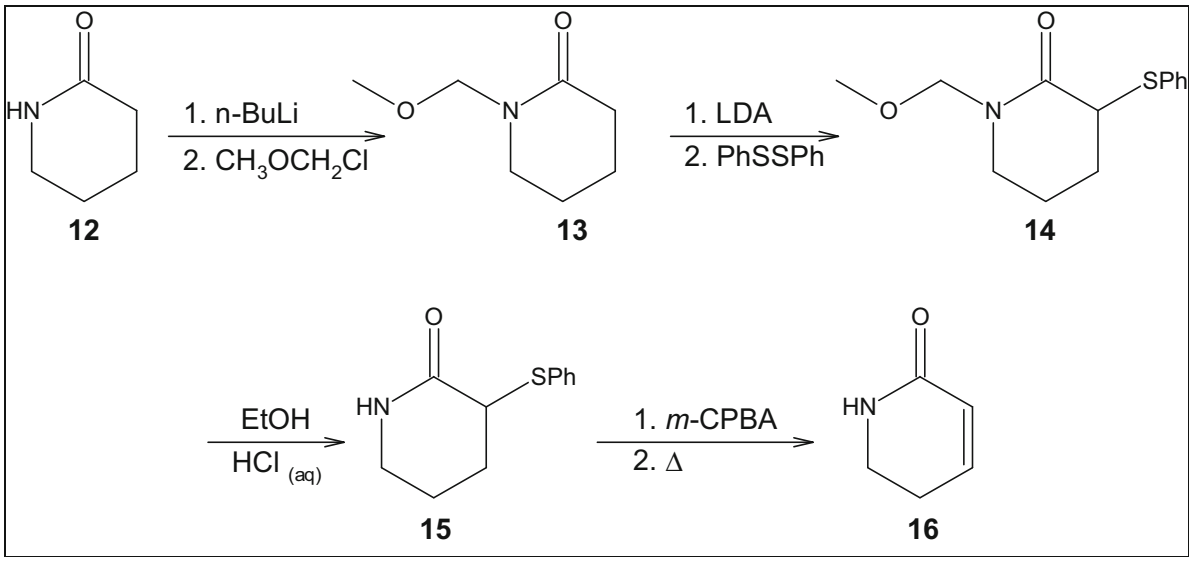
1-(Methoxymethyl)-3-(phenylthio)piperidin-2-one (14) A 250-ml three-necked round bottom was oven dried, fitted with an addition funnel and a septum, and flushed under N_2. Distilled THF (30 ml) and diisopropylamine (8 ml) were added to the flask and the solution stirred on ice for 10 min. 2.25 M n-BuLi (24 ml) was added through the septum via syringe and the mixture stirred on ice for 10 min. The mixture was cooled to −78°C in a dry ice/isopropanol bath. Compound **13** (4 g, 27.9 mmol) was dissolved in distilled THF (15 ml) and added to the reaction through the dropping funnel over 10 min. The mixture stirred at −78°C for an additional 45 min. A mixture of phenyldisulfide (6.01 g) and HMPA (4.8 ml) in distilled THF (15 ml) was added to the reaction mixture over 20 min. The reaction stirred at −78°C for an additional 40 min and was then allowed to warm to room temperature. The reaction mixture was transferred to a separatory funnel with water (60 ml) and extracted with diethyl ether (3× 60 ml). The combined ether layers were washed with 3 M NaOH (1×40 ml), water (1×40 ml), 3 M HCl (1×40 ml), water (1×40 ml), and brine (1×40 ml). The solution was dried over magnesium sulfate, filtered, and evaporated to yield **14** as an orange oil [6.25 g, 89% yield; ^{1}H NMR ($CDCl_3$): δ 7.54 (2H, m), δ 7.29 (3H, m), δ 4.83 (2H, q, *J*=



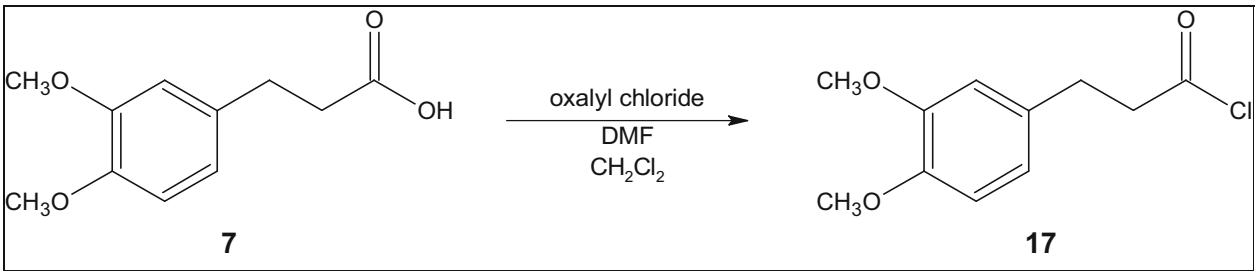
Scheme 1 Convergent retrosynthesis of "*Piper* amides" isolated from *P. melanocladum*

10 Hz), δ 3.89 (1H, t, *J*=5.5 Hz), δ 3.39 (2H, m), δ 3.30 (3H, s), δ 2.06 (3H, m), δ 1.7 (1H, m). ^{13}C NMR ($CDCl_3$): δ 169.8, δ 134.4, δ 132.7, δ 129.1, δ 127.7, δ 77.5, δ 56.2, δ 49.0, δ 45.9, δ 28.4, δ 20.4].

3-(Phenylthio)piperidin-2-one (15) Compound **14** (10 g) was placed in a 1-l round bottom flask with 95% ethanol (270 ml) and conc. HCl (54 ml). The reaction mixture was heated at reflux for 6 h. The volume was reduced to 100 ml



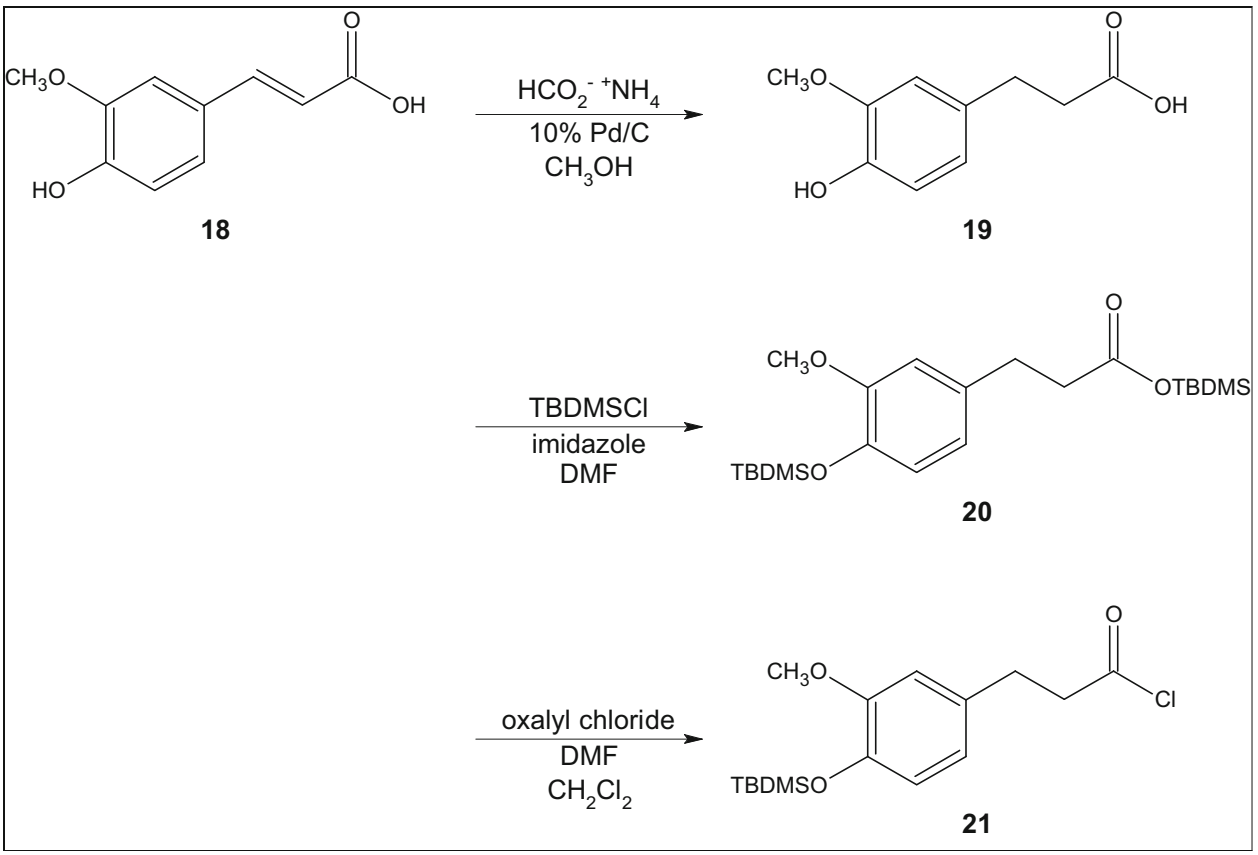
Scheme 2 The dihydropyridinone moiety was prepared from δ-valerolactam (**12**). The lactam nitrogen was protected (**13**) by treating the lithium salt of the lactam with chloromethyl methyl ether (MOMCl). The enolate of compound **13** was generated by treating the protected lactam with two equivalents of LDA in THF at −78°C and sulfenylated in the α-position with phenyl disulfide (PhSSPh). After deprotection of the sulfenylated lactam (**14**), the sulfide was oxidized to the sulfoxide and subjected to thermal elimination to produce 5,6-dihydropyridin-2(1*H*)-one (**16**)



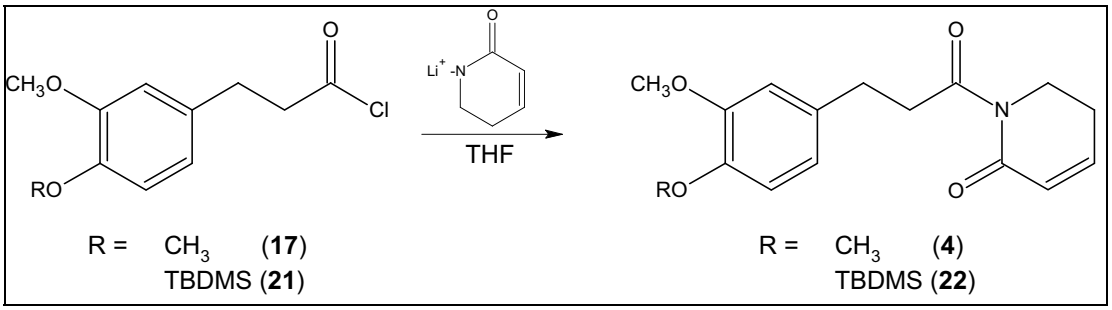
Scheme 3 The hydrocinnamoyl derivatives were prepared from appropriate carboxylic acid precursors. Consequently, commercially available 3,4-dimethoxyhydrocinnamic acid (**7**) was converted to 3,4-dimethoxyhydrocinnamoyl chloride (**17**) by treating the acid with oxalyl chloride in dichloromethane using catalytic amounts of dry DMF

under reduced pressure, and the mixture was extracted with CH_2Cl_2 (2×250 ml). The acid layer was neutralized with 6 M NaOH and extracted with CH_2Cl_2 (2×250 ml). Each CH_2Cl_2 layer was washed with saturated $NaHCO_3$ (2× 80 ml) and brine (1×80 ml). The combined organic layers were dried over sodium sulfate, filtered, and evaporated to yield a brown residue (7.4 g). The residue was recrystallized from ethyl acetate/hexane to yield **15** as an off-white solid [5.3 g, 64% yield; ^{1}H NMR ($CDCl_3$): δ 7.54 (2H, m), δ 7.31 (3H, m), δ 6.05 (1H, broad s), δ 3.83 (1H, t, *J*=6 Hz), δ 3.32 (2H, t, *J*=4 Hz), δ 2.03 (3H, m), δ 1.74 (1H, m)]. ^{13}C NMR ($CDCl_3$): δ 170.4, δ 134.1, δ 132.9, δ 129.1, δ 127.8, δ 48.4, δ 42.5, δ 28.3, δ 20.2.

5,6-Dihydropyridin-2(1H)-one (16) Compound **15** (3.0 g) was weighed into a 250-ml round bottom flask. CH_2Cl_2 (100 ml) and saturated $NaHCO_3$ (20 ml) were added to the flask, which was then cooled on an ice bath for 10 min.



Scheme 4 In the case of the 4-hydroxy-3-methoxy derivative, the appropriate acid (**19**) was first obtained via catalytic transfer hydrogenation of commercially available ferulic acid (**18**) with ammonium formate using 10% Pd/C in methanol. The free phenolic hydroxyl group was then protected as the TBDMS ether (**20**) using *t*-butyldimethylsilyl chloride and imidazole in dry DMF. Protection was necessary to avoid polymerization during and after the formation of the acyl halide. While this step also introduced a TBDMS group at the carboxyl oxygen, this group was directly converted to the acyl chloride (**21**) by treating the silyl ester with oxalyl chloride in dichloromethane using catalytic amounts of DMF



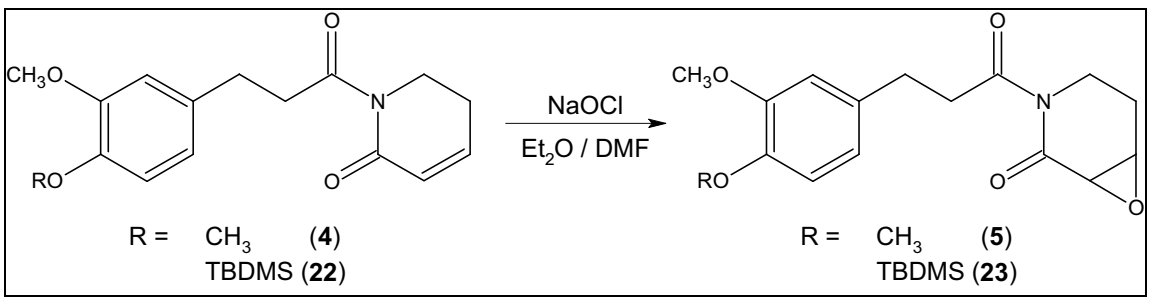
Scheme 5 In all cases, the next step involved the coupling of compound **16** with the appropriate acyl halide. Thus, the lithium salt of compound **16** was generated by treatment with KHMDS in dry THF and condensed with ether **17** or **21** to yield **4** or **22**, respectively

Seventy-seven percent 3-chloroperbenzoic acid (3.1 g) was added in five portions over 10 min. The mixture was vigorously stirred on an ice bath 1 h. The reaction mixture was transferred to a separatory funnel with CH_2Cl_2 (200 ml) and washed with saturated $NaHCO_3$ (2×30 ml). The solution was dried over sodium sulfate, filtered, and evaporated to yield a brown residue (3.2 g). Toluene (50 ml) was added to the brown residue and the solution was heated at reflux for 1 h. The solvent was removed under reduced pressure, and the brown oil was purified via flash chromatography (EtOAc) to yield compound **16** [1.25 g, 90% yield; ^{1}H NMR ($CDCl_3$): δ 6.64 (1H, m) δ 6.45 (1H, broad s), δ 5.89 (1H, m), δ 3.42 (2H, m), δ 2.34 (2H, m)]. ^{13}C NMR ($CDCl_3$): δ 166.6, δ 141.9, δ 129.2, δ 39.7, δ 23.9.

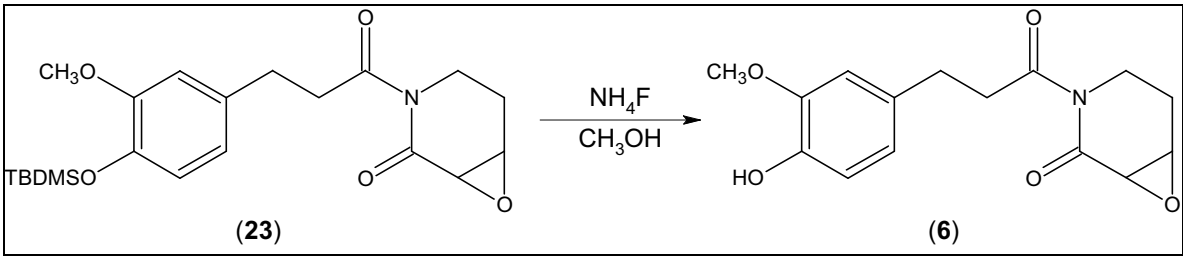
3-(3,4-Dimethoxyphenyl)propanoyl chloride (17) A 100-ml round-bottomed flask was flushed with N_2 and charged with **7** (2.1 g, 10 mmol), freshly distilled dichloromethane (40 ml), and dry DMF (four drops). Oxalyl chloride (1.75 ml, 20 mmol) was added slowly via pipet. The flask was fitted with a drying tube ($CaCl_2$), and the mixture was stirred at RT for 22 h. The volatiles were removed under reduced pressure to yield **17** as a pale yellow oil sufficiently pure for use [2.3 g, 100%; ^{1}H NMR ($CDCl_3$): δ 6.82–6.69 (3H, m, ArH), δ 3.87 (3H, s, -OCH_3), δ 3.86 (3H, s, -OCH_3), δ 3.19 (2H, t, J=7.4 Hz, -CH_2-), δ 2.96 (2H, t, J= 7.4 Hz, -CH_2-). ^{13}C NMR ($CDCl_3$): δ 173.22, δ 149.09, δ 147.94, δ 131.22, δ 120.30, δ 111.61, δ 111.43, δ 55.99, δ 55.94, δ 48.92, δ 30.75].

3-(4-Hydroxy-3-methylphenyl)propanoic acid (19) A mixture of **18** (2.0 g, 10 mmol), ammonium formate (2.0 g, 32 mmol), and 10% Pd/C (0.1 g) in methanol (50 ml) was stirred under N_2 at RT for 24 h. Celite filter aid (0.5 g) was added, and the mixture was vacuum filtered. The volatiles were removed under reduced pressure. The residue was transferred to a separatory funnel with the aid of 1 M HCl (50 ml) and dichloromethane (50 ml). The organic layer was isolated, and the aqueous solution was extracted with more dichloromethane (1×50 ml). The combined organic layers were washed with brine, dried over anhydrous $MgSO_4$, and filtered. The volatiles were removed under reduced pressure to yield **19** as a pure white solid [1.9 g, 95%; ^{1}H NMR ($CDCl_3$): δ 11.17 (1H, br s), δ 6.84 (1H, d), δ 6.71 (2H, m), δ 5.54 (1H, br s), δ 3.87 (3H,s), δ 2.89 (2H, t), δ 2.65 (2H, d). ^{13}C NMR ($CDCl_3$): δ 179.11, δ 146.50, δ 144.14, δ 132.14, δ 120.91, δ 114.47, δ 110.97, δ 55.94, δ 36.07, δ 30.41].

tert-Butyldimethylsilyl 3-(4-t-butyldimethylsilyloxy-3-methyoxyphenyl)propanoate (20) A mixture of **19** (2.7 g, 14 mmol), TBDMSCl (4.52 g, 30 mmol) and imidazole (4.1 g, 60 mmol) in dry DMF (12 ml) was stirred under N_2 at RT for 5 days. The mixture was poured into a separatory funnel with diethyl ether (75 ml) and hexanes (25 ml). The mixture was washed with water (1×50 ml), saturated $NaHCO_3$ (1×50 ml), and water (1×50 ml), and brine (1× 50 ml). The organic solution was dried over anhydrous $MgSO_4$ and filtered. Solvents were removed under reduced



Scheme 6 The corresponding epoxides (**5** and **23**) were prepared via epoxidation of the respective alkenes by treatment with NaOCl in a mixture of diethyl ether and DMF



Scheme 7 Finally, compound **6** was prepared after deprotection of **23** with methanolic NH_4F

pressure to yield a colorless oil. The remaining volatiles were removed under high vacuum to yield **20** as a pure, pale yellow oil [5.77 g, 99%; ^{1}H NMR ($CDCl_3$): δ 6.74 (1H, d), δ 6.68 (1 H, d), δ 6.63 (1H, d of d), δ 3.78 (3H, s), δ 2.85 (2H, t), δ 2.62 (2H, t), δ 0.98 (9 H, s), δ 0.90 (9H, s), δ 0.23 (6H, s), δ 0.13 (6H, s). ^{13}C NMR ($CDCl_3$): δ 173.57, δ 150.81, δ 143.36, δ 134.22, δ 120.84, δ 120.35, δ 112.38, δ 55.53, δ 37.78, δ 30.88, δ 25.82, δ 25.63, δ 18.52, δ 17.67, δ −4.58, δ −4.74].

3-(4-t-butyldimethylsilyloxy-3-methyoxyphenyl)propanoyl chloride (21) A 250-ml round-bottomed flask was flushed with N_2 and charged with **20** (5.768 g, 13.6 mmol), freshly distilled dichloromethane (25 ml), and dry DMF (five drops). Oxalyl chloride (1.80 ml, 20.6 mmol) was added slowly via pipet. The flask was fitted with a drying tube ($CaCl_2$), and the mixture was stirred at RT for 20 h. The volatiles were removed under reduced pressure to yield **21** as a pale yellow oil sufficiently pure for further use [2.3 g, 100%; ^{1}H NMR ($CDCl_3$): δ 6.77 (1H, d), δ 6.66 (1H,d), δ 6.62 (1H,d), δ 3.79 (3H, s), δ 3.18 (2H, t), δ 2.93 (2H, t), δ 0.98 (9H, s), δ 0.13 (6H, s). ^{13}C NMR ($CDCl_3$): δ 173.25, δ 151.03, δ 143.91, δ 132.09, δ 121.09, δ 120.45, δ 112.38, δ 55.58, δ 48.95, δ 30.84, δ 25.80, δ 18.52, δ −4.56].

1-[3-(3,4-Dimethoxyphenyl)propanoyl]-5,6-dihydropyridin-2(1H)-one (4) A 100-ml three-necked, round-bottomed flask was oven dried and flushed with N_2 while cooling. Freshly distilled THF (15.6 ml) and a solution of KHMDS (8.2 ml, 0.5 M in toluene) were added to the flask and cooled on ice for 10 min. Compound **16** (0.4 g, 4 mmol) was dissolved in THF (4.5 ml) and added dropwise to the reaction mixture, which was then stirred on ice for 10 min. Compound **17** (1.0 g, 4.4 mmol) was added to the reaction mixture, which was then stirred on ice for 10 min. The reaction mixture was transferred to a separatory funnel with water (20 ml), ethyl acetate (30 ml), and hexane (10 ml). The organic layer was isolated and washed with saturated $NaHCO_3$ (2×20 ml), 0.1 M HCl (1×20 ml), and brine (1× 20 ml). The organic solution was dried over sodium sulfate, filtered, and the solvent was evaporated to yield an orange oil (800 mg). The oil was purified via flash chromatography (4:1 EtOAc/hexane) to yield compound **4** as a white solid [470 mg, 40% yield; ^{1}H NMR ($CDCl_3$): δ 6.86 (1H, d of t), δ 6.78 (3H, s), δ 5.97 (1H, d of t), δ 3.96 (2H, t), δ 3.86 (3H, s), δ 3.84 (3H, s), δ 3.24 (2H, t), δ 2.93 (2H, t), δ 2.37 (2H, m); ^{13}C NMR ($CDCl_3$): δ 175.7 (s), δ 165.5 (s), δ 149.0 (s), δ 147.5 (s), δ 145.3 (d), δ 133.8 (s), δ 126.0 (d), δ 120.4 (d), δ 111.9 (d), δ 111.2 (d), δ 56.0 (q), δ 55.9 (q), δ 41.2 (t), δ 41.1 (t), δ 30.8 (t), δ 24.7 (t). LREIMS *m/z* (rel. int.): 289 (37), 192 (51), 164 (100), 151 (50). HRMS: Found *m/z* 289.1315, calculated for $C_{16}H_{19}O_4N$ 289.1314. Anal. Calcd. for $C_{16}H_{19}NO_4$: C, 66.42; H, 6.62; N, 4.84. Found: C, 66.52; H, 6.62; N, 4.65.

3-[3-(3,4-dimethoxyphenyl)propanoyl]-7-oxa-3-azabicyclo [4.1.0]heptan-2-one (5) A 250-ml round-bottomed flask was charged with **4** (3.47 g, 12 mmol), DMF (100 ml) and Et_2O (100 ml). The mixture was cooled to 5°C on an ice bath. Cold, 6% $NaOCl_{(aq)}$ (Clorox, 20 ml, 24 mmol) was added, and the resultant mixture was vigorously stirred for 10 min. The reaction mixture was then transferred to a separatory funnel with 5% $Na_2S_2O_3$ (100 ml) and Et_2O (50 ml). The layers were mixed and separated. The aqueous layer was further extracted with Et_2O (2×100 ml). The combined organic layers were then washed with brine (1× 100 ml) and dried over anhydrous $MgSO_4$. The volatiles were evaporated under reduced pressure to yield **5** as a pale solid that was sufficiently pure for further use (1.23 g, 34%). Further purification can be accomplished via recrystallization from 95% EtOH [^{1}H NMR ($CDCl_3$): δ 6.77 (3H, br s), δ 4.32 (3H, dddd), δ 3.87 (3H, s), δ 3.85 (3H, s), δ 3.68 (1H, dd), δ 3.56 (1H, d), δ 3.21 (2H, m), δ 3.15 (1H, dd), δ 2.90 (2H, t), δ 2.41 (1H, dm), δ 1.98 (1H, dddd). ^{13}C NMR ($CDCl_3$): δ 174.8 (s), δ 169.7 (s), δ 148.8 (s), δ 147.4 (s), δ 133.5 (s), δ 120.4 (d), δ 111.9 (d), δ 111.2 (d), δ 56.0 (q), δ 55.9 (q), δ 53.6 (d), δ 52.5 (d), δ 41.5 (t), δ 35.8 (t), δ 30.6 (t), δ 24.0 (t). LREIMS *m/z* (rel. int.): 305 (24), 192 (35), 164 (100), 151 (62). Anal. Calcd. for $C_{16}H_{19}NO_5$: C, 62.94; H, 6.27; N, 4.59. Found: C, 63.05; H, 6.08; N, 4.54.

1-[3-(4-t-Butyldimethylsilyloxy-3-methyoxyphenyl)-propanoyl]-5,6-dihydropyridin-2(1H)-one (22) A 250-ml three neck, oven dried, round bottom was cooled while flushing with N_2. KHMDS (0.5 M in toluene, 28.4 ml) was

added and the flask cooled on an ice bath for 10 min. Compound **16** (1.38 g, 14 mmol) was dissolved in THF (30 ml) and added dropwise to the reaction mixture which then stirred for 10 min. Compound **21** (4.5 g, 14 mmol) was added to the reaction which then stirred for 10 min. The reaction mixture was transferred to a separatory funnel with water (50 ml), ethyl acetate (75 ml), and hexanes (15 ml). The water layer was removed and the organic layer was washed with saturated $NaHCO_3$ (2×50 ml), 0.1M HCl (1× 50 ml), and brine (1×50 ml). The solution was dried over sodium sulfate, filtered, and evaporated to yield a brown oil (5.2 g). The oil was purified via flash chromatography (1:1 EtOAc/hexanes) to yield compound **22** [2.2 g, 42% yield; ^{1}H NMR ($CDCl_3$): δ 6.86 (1H, d of t), δ 6.70 (3H, m), δ 5.97 (1H, d of t), δ 3.95 (2H, t), δ 3.77 (3H, s), δ 3.21 (2H, t), δ 2.90 (2H, t), δ 2.36 (2H, m), δ 0.97 (9H, s), δ 0.12 (6H, s). ^{13}C NMR ($CDCl_3$): δ 175.81, δ 165.44, δ 150.73, δ 145.26, δ 143.26, δ 134.64, δ 125.97, δ 120.72, δ 120.66, δ 112.68, δ 55.55, δ 41.14, δ 41.08, δ 30.94, δ 25.82, δ 24.72, δ 18.51, δ 14.28, δ -4.55].

3-[3-(4-t-Butyldimethylsilyloxy-3-methoxyphenyl)-propanoyl]-7-oxa-3-azabicyclo[4.1.0]heptan-2-one (23) A 250-ml round-bottomed flask was charged with **22** (1.34 g, 3.44 mmol), DMF (36 ml) and Et_2O (36 ml). The mixture was cooled to 5°C on an ice bath. Cold, 6% $NaOCl_{(aq)}$ (Clorox, 20 ml, 17.2 mmol) was added, and the resultant mixture was vigorously stirred for 1 h. The reaction mixture was then transferred to a separatory funnel with 5% $Na_2S_2O_3$ (180 ml) and Et_2O (90 ml). The layers were mixed and separated. The aqueous layer was further extracted with Et_2O (2×90 ml). The combined organic layers were then washed with brine (1×180 ml) and dried over anhydrous $MgSO_4$. The volatiles were evaporated under reduced pressure to yield **23** as a pale orange oil that was sufficiently pure for further use (1.27 g, 91%). Further purification can be accomplished via flash chromatography on silica [1:1 EtOAc/hexanes; ^{1}H NMR ($CDCl_3$): δ 6.75–6.64 (3H, m), δ 4.32 (1H, dddd), 3.78 (3H, s), 3.68 (1H, dd), 3.55 (1H, d), 3.18 (2H, m), 3.13 (1H, dd), 2.34 (1H, dm), δ 1.98 (1H, dddd), 0.98 (9H, s), 0.13 (6H, s)].

3-[3-(4-hydroxy-3-methoxyphenyl)propanoyl]-7-oxa-3-aza-bicyclo[4.1.0]heptan-2-one (6) A mixture of **23** (1.27 g, 3.12 mmol), ammonium fluoride (0.579 g, 15.6 mmol), and methanol (75 ml) was heated at reflux for 5 min. The reaction mixture was transferred to a separatory funnel with the aid of water (90 ml). The aqueous layer was extracted with CH_2Cl_2 (2×90 ml). The combined organic extracts were washed with brine (1×90 ml) and dried over anhydrous $MgSO_4$. The volatiles were evaporated under reduced pressure to yield a tan solid. This was recrystallized from EtOAc-hexanes to yield **6** as an off-white solid [0.41 g, 48%; ^{1}H NMR ($CDCl_3$): δ 6.83–6.70 (3H,m), δ 5.47 (1H,s), δ 4.35 (1H, ddt), δ 3.87 (3 H, m), δ 3.68 (1 H, br t), δ 3.56 (1H, d), δ 3.21 (2H, t), δ 3.21 (1H, m), δ 2.90 (2 H, t), δ 2.40 (1H, m), δ 1.98 (1H, ddd). ^{13}C NMR ($CDCl_3$): δ 175.1 (s), δ 169.9 (s), δ 146.6 (s), δ 144.2 (s), δ 133.0 (s), δ 121.2 (d), δ 114.3 (d), δ 111.2 (d), δ 56.0 (q), δ 53.5 (d), δ 52.4 (d), δ 41.5 (t), δ 35.7 (t), δ 30.6 (t), δ 23.9 (t). LREIMS *m/z* (rel. int.): 291 (13), 178 (23), 150 (100), 137 (50). ES(+)MS *m/z* 292.1 [M+H], 314.1 [M+Na]. Anal. Calcd. for $C_{15}H_{17}NO_5$: C, 61.85; H, 5.88; N, 4.81. Found: C, 62.16; H, 6.11; N, 4.81.

Other potential compounds All three species were screened for basic alkaloids using a standard acid/base partitioning technique followed by TLC with visualization using the alkaloid specific spray reagent, iodoplatinic acid, and found to be negative. Each species was examined by GC-MS using the same conditions described above for imide/amide quantitation. *Piper cenocladum* and *P. melanocladum* were found to be devoid of compounds other than the imides/amides described (other peaks in the chromatograms had areas at least 1,000 times less than the imides/amides). *Piper imperiale* was found to contain a series of seven peaks with areas similar to or greater than the imides described. The mass spectra of all of these compounds are consistent with sesquiterpenes or mono-oxygenated sesquiterpenes. Five peaks had mass spectra that were excellent matches to the library spectra of copaene, cyclopropylazulene, (-) spathulenol, ledrol, and caryophyllene oxide. The peak for caryophyllene oxide was the largest in the chromatogram. Lack of standards for these compounds precluded confirmation of their identities and quantification. Other classes of natural products were not actively pursued.

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