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Two polyisoprenylated benzophenones from the trunk latex of *Clusia grandiflora* (Clusiaceae)

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Abstract

The polyisoprenylated benzophenones, chamones I and II, were isolated from the trunk latex of *Clusia grandiflora* (Clusiaceae) growing in southeastern Venezuela. A third benzophenone, nemorosone II, was isolated from the pollinator reward resin of the female flowers of the same plant. Chamone I and nemorosone II are structurally similar, differing only in the degree of prenylation. Bioassays of chamone I and nemorosone II using the honeybee pathogens, *Paenibacillus larvae* and *Paenibacillus alvei*, demonstrate that both have potent antibacterial activity, and that their structural differences affect both their bactericidal efficacies and their aqueous mobilities.

Keywords: *Clusia grandiflora*; Clusiaceae; Benzophenone; Nemorosone II; Chamone I; Chamone II; Antibacterial pollinator reward; Antibacterial latex; Pollinator reward evolution

1. Introduction

Both male and female plants of the dioecious, neotropical tree species *Clusia grandiflora* (Clusiaceae) secrete a viscous, hydrophobic resin from specialized floral tissues (Bittrich and Amaral, 1997). This substance serves as the pollinator reward for numerous apid-bee species that visit *C. grandiflora*. Resin is not a nutrititive reward; rather, bees use it as nest construction material. This type of pollinator reward system is rare, being known from only three angiosperm genera. Since the resins produced in these systems are derived from secondary metabolic pathways, they provide an opportunity to trace the evolutionary origins of a pollinator reward system that primary metabolic rewards do not. An earlier paper (Lokvam and Braddock, 1999) reported on the biological properties of *C. grandiflora* pollinator reward resins. It was found that the resins of both sexes contain potent bactericidal substances that are particularly toxic to the honeybee pathogens *Paenibacillus larvae* and *Paenibacillus alvei*.

In addition to the resin pollinator reward, *C. grandiflora* plants, like most clusiaceous taxa, maintain a second prominent pool of natural products in the form of a defensive latex. Latex is produced constitutively by the plant in a system of canals that run through all plant tissues. When tissues are damaged, latex oozes from the wound and forms a barrier against insect and possibly microbial invasion. Numerous studies have shown that the genus *Clusia* is a rich source of polyisoprenylated benzophenones. Several compounds from this class have been isolated from *C. grandiflora* floral resins (de Oliveira et al., 1996). To date, there have been no benzophenones identified from *C. grandiflora* latex. As part of an investigation of the evolutionary origins of the resin pollinator reward system, we take a bioassay-guided approach to explore the chemical and biological similarities between resin and

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latex components. We report on the isolation of a known benzophenone from female *C. grandiflora* resin and on the structures of two novel benzophenones from *C. grandiflora* latex. In addition, data are presented on the relative bioactivities of resin and latex benzophenones and demonstrate that these compounds are the agents responsible for the bioactivity of the *C. grandiflora* pollinator reward.

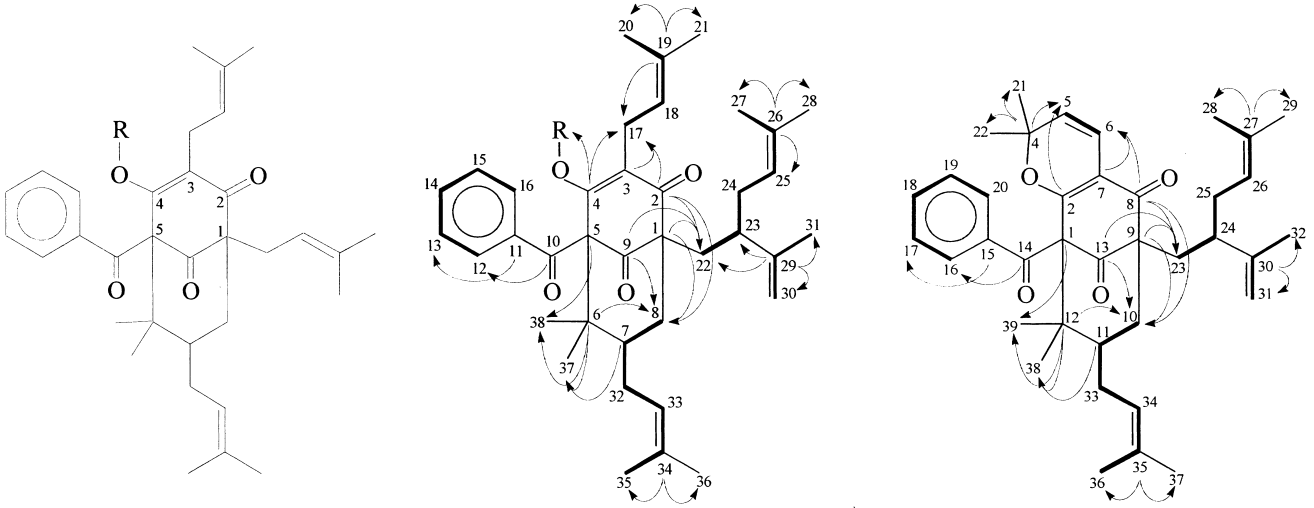
2. Results and discussions

A thin-layer chromatographic assay (Lokvam and Braddock, 1999) was used to visualize bactericidal components in *C. grandiflora* resins and latex. This led us to the characterization of benzophenones **1**, from female resin (Fig. 1), and **2**, from latex. Compound **3**, though biologically inactive, appeared to be a benzophenone and was isolated along with **2**. Compounds **1** and **2** are unstable when purified in their native state. Their study was expedited by derivitization with CH_2N_2 and both **1** and **2** were, therefore, isolated as their methyl ethers **1a** and **2a**.

All the three compounds share several spectral characteristics with other benzophenones and, as a result, our structural analyses were aided by earlier studies (de Oliveira et al., 1996; Delle Monache et al., 1991). ^{13}C NMR spectra show the presence of two conjugated and one unconjugated carbonyl carbons in each of the compounds. In the MS, all the three compounds have a base peak at m/z 105 indicating the presence of a unsubstituted benzoyl moiety. This is further substantiated by the presence of the multiplets of an AB_2X_2 spin system in the aromatic region of the ^1H NMR spectra. ^1H–^1H COSY and HMBC show that all the three compounds are extensively prenylated.

Compound **1a** was isolated from female *C. grandiflora* resin following treatment of the crude material with diazomethane. It was identified by analysis of HR-EIMS and NMR spectra as the methyl ether of nemorosone II, previously described from two other *Clusia* species (de Oliveira et al., 1999), but was not reported in an earlier study of *C. grandiflora* resins. Separation on silica gel yielded both the 3-ene-4-methoxy and 2-ene-2-methoxy tautomeric forms in the ratio of ca. 5:3.

Compound **2a** was isolated as a colorless oil from the diazomethane-treated crude latex. A total of 39 carbons were found in the ^{13}C NMR spectrum (Table 1). DEPT showed the presence of 10 methyl carbons as well as five methylene carbons, four of which were sp^3 and one sp^2. There were also two sp^3 and eight sp^2 methine carbons. By inference, **2a** has 13 quaternary centers, three of which are sp^3 and the remaining 10 sp^2 carbons, including two conjugated and one unconjugated carbonyls. HR-EIMS gave a molecular ion peak of m/z equal to 584.3863, confirming the molecular formula of $C_{39}H_{52}O_4$. The molecule thus exhibited 14 double-bond equivalents. In contrast to compound **1**, only a single tautomer of **2** resulted from treatment with diazomethane. Clues to the structural differences between **1a** and **2a** included the presence of an additional prenyl group and a terminal



1-R = H
1a-R = Me

2-R = H
2a-Me

3

Fig. 1. —= ^1H–^1H COSY connectivity; →= long range C–H correlation.

methylene carbon in compound **2a**, suggesting a second isopentenyl branching. ^{1}H–^{1}H COSY and HMBC showed that this branching occurs at C-23, and is accompanied by a double-bond isomerization to C-29 (Fig. 1).

The relative configuration of **2a** was determined using gradient 1D NOESY pulse sequences. Selective irradiation of the C-4 methoxide gave a positive NOE of 0.3% at the C-37 (equatorial) methyl and no increment at the axial methyl. Molecular modeling showed that if the B ring were in a boat conformation, it would be the axial gem-dimethyl in closer proximity to the methoxide. Since no axial-methyl NOE was observed here, we concluded that the B ring is in the chair conformation. The chair conformation would not be observed if the C-7 isopentenyl group were in an axial position owing to the strong 1,3-diaxial interactions with the A ring that would result. Moreover, C-4 methoxide irradiation gave positive NOEs at C-17 (1%) and C-18 (1%). No NOEs were observed at C-32 or C-33, as would be expected if the isopentenyl group were axial. These results indicated that C-7 hydrogen was axial and the relative configuration of the molecule was S^{*}. Though no configuration has been pre-

Table 1
NMR spectral data for *O*-methyl-chamone I (**2a**) and chamone II (**3**)[a]

C(O) #	*O*-methyl-chamone I (**2a**)		Chamone II (**3**)	
	$\delta^{13}C$	$\delta^{1}H$	$\delta^{13}C$	$\delta^{1}H$
1	63.8	–	72.5	–
2	197.3	–	166.2	–
3	123.4	–	–	–
4	169.7	–	83.5	–
5	74.4	–	123.9	5.21 (1H, *d*, $J = 10.3$ Hz)
6	47.9	–	114.5	6.47 (1H, *d*, $J = 10.3$ Hz)
7	42.3	1.63 (1H, *o*)	113.2	–
8	45.8	1.92 (1H, *dd*, $J = 12.8$, 3.5 Hz), 1.45 (1H, *o*)	192.2	–
9	207.4	–	64.3	–
10	193.5	–	44.8	1.96 (1H, *o*, eq), 1.45 (1H, *o*, ax)
11	137.4	–	43.0	1.66 (1H, *o*)
12	128.5	7.72 (1H, *dm*, $J = 7.2$ Hz)	48.7	–
13	128.1	7.32 (1H, *tm*, $J = 2.2$ Hz)	206.2	–
14	132.2	7.46 (1H, *tt*, $J = 7.2$, 1.2 Hz)	193.2	–
15	128.1	7.32 (1H, *tm*, $J = 7.2$ Hz)	137.3	–
16	128.5	7.72 (1H, *dm*, $J = 7.3$ Hz)	128.7	7.74 (1H, *dm*, $J = 7.1$ Hz)
17	23.4	3.28 (2H, *dd*, $J = 6.4$, 1.0 Hz)	128.1	7.33 (1H, *tm*, $J = 7.1$ Hz)
18	121.8	5.01 (1H, *o*)	132.3	7.45 (1H, *tt*, $J = 7.1$, 1.0 Hz)
19	133.1	–	128.1	7.33 (1H, *tm*, $J = 7.2$ Hz)
20	18.3	1.67 (3H, *o*)	128.7	7.72 (1H, *dm*, $J = 7.2$ Hz)
21	26.0	1.68 (3H, *o*)	30.2	1.34 (3H, *s*)
22	35.1	2.16 (1H, *dd*, $J = 14.1$, 11.7 Hz), 1.76 (1H, *dd*, $J = 14.1$, 5.0 Hz)	28.7	0.61 (3H, *s*)
23	44.3	2.52 (1H, *m*)	34.9	2.16 (1H, *dd*, $J = 14.3$, 11.3 Hz), 1.77 (1H, *dd*, $J = 14.3$, 4.8 Hz)
24	33.2	2.00 (2H, *dd*, $J = 7.2$, 7.2 Hz)	44.3	2.54 (1H, *m*)
25	123.2	5.01 (1H, *o*)	33.3	2.02 (2H, *o*)
26	131.7	–	123.2	5.02 (1H, *o*)
27	18.3	1.60 (3H, *s*)	131.8	–
28	26.0	1.68 (3H, *o*)	18.2	1.60 (3H, *br s*)
29	148.6	–	26.1	1.68 (3H, *o*)
30	112.4	4.67 (1H, *dd*, $J = 2.9$, 1.6 Hz), 4.58 (1H, *br d*, $J = 2.9$ Hz)	148.6	–
31	18.1	1.56 (3H, *s*)	112.5	4.67 (1H, *br q*, $J = 2.9$ Hz), 4.61 (1H, *br d*, $J = 12$ Hz)
32	27.9	2.07 (1H, *o*), 1.63 (1H, *o*)	18.2	1.57 (3H, *br s*)
33	122.7	4.92 (1H, *m*)	27.9	2.12 (1H, *o*), 1.68 (1H, *o*)
34	133.4	–	122.5	4.96 (1H, *o*)
35	18.1	1.55 (3H, *s*)	133.6	–
36	26.1	1.67 (3H, *o*)	18.3	1.55 (3H, *br s*)
37	24.7	1.30 (3H, *s*, eq)	26.2	1.68 (3H, *o*)
38	16.4	1.17 (3H, *s*, ax)	16.6	1.19 (3H, *s*, ax)
39	–	–	24.7	1.36 (3H, *s*, eq)
OMe	61.6	3.43	–	–

[a] Key: *s* = singlet; *d* = doublet(s); *t* = triplet(s); *q* = quartet; *m* = multiplet(s); *o* = overlap; *br* = broad; ax = axial; eq = equatorial.

viously assigned to nemorosone II, 1D NOESY data from *O*-methyl nemorosone II isolated by us showed that compound **2** and nemorosone II have the same relative stereochemistry.

Chamone II (**3**) was obtained as a yellowish oil from the diazomethane-treated crude latex. A total of 38 carbons were found in the ^{13}C NMR spectrum, again with two aromatic peaks and one methyl peak at double intensity (Table 1). DEPT showed the presence of nine methyl carbons, four sp^3 and one sp^2 methylene carbons, and two sp^3 methine carbons. In addition, there were nine sp^2 methine carbons. By inference, there are 13 quaternary carbons; four are sp^3 and the remaining nine are sp^2 carbons, including two conjugated and one unconjugated carbonyls. HR-EIMS gave a molecular ion peak of m/z equal to 568.3558, confirming the molecular formula of $C_{38}H_{48}O_4$ and indicating 15 double-bond equivalents. The absence of an oxygen-bonded methyl showed that the molecule had not reacted in the presence of diazomethane. This was confirmed in side-by-side TLC comparisons of crude and diazomethane-treated latex, where the molecule had identical R_f values and the same characteristic violet fluorescence under UV light (254 nm). Comparison of the ^1H and HMBC spectra of **3** showed two pairs of sharp, upfield methyl singlets at δ 0.61/1.34 and 1.19/1.36, each of which correlated through an sp^3 quaternary carbon, indicating the presence of two pairs of ring-bonded gem-dimethyls. The downfield pair had an analogue in **2** whereas the upfield pair did not. The latter also had long range correlations with coupled olefinic protons at δ 6.47 (*d*, $J = 10.2$ Hz) and 5.21 (*d*). The additional sp^3 quaternary center and the lack of reactivity with diazomethane suggested that the C-3 isopentenyl group, which remains open in compound **2**, had ring closure at the C-2 enolic hydroxyl in compound **3**. ^1H–^1H COSY and HMBC confirmed this to be the case (Table 1 and Fig. 1).

Both compounds **1** (nemorosone II) and **2** (chamone I) showed pronounced activity in TLC bioassays. These two compounds serve entirely different functions with respect to the ecology of the plant, and are, therefore, likely to have evolved under selection for different functional characteristics. One of these is antimicrobial activity. We compared the bactericidal efficacies of **1** and **2** in side-by-side bioassays, using a previously described disk-diffusion assay technique (Lokvam and Braddock, 1999) and the honeybee pathogens *P. larvae* and *P. alvei* as assay microorganisms. From earlier TLC bioassays, it was known that neither compound **3** nor the methyl ethers of **1** or **2** were bactericidal. This suggests that the presence of an enol functionality is necessary for antibacterial activity in these molecules. No further assays were performed with either the methyl ethers or **3**.

The results of the bioassays of **1** and **2** are presented in Table 2. Both compounds are clearly toxic to the *Paenibacillus* honeybee pathogens. Given that the assays were run at equal test masses (5 μg/disk) it appears that nemorosone II (**1**), in contrast to chamone I (**2**), is more diffusible in the semi-aqueous agar medium and more effective as a bactericide. This is most evident in the *P. larvae* assay; equivalent zones of total inhibition were observed surrounding both nemorosone II and chamone I disks but nemorosone II disks had a further zone of partial inhibition. In the *P. alvei* assay, both compounds appear to be less toxic to the assay organism. The greater diffusibility of nemorosone II is evidenced by its dilution to the point where a zone of total inhibition no longer exists after 35 h of incubation. This is in contrast to chamone I, where there is a persistent concentrated core free of bacterial growth. These data indicate that even minor changes in the prenylation pattern of these molecules produce readily observable effects on their biological activity.

TLC bioassays show that a single component, now identified as nemorosone II, is the basis of nearly all the bioactivity in female *C. grandiflora* resin in Venezuela (Lokvam and Braddock, 1999). This compound comprises more than 50% of the mass of female *C. grandiflora* resin and is, therefore, a major portion of the pollinator reward produced by this plant. By contrast, chamone I comprises just 1–2% of the mass of latex. The structural data presented here point toward a common biogenetic origin for the benzophenones that are found in these two pools of natural products. Given that the latex defensive system occurs in many clusiaceous genera and that the resin pollinator reward occurs in only two, latex defense appears to be evolutionarily ancestral to the pollinator reward system

Table 2
Disk-diffusion assay results: *Clusia grandiflora* benzophenones **1** and **2** vs. *Paenibacillus larvae* (*P. l.*) and *Paenibacillus alvei* (*P. a.*)

5 μg/disk	Inhibition zone diameter (mm)			
	Compound **1**		Compound **2**	Control
	Total	Partial	Total	
P. l. @				
35 h				
Mean	19.5	27.3	19.7	0
S.E.	1.6	0.2	0.3	–
P. a. @				
20 h				
Mean	19.3	–	13.3	0
S.E.	0.7	–	1.2	–
35 h				
Mean	2.3	17.3	10.0	0
S.E.	2.3	1.5	0.6	–

found in *C. grandiflora* and other *Clusia* species. Resin-collecting bees are known to be attracted to the latexes of clusiaceous species. The mechanical properties of latex, malleability and hydrophobicity, no doubt make it a useful nest building material. But the antibacterial activity of the benzophenones in latex may also contribute to its attractiveness to apids. As has been suggested by Bittrich and Amaral (1997), early apids may have visited the ancestors of modern *Clusia* to wound floral tissues and remove latex. Over evolutionary time, plants responded to selection by these insects with the development of secretory structures, thereby recruiting a group of faithful pollinators in the resin-collecting bees. The abundance of bioactive benzophenones in the resin compared to latex suggests that these compounds are of benefit to bees, and that their greatly amplified expression in resin is similarly the result of pollinator selection.

3. Experimental

3.1. General

IR absorptions were measured on a Nicolet 560 FTIR spectrometer. Mass spectra were determined on a Finnigan MAT 95 spectrometer operating at a resolution of 8780. ^{1}H and ^{13}C spectra were acquired on a Varian 300 MHz spectrometer using $CDCl_3$ as solvent and TMS (^{1}H) and $CDCl_3$ (^{13}C) as internal references. HMBC acquisitions were optimized for $J_{CH} = 8$ Hz.

3.2. Plant material

Resin and latex from *C. grandiflora* were collected in the Parque Nacional Canaima in southeastern Venezuela in October and November 1997. Voucher specimens of both male and female plants are deposited with the Herbario Nacional de Venezuela in Caracas, Venezuela (accession numbers VEN 294991, VEN 294992) and at the Herbario Regional de EDELCA in San Ignacio Yuruaní, Bolívar, Venezuela. The bioassay bacteria, acquired from the American Type Culture Collection, were *P. larvae* (ATCC #25923) and *P. alvei* (ATCC #9545).

3.3. Collection, bioassay-guided fractionation and isolation

Details of resin collection have been previously reported (Lokvam and Braddock, 1999). Latex was collected by scoring trunk tissue and then scraping the latex as it oozed from the wound. The material was transferred to Teflon-stoppered glass vials and returned to the University of Alaska Fairbanks for analysis. Methylations of **1** and **2** were performed by dissolving crude resin or latex in a CH_2N_2-saturated ether solution. A previously described TLC bioassay technique (Lokvam and Braddock, 1999) was used to screen for bioactivity in specific components in underivatized resin and latex. Mobility differences of derivatized/underivatized components were determined by TLC comparisons. Separation of derivatized compounds were performed by CC using 40 μm mesh silica gel and 100% benzene for resin and 85% petroleum ether:7.5% acetone:7.5% EtOAc for latex.

3.4. Disk-diffusion bioassays

CC (40 μm mesh silica gel) was used to isolate the pure, underivatized benzophenones from resin and latex. Nemorosone II was separated using dichloromethane with 1% acetone. Chamone I was separated using 85% petroleum ether:7.5% acetone:7.5% EtOAc. The latter solvent system was used to apply 5 μg of both compounds **1** and **2** and the solvent control to the filter paper disks using a 10 μl Hamilton syringe. The solvent control was prepared using Tween 80 at 10 mg/ml of solvent. The pure benzophenones were stabilized following separation by collecting fractions into tubes held in a dry ice/ethanol bath. Those containing the compounds of interest were concentrated and stored at −80°C while the assay was prepared. Details of the disk-diffusion assay protocol are elaborated elsewhere (Lokvam and Braddock, 1999). Assays were run in triplicate. *P. larvae* plates were incubated at 37°C and *P. alvei* plates at 30°C.

Chamone I: [7-(3-methylbut-2-enyl)-1-[5-methyl-2-(1-methylvinyl)hex-4-enyl]-(3-methylbut-2-enyl)-4-methoxy-6,6-dimethyl-5-(phenylcarbonyl)bicyclo[3.3.1]non-3-ene-2,9-dione]. Oil, IR; (KBr) ν_{max} cm^{-1}: 2975, 2972, 1724, 1704, 1658, 1606, and 1448, HR-EIMS 70 eV, *m/z* (rel. int.): 584.3863 $[M]^-$, 515 (20), 448 (45), 325 (100), 269 (50), 105 (55), and 69 (29).

Chamone II: [11-(3-methylbut-2-enyl)-9-[5-methyl-2-(1-methylvinyl)hex-4-enyl]-4,4,12,12-tetramethyl-3-oxa-1-(phenylcarbonyl)tricyclo[7.3.1.0 $<2,7>$]trideca-2(7),5-diene-8,13-dione]. Oil, IR; (KBr) ν_{max} cm^{-1}: 3023, 2405, 1723, 1647, 1592, 1424, and 1218; HR-EIMS 70 eV, *m/z* (rel. int.): 568.3558 $[M]^-$, 499 (5), 434 (25), 417 (44), 309 (100), 189 (17), 105 (38), and 69 (24).

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