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Cenocladamide, a dihydropyridone alkaloid from *Piper cenocladum*

Craig D. Dodson[a,*], Lee A. Dyer[a], Justin Searcy[a], Zane Wright[a], Deborah K. Letourneau[b]

[a]*Department of Physical and Environmental Sciences, Western Colorado Center for Tropical Research, Mesa State College, PO Box 2647, Grand Junction, CO 81502-2647, USA*

[b]*Department of Environmental Studies, 214 College Eight, UCSC, Santa Cruz, CA 95064, USA*

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Abstract

A dihydropyridone alkaloid, cenocladamide, and a derivative of piplartine, 4′-desmethylpiplartine were isolated along with piplartine from the leaves of *Piper cenocladum*. The structures of the new compounds were determined by spectroscopic methods and by comparison to piplartine. Concentrations of these amides in plants with and without ant mutualists, are compared.

Keywords: *Piper cenocladum*; Piperaceae; Leaves; Dihydropyridone alkaloids; *N*-(3′,4′,5′-trimethoxycinnamoyl)-Δ^3-pyridin-2-one; *N*-(4′-hydroxy-3′,5′-dimethoxycinnamoyl)-Δ^3-pyridin-2-one; *N*-(4′-hydroxy-3′,5′-dimethoxycinnamoyl)-Δ^2-pyridin-4-one; Piplartine; 4′-desmethylpiplartine; Cenocladamide

1. Introduction

Piper cenocladum C. DC. is an understory shrub (usually less than 4 m tall) common in wet forests throughout the Atlantic lowlands of Costa Rica. This species of *Piper* forms a mutualism with the ant *Pheidole bicornis* Forel which lives in the hollow petioles and stems of the plant. The ants feed virtually entirely on the amino acid and lipid rich opalescent food bodies produced on the adaxial sides of the petioles. These ant colonies remove insect eggs, some vines and small phylloplane particles from the leaves, thus providing a mechanical means of defense. The production of food bodies is induced by the presence of this species of ant and stops within 48 h of the removal of ant colonies (Letourneau & Dyer, 1998). While the majority of plants in the field support ant colonies, the plants can survive without their ant mutualists, but they are less fit in terms of biomass, leaf area, and asexual reproductive success (Dyer & Letourneu, 1999a, 1999b). In an effort to assess the relative costs/benefits of chemical versus mechanical defenses and explore redundancy in plant defenses, we set out to determine what secondary metabolites are present in *Piper cenocladum*. We report herein, the isolation and characterization of three dihydropyridone alkaloids, the known compound piplartine (**1**) and two previously unreported compounds, 4′-desmethylpiplartine (**2**) and cenocladamide (**3**). To the best of our knowledge, the substitution pattern of the dihydropyridone ring in cenocladamide (**3**) is novel. Piplartine is known to be cytotoxic in vitro, and two simple derivatives of piplartine, 3′-desmethoxypiplaritine and its 3,4-epoxy derivative are known to be repellant to the leaf cutter ant, *Atta cephalotes* (Duh, Wu & Wang,

* Corresponding author.

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Table 1
^{1}H-NMR spectral data for compounds **2** and **3** (270 MHz, $CDCl_3$, residual protio-chloroform signal assigned 7.24 ppm)[a]

H	2	3
2	–	7.97 *br d* (8.4)[b]
3	6.03 *dt* (9.6, 2.0)	5.43 *d* (8.4)
4	6.93 *dt* (9.6, 4.2)	–
5	2.56 *m*	2.62 *t* (7.4)
6	4.03 *t* (6.6)	4.16 *t* (7.4)
8′	7.38 *d* (15.6)	6.76 *d* (15.3)
7′	7.68 *d* (15.6)	7.73 *d* (15.3)
2′	6.81 *s*	6.79 *s*
6′	6.81 *s*	6.79 *s*
-OMe	3.91 *s*	3.82 *s*

[a] All assignments were confirmed by ^{1}H–^{1}H 2D COSY, and comparison to piplartine.
[b] Chemical shifts are in ppm, coupling constants in Hz are in parentheses.

1990; Capron & Wiemer, 1996). Many amides of similar structure to (**1**), (**2**) and (**3**) from other species of *Piper* have insect antifeedant activity (Gbewonyo, Candy & Anderson, 1993; Su & Horvat, 1981; Miyakado, Nakayama & Ohno, 1989). The concentrations of (**1**), (**2**) and (**3**) increase approximately three-fold when the ant mutualists are removed from the plant.

Table 2
^{13}C-NMR spectral data for compounds **2** and **3** (68 MHz, $CDCl_3$, residual protio-chloroform signal assigned 77.0 ppm)[a]

C	2	3
2	166.0 *s*[b,c]	143.3 *d*
3	126.1 *d*	108.2 *d*
4	145.6 *d*	194.1 *s*
5	24.5 *t*	35.7 *t*
6	41.4 *t*	42.2 *t*
9′	169.3 *s*	165.9 *s*
8′	119.8 *d*	112.2 *d*
7′	144.6 *d*	147.9 *d*
1′	126.9 *s*	125.9 *s*
2′	105.5 *d*	105.6 *d*
3′	147.5 *s*	147.7 *s*
4′	137.5 *s*	138.2 *s*
5′	147.5 *s*	147.7 *s*
6′	105.5 *d*	105.6 *d*
-OMe	56.2 *q*	56.3 *q*

[a] Assignments of carbons bearing hydrogen atoms were confirmed by the ^{13}C–^{1}H HETCOR experiment and by comparison to piplartine.
[b] Assignments for C2 and C9′ are interchangeable.
[c] DEPT multiplicity.

2. Results and discussion

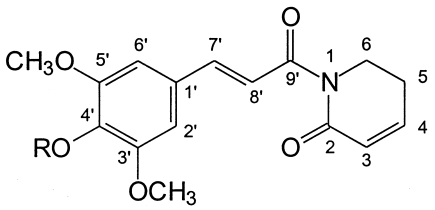
Ground leaves of *P. cenocladum* were extracted overnight, twice, at room temperature with 95% ethanol. The crude residue of this extract was dissolved in 3:1 water/ethanol and the aqueous ethanol was sequentially extracted with hexane, chloroform and finally ethyl acetate. TLC of the hexane and chloroform partitions indicated that they contain three yellow compounds. The NMR of these fractions showed similar aromatic, methoxyl and double bond resonances. The chloroform partition was subjected to column chromatography on silica gel followed by preparative layer TLC. This produced three dihydropyridone alkaloids. The highest R_f compound yielded NMR and mass spectral data that match that of the known compound, piplartine (**1**) (Duh et al., 1990). The illustrated arrangement of carbonyls about the imide functionality is based upon the X-ray crystal structure (Banerjee & Chaudhuri, 1986). The mass spectrum of the mid R_f compound (**2**) indicated a molecular mass of 303 a.m.u. which is 14 amu (CH_2) less than that of piplartine (**1**). The ^{1}H-NMR spectrum of this compound (Table 1) is identical to that of piplartine with the exception that it possesses a single methoxyl resonance that integrates for 6 protons. In addition, the ^{13}C-NMR spectrum (Table 2) is also quite similar to piplartine with one of piplartine's two methoxyl resonances missing. This compound exhibits a bathochromic base shift in the UV spectrum upon addition of strong base indicating the presence of a phenolic functionality. These data suggest a symmetrically substituted hydroxy-dimethoxycinnamoyl moiety, and a structure consistent with 4′-desmethylpiplartine (**2**). High resolution mass spectral data of the low R_f compound (**3**) indicates a molecular mass of 303.1084 and a molecular formula of $C_{16}H_{17}O_5N$, thus it is a structural isomer of 4′-desmethylpiplartine (**2**). The low resolution E.I. mass spectra of both, this compound and (**2**) have a base peak of m/z 207, corresponding to $C_{11}H_{11}O_4$, suggesting that they share a common cinnamoyl moiety. In addition, the resonances in the ^{1}H-NMR spectrum (Table 1) that correspond to the cinnamoyl portion of the molecule are identical to those of 4′-desmethylpiplartine (**2**) indicating the presence of a 4′-hydroxy-3′, 5′-dimethoxy-*trans*-cinnamoyl group. The ^{1}H-NMR resonances for the dihydropyridone ring are completely different from those of either (**2**) or (**1**) and consist of two isolated spin systems. One spin system consists of a broad doublet at 7.97 ppm coupled to a doublet at 5.43 ppm with a 8.4 Hz coupling constant, and each integrates for one proton. This is consistent with an isolated *cis*-double bond with the proton resonating at 7.97 ppm, adjacent to the nitrogen and exhibiting quadruple broadening. The other isolated spin system consists of a pair of triplets at 4.16 and 2.62

Table 3
Amide concentrations with and without ant mutualists (The error term for the *t*-test values was determined using the variance of amide concentrations in 24 randomly selected plants with and without ants)

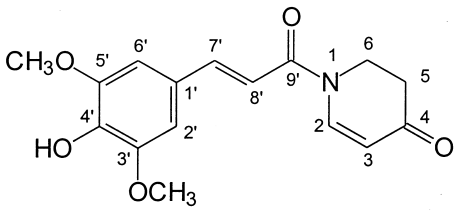
	Piplartine	4′-Desmethylpiplartine	Cenocladamide
With ants	0.14[a]	0.18	0.09
Without ants	0.58	0.45	0.33
t, (degrees of freedom)	18.0, (22)	9.7 (22)	5.4, (22)
P	< 0.001	< 0.001	< 0.001
Increase ratio Without ants/with ants	4.1	2.5	3.7

[a] % by dry mass.

ppm coupled only to each other with a 7.4 Hz coupling constant and integrating for two protons each. These data support a dihydropyridone ring in which the carbonyl carbon is at C4, with a pair of coupled methylene groups on one side of the ring, and a *cis*-double bond on the other side. The arrangement about the amide functionality is inferred by the close similarity in the ^{1}H-NMR chemical shifts of H5 and H6 for (**2**) and (**3**). These data support the structure illustrated, for which we propose the trivial name cenocladamide (**3**). This substitution pattern for the dihydropyridone ring has not been observed in the amides/alkaloids isolated thus far from members of the genus *Piper* (Parmar et al., 1997). The GC/MS analysis of a composite sample made of one leaf from each of 12 individual plants with ant colonies found concentrations of each amide that were between 0.1 and 0.2% by mass (Table 3). A similarly obtained composite sample for plants without ant colonies revealed individual amide concentrations between 0.3 and 0.6%. This is a statistically significant increase of approximately three-fold in the absence of ant mutualists and the necessity of producing food bodies to feed them (Table 3). The increase in amide concentrations in the absence of ant mutualists shows that this chemical defense can be induced in the absence of mechanical defenses. The fact that plants without ant colonies are less fit and fail to thrive suggests that the metabolic cost of relatively low amide levels and the production of food bodies is less than the cost of amides at three times that concentration. The presence of substantial amounts (0.1–0.2%) of each amide in the presence of ant mutualists suggests that mechanical means of defense alone are inadequate against all the various herbivores and pathogens attacking *P. cenocladum*. This indicates that the redundancy observed in chemical defenses also extends to systems where mutualism provides a mechanical means of defense (Romeo, Saunders & Barbosa, 1996).



1 R = CH_3
2 R = H



3

3. Experimental

Mps: uncorr. ^{1}H-NMR (270 MHz): $CDCl_3$. ^{13}C-NMR (68 MHz): $CDCl_3$. GC/MS: Chromatographed on a 5% phenylsilicon column ramped at 20°/min from 50 to 275°C with 70 eV electron impact ionization. HRMS: FAB (+) ion mode. UV: MeOH. IR: thin film from evaporated chloroform solution. Preparative separations carried out on silica gel G. TLC spots detected under UV (254 nm).

3.1. Plant material

Piper cenocladum C. DC. was collected at La Selva Biological Research Station in Costa Rice during June 1996 and again in June 1997. Voucher specimens are

kept in the herbarium at La Selva Biological Research Station.

3.2. Extraction and fractionation

Air-dried, milled leaves (20 g) were extracted overnight twice in 95% EtOH. After removal of solvent in vacuo, the residue was redissolved in 3:1 water/EtOH and the aqueous ethanol was sequentially partitioned against hexane, chloroform and ethyl acetate. The residue of the chloroform partition (195 mg) was subjected to column chromatography on silica gel using a gradient of 2:1–1:2 toluene:ethyl acetate. All fractions containing UV absorbing, visibly yellow spots were combined (88 mg) and streaked on a 2 mm preparative silica TLC plate. The PTLC was double developed in 2:1 toluene:ethyl acetate and three visibly yellow colored, UV absorbing bands corresponding to (**1**), (**2**) and (**3**) were recovered.

3.3. Quantitation of amides

One leaf from 12 individual plants with ant colonies and one leaf from 12 individual plants without ant colonies were collected and air dried. All leaves from plants with ants were combined and milled to produce a 146 g composite sample. Leaves from plants without ants were similarly treated to produce a 46 g composite sample. Each sample was extracted and solvent partitioned as described above. The hexane and chloroform partitions of each sample were analyzed separately by GC/MS using commercially available piperine as an internal standard at the 80 ug/ml level. Five point calibrations (50, 100, 200, 300 and 500 ug/ml) using isolated piplartine ($r^2 = 0.992$) and 4′-desmethylpiplartine ($r^2 = 0.986$) were prepared. Cenocladamide was unstable in solution and its structural isomer (**2**) was used as a standard for it, thus, the concentration of (**3**) is an estimate. Quantitation was by ion current in the molecular ion of each compound, m/z 317 for piplartine and m/z 303 for 4′-desmethylpiplartine and cenocladamide. Percent relative standard deviations (%RSD) for analytical triplicates of each amide in every hexane and chloroform partition analyzed, ranged from 0.3 to 8% (see Table 3).

3.3.1. *N-(4′-hydroxy-3′, 5′-dimethoxycinnamoyl)-Δ^2-pyridin-4-one, cenocladamide (**3**)*

Obtained as a pale yellow oil (17 mg). UV λ_{max} nm (log ε): 220 (4.70), 300 *sh* (4.53), 350 (4.67), 660 (0.39). IR ν_{max} cm^{-1}: 3400, 2950, 2870, 1730, 1670, 1605, 1525, 1470, 770. ^{1}H-NMR spectral data (see Table 1); ^{13}C-NMR spectral data (see Table 2). MS m/z (rel. int.): 303 $[M]^+$ (10), 207 (100), 175 (20); HRMS: Found 303.1084 (M^+), calculated for $C_{16}H_{17}O_5N$ 303.1107.

3.3.2. *N-(4′-hydroxy-3′,5′-dimethoxycinnamoyl)-Δ^3-pyridin-2-one, 4′-desmethylpiplartine (**2**)*

Obtained as a yellow oil (29 mg). UV λ_{max} nm (log ε): 220 *sh* (4.74), 270 (4.62), 345 (4.50). IR ν_{max} cm^{-1}: 3200, 3030, 2960, 2860, 1690 *br*, 1625 *br*, 1530, 1130, 895, 775. ^{1}H-NMR spectral data (see Table 1); ^{13}C-NMR spectral data (see Table 2). MS m/z (rel. int.): 303 (98), 275 (25), 207 (100), 175 (50); HRMS: Found 303.1105, calculated for $C_{16}H_{17}O_5N$ 308.1107.

3.3.3. *N-(3′,4′,5′-trimethoxycinnamoyl)-Δ^3-pyridin-2-one, piplartine (**1**)*

Obtained as pale yellow needles after recrystallization from MeOH (9.7 mg), mp 124–126°C (literature Capron & Wiemer, 1996, 121–123°C). Identified by spectral comparison (IR, UV, ^{1}H-NMR, ^{13}C-NMR and MS) with literature (Duh et al., 1990; Banerjee & Chaudhuri, 1986; Joshi, Kamat & Saksena, 1968).

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