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6-Alkyl-3,4-dihydro-2*H*-pyrans: Chemical Secretion Compounds in Neotropical Harvestmen

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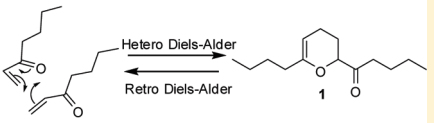
Supporting Information

ABSTRACT: The defensive secretions of five neotropical species of harvestmen (Opiliones: Gonyleptidae) from the Brazilian Atlantic Forest were analyzed and chemically characterized by GC-MS and NMR methods. Three of the species, *Cobania picea, Roweria virescens*, and *Serracutisoma proximum*, secrete a mixture of 2,3-dimethyl-1,4-benzoquinone and 2-ethyl-3-methyl-1,4-benzoquinone. The secretions produced by the other two species, *Iporangaia pustulosa* and *Neosadocus maximus*, contain 1-hepten-3-one, 5-methyl-1-hexen-3-one, and 1-(6-butyl-3,4-dihydro-2*H*-pyran-2-yl)pentanone (**1**) as major components, as well as 2,3-dimethyl-1,4-benzoquinone and 2-ethyl-3-methyl-1,4-benzoquinone as minor constituents. The dihydropyran 1-(6-butyl-3,4-dihydro-2*H*-pyran-2-yl)pentanone (**1**) is a new natural product, composed of two 1-hepten-3-one subunits formally linked in a hetero-Diels−Alder reaction. The natural product was proven to be racemic, and its biogenetic origin is discussed.



The order Opiliones, popularly known as harvestmen, constitutes the third major group of arachnids, with nearly 6000 known species distributed worldwide.[1] Harvestmen are divided into four suborders (Laniatores, Dyspnoi, Eupnoi, and Cyphophthalmi) that contain 45 recognized families and about 1500 genera. These four suborders are recognized as monophyletic groups, although their relationship is still under discussion.[2] One of the 26 famililies within the Laniatores suborder is Gonyleptidae, a large neotropical family comprising almost 900 species that occur in Central and South America and englobes about 97% of the Brazilian species. All species of Opiliones possess a pair of exocrine glands called "scent glands", which are located at the laterofrontal angles of the prosoma and produce volatile compounds.[2] These exudates tend to have a strong smell and are known to protect the individuals against predators such as ants, frogs, lizards, and spiders.[3−5] The scent gland exudates of ca. 60 species of harvestmen have been characterized, indicating that a few species release only one compound in their scent gland exudates, while most species release a blend that varies from species to species.[2,6,7] Blends of benzoquinones, phenols, and alkyl vinyl ketones are usually present in the secretions of Opiliones from the Laniatores suborder.[2,6] Within the family Gonyleptidae (Laniatore) the chemical defense of ca. 30 species was investigated, and the chemical data did not fit into the phylogenetic lineages. However analysis of a larger number of subfamilies and the study of the relationships among groups of compounds might provide clue compounds to build a chemical phylogenetic tree.[6] In the present study, scent gland exudates of five harvestman species, belonging to the family Gonyleptidae, were characterized: *Cobania picea* (Cobaniinae), *Iporangaia pustulosa* (Progonyleptoidellinae), *Neosadocus maximus* (Gonyleptinae), *Roeweria virescens* (Pachylinae), and *Serracutisoma proximum* (Goniosomatinae). The structures of the compounds released from the scent glands of *I. pustulosa*, *N. maximus*, and *R. virescens* have been previously identified by GC-MS,[6] but for *S. proximum* and *C. picea* there was no prior information on the chemistry of the scent gland exudates. Moreover, a constituent reported as unknown[6] was identified in exudates of *I. pustulosa* and *N. maximus* whose structure and synthesis are described herein.

RESULTS AND DISCUSSION

Like most of the representatives of the suborder Laniatores,[2] scent gland exudates of *C. picea* (Figure 1A), *I. pustulosa*, *N. maximus*, *R. virescens*, and *S. proximum* are mixtures of volatile compounds, which were analyzed by GC-MS and ^{1}H and ^{13}C NMR. The GC-MS analyses of these exudates can be roughly separated into two groups. The first group of secretions is mainly composed by two benzoquinones produced by *C. picea, R. virescens*, and *S. proximum*. Their fragmentation patterns by EIMS showed molecular ions at *m*/*z* 136 and 150, both compounds with an intense peak at *m*/*z* 54. Their ^{1}H and ^{13}C NMR spectra

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were identical to those of 2,3-dimethyl-1,4-benzoquinone and 2-ethyl-3-methyl-1,4-benzoquinone from *Goniosoma longipes*, previously characterized.[5] The second group of exudates secreted by *I. Pustulosa* and *N. maximus* (Table 1) were shown by GC-MS to have predominance of a vinyl ketone of seven carbons with a molecular ion at *m/z* 112 and a fragmentation pattern consistent with 5-methyl-1-hexen-3-one, 4-methyl-1-hexen-3-one, or 1-hepten-3-one. Hara et al.[6] reported chromatographic retention times of 3.68 min (5-methyl-1-hexen-3-one), 3.74 min (4-methyl-1-hexen-3-one), and 4.63 min (1-hepten-3-one) and did not report relative retention indices, which prevented a structural definition by comparison. Characterization by ^{1}H NMR revealed the presence of only one methyl group (a triplet) at 0.91 ppm, assigned to methyl C-7 of 1-hepten-3-one (Figure 2) as the major constituent of this second group of exudates. The ^{1}H and ^{13}C NMR chemical shifts were fully assigned and were identical to those of a synthetic sample previously synthesized in our laboratories and also reported by others.[8] According to Hara et al.[6] 5-methyl-1-hexen-3-one was expected to be the major compound in *N. maximus* scent gland exudates, but in our analyses this compound was present at only 1.7% (Table 1), revealing that the qualitative exudate composition can change from individual to individual.

Another major constituent of the scent gland exudates in the group of *I. pustulosa* and *N. maximus* had a molecular ion at *m/z* 224 (Table 1). This compound was previously reported as unknown by Hara et al.[6] Analysis of the EIMS pattern was

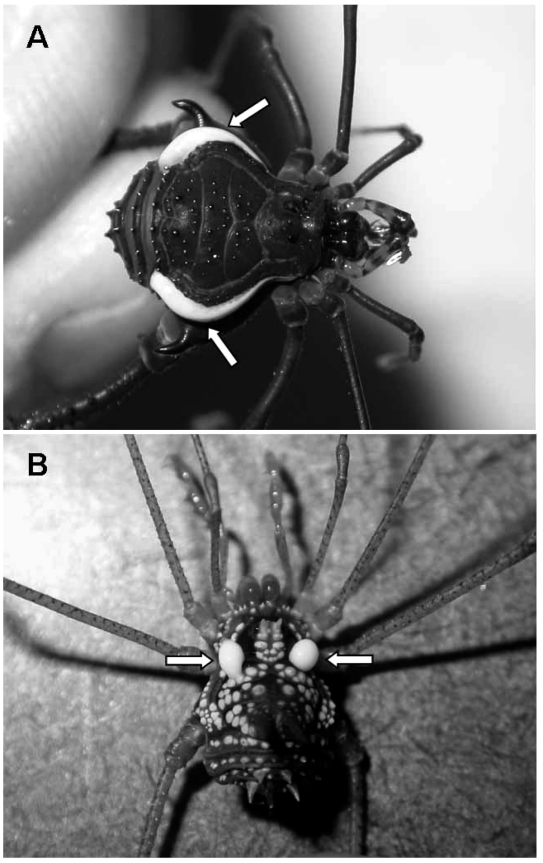

Figure 1. (A) Male of *Cobania picea* releasing scent gland exudates, which flow along the lateral margins of the dorsal scute and accumulate at the lateroposterior area of the body (white arrows). (B) Male of *Iporangaia pustulosa* with two droplets of exudate on the gland openings (white arrows).

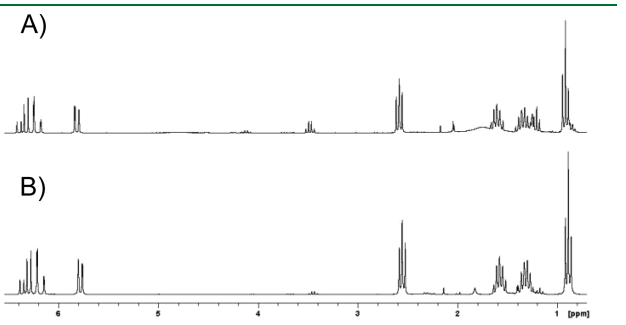

Figure 2. ^{1}H NMR ($CDCl_3$, 250.13 MHz) spectra of 1-hepten-3-one: (A) natural product and (B) synthetic product.

Table 1. GC-MS Analyses of the Scent Gland Exudates of *Cobania picea, Iporangaia pustulosa, Neosadocus maximus, Roeweria virescens*, and *Serracutisoma proximum*

compound	ret. index	characteristic ions (m/z)	species	relative abundance
5-methyl-1-hexen-3-one	758	112[M]$^+$, 97, 84, 70, 55, 41	*N. maximus*	1.7%
1-hepten-3-one	801	112[M]$^+$, 97, 83, 70, 55, 41	*I. pustulosa*	79.0%
			N. maximus	79.2%
2,3-dimethyl-1,4-benzoquinone	1098	136[M] $^+$,107, 82, 79, 65, 54	*I. pustulosa*	3.0%
			N. maximus	2.0%
			S. proximum	67.1%
			R. virescens	72.0%
			C. picea	57.0%
2-ethyl-3-methyl-1,4-benzoquinone	1185	150[M]$^+$, 122, 121, 107, 82, 79, 67, 54	*I. pustulosa*	4.4%
			S. proximum	32.9%
			R. virescens	28.0%
			C. picea	43.0%
1	1691	224[M]$^+$,139, 182, 97, 95, 85, 79, 69, 55, 41	*I. pustulosa*	13.6%
			N. maximus	15.6%
not identified	1858	156[M]$^+$, 155, 127, 85, 57, 55, 42, 41	*N. maximus*	1.5%

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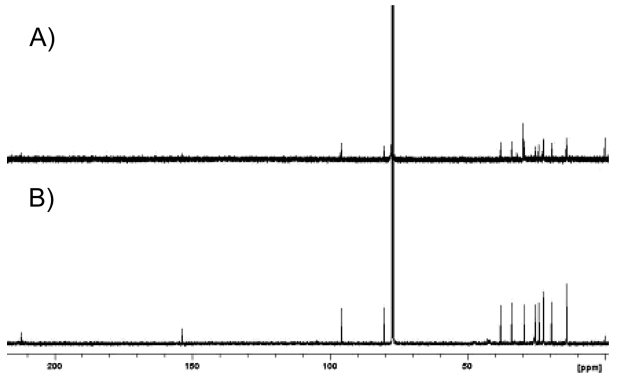


Figure 3. ^{13}C NMR ($CDCl_3$, 125.71 MHz) spectra of **1**: (A) natural product and (B) synthetic product.

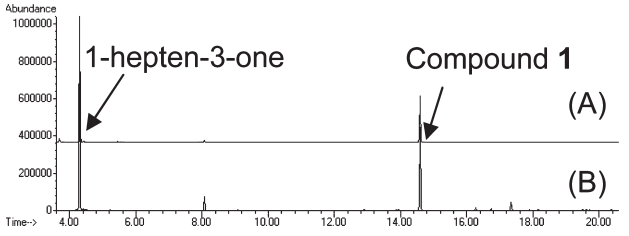
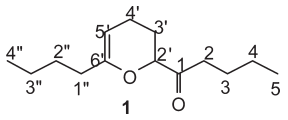


Figure 4. Chromatograms of (A) co-injection of synthetic 1-hepten-3-one and **1** mixed with *N. maximus* exudate and (B) *N. maximus* exudate.

consistent with 1-(6-butyl-3,4-dihydro-2*H*-pyran-2yl)pentanone (**1**), which is the dimer of 1-hepten-3-one. This suggestion required a pure sample for better characterization. Taking into consideration that the *N. maximus* scent gland exudate was mainly comprised of the relative low-boiling-point 1-hepten-3-one and the high-boiling **1**, a nitrogen stream was blown over the exudate of *N. maximus,* leaving **1** almost pure. The ^{13}C NMR spectrum revealed the presence of 14 signals (two methyl, eight methylenes, one methine, two olefinic carbons attached to a trisubstituted double bond, and one carbonyl group). Data for the olefinic carbons at 153.5 and 95.6 ppm, the methine at 80.1 ppm, and the two methylenes at 19.3 and 37.8 ppm are in accordance with those reported for 1-(6-methyl-3,4-dihydro-2*H*-pyran-2-yl)-1-ethanone.[9] Total assignment of ^1H and ^{13}C NMR signals based on homonuclear and heteronuclear correlations, comparing natural product **1** with a synthetic standard obtained by dimerization of 1-hepten-3-one in a hetero-Diels–Alder reaction, revealed that **1** was 1-(6-butyl-3,4-dihydro-2*H*-pyran-2yl)pentanone (Figures 3 and 4, Table 2).

We suspected that the biogenetic origin of compound **1** was an enzyme-promoted hetero-Diels–Alder reaction between two 1-hepten-3-one subunits (Scheme 1). Biosynthetic transformations involving hetero-Diels–Alder cycloadditions have been recognized in many known classes of natural products such as polyketides, isoprenoids, phenylpropanoids, and alkaloids.[10] To investigate this hypothesis we analytically screened the presence of 1-hepten-3-one and the dimer **1** directly in the gland sac and in the exudate (Table 3) and found the mixture to be similar in both sources. We, therefore, conclude that the dimer does not release the monomer due to a retro-hetero Diels–Alder reaction and that dimer **1** formation is not a strategy to store more 1-hepten-3-one. We further expected that formation of **1** involving an enzymatic reaction should produce a nonracemic dimer with high predominance of one enantiomer. Consequently, we used

Table 2. ^1H and ^{13}C NMR ($CDCl_3$) Data for Natural and Synthetic 1-(6-Butyl-3,4-dihydro-2*H*-pyran-2-yl)-pentanone (1)



	synthetic **1**[a]		natural **1**[b]
position	δ_C, mult.	δ_H (J in Hz)	δ_C
1	211.8, C		211.8
2	37.8, CH_2	2.65, 2.55, dd (17.7, 7.4)	37.8
3	25.2, CH_2	1.56, m	25.2
4	22.3[c], CH_2	1.30, m	22.3[c]
5	13.9[d], CH_3	0.91 (7.3)	13.9[d]
2′	80.2, CH	4.25, dd, (8.6, 3.1)	80.1
3′	24.0, CH_2	1.82, 1.98, m	24.0
4′	19.3, CH_2	1.98, 2.00, m	19.3
5′	95.6, CH	4.52, t (3.4)	95.6
6′	153.5, C		153.5
1″	33.8, CH_2	2.06, t (7.7)	33.8
2″	29.1, CH_2	1.48, m	29.7
3″	22.4[c], CH_2	1.30, m	22.4[c]
4″	14.0[d], CH_3	0.91, t (7.3)	13.9[d]

[a] Results from ^1H and ^{13}C NMR (fully decoupled and DEPT-90 and DEPT-135) and 2D NMR gCOSY ($^1H-^1H$) and HSQC ($^1H-^{13}C$ ^1J) experiments. [b] *I. pustulosa* exudate after removing 1-hepten-3-one. [c,d] Interchangeable assignments within the table column.

enantioselective gas chromatography to answer this question. The synthetic racemate **1** (obtained through Diels–Alder dimerization of 1-hepten-3-one) was resolved on a Lipodex E capillary column, and comparison of the natural and synthetic racemate revealed the presence of two peaks in a 1:1 ratio in both samples (Figure 5), proving the natural product to be racemic. This suggested that either the catalytic enzymatic reaction is not necessary for the dimerization of 1-hepten-3-one *In Vivo* or racemization of **1** (via the enolate) is spontaneous.

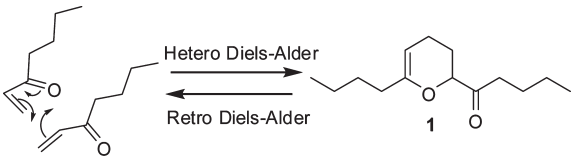
Our corresponding investigations showed that the synthetic dimer was formed only under special conditions of pressure and temperature and with catalytic amounts of hydroquinone under an inert atmosphere, but never occurred spontaneously. The only spontaneous reaction at room temperature involving the natural and the synthetic **1** was the retro-Diels–Alder reaction. The dimerization of 1-hepten-3-one was carefully followed under ordinary conditions; however, at the most, only traces of **1** were observed (see Supporting Information). We, therefore, conclude that a Diels–Alderase is involved in the biosynthesis of the new dihydropyran.

Our chemical analyses was merged into Hara's cladogram,[6] and a preliminary phylogenetic pattern emerges. The presence of the ketones, 1-hepten-3-one and 5-methyl-1-hexen-3-one, and other vinyl alkyl ketones, seems to occur in representatives of a monophyletic clade composed of the subfamilies Gonyleptinae, Hernandariinae, Sodreaninae, Progonyleptoidellinae, and Caelopyginae. This suggests that these ketones may be regarded as a

Table 3. GC-MS Analyses of Gland Sacs and Exudate of *Iporangaia pustulosa* and *Neosadocus maximus* (samples collected from different individuals)

	I. pustulosa		*N. maximus*	
compound (ret. time (min))	exudate (%)	gland sac (%)	exudate (%)	gland sac (%)
5-methyl-1-hexen-3-one (3.8)		1.6	1.7	9.9
1-hepten-3-one (4.2)	79.0	74.1	79.2	68.7
3-heptanone (4.3)		1.6		1.0
2,3-dimethyl-1,4-benzoquinone (7.8)	3.0	2.7	2.0	
2-ethyl-3-methyl-1,4-benzoquinone (8.8)	4.4	7.5		
(C_3H_7)-1,4-benzoquinone (9.4)		0.7		
2,6-diethyl-1,4-benzoquinone (10.3)		1.6		
unknown (13.5)		0.5		
isomer of **1** (13.5)				0.4
isomer of **1** (13.6)				0.6
1 (14.3)	13.6	9.7	15.6	19.4
unknown (16.4)			1.5	

Scheme 1. Dimerization Reaction of 1-Hepten-3-one Yielding Compound 1



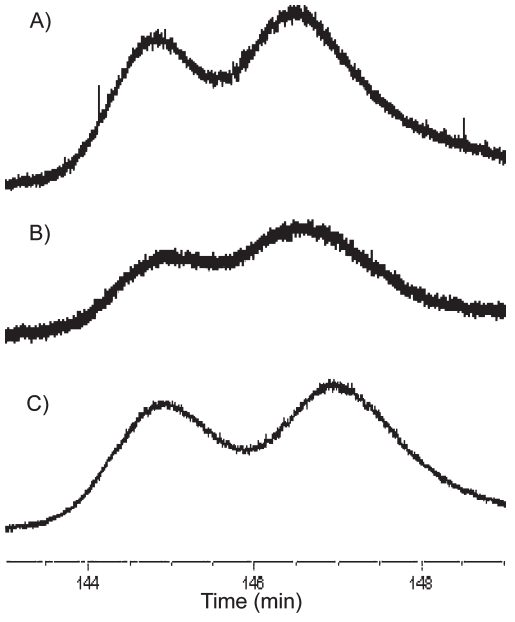


Figure 5. GC-FID (using a chiral column similar to Lipodex-E) of (A) synthetic compound **1**, (B) *Neosadocus maximus* exudate, and (C) *Iporangaia pustulosa* exudate.

synapomorphic trait of this group of subfamilies. Here, 2,3-dimethyl-1,4-benzoquinone and 2-ethyl-3-methyl-1,4-benzoquinone are found in the scent gland exudates of *R. virescens*, *S. proximum*, and *C. picea* as major compounds, and also in *I. pustulosa* exudates as a minor constituent for the first time (Table 1), despite being distantly related species.[11] In the family Cosmetidae, a sister group of the family Gonyleptidae,[12,13] 2,3-dimethyl-1,4-benzoquinone has been recorded in four species: *Eucynortula nannocornuta*,[14] *Paecilaema eutypa*,[15] *Paecilaemana quadripunctata*,[15] and *Vonones sayi*.[16] Given its widespread occurrence among representatives of Cosmetidae and Gonyleptidae,[5,6] including the basal subfamily Cobaniinae,[12] benzoquinones can be ancient components of the scent gland exudates in these two families.

Our chemical results are in accordance with the group phylogeny and a larger number of species should be analyzed to recognize groups using analogous biosynthetic pathways to produce their chemical defense. Moreover, a new harvestmen secretion constituent, the dihydropyran **1**, was identified by MS, ^{1}H and ^{13}C NMR spectroscopies, and synthesis.

EXPERIMENTAL SECTION

Secretion Sampling. Individuals of *S. proximum*, *I. pustulosa*, *N. maximus*, and *R. virescens* were collected in an Atlantic Forest fragment at Intervales State Park (24°14′ S; 48°04′ W; 800 m alt.), state of São Paulo, southeastern Brazil (for a detailed description of this site, see ref 17). Individuals of *C. picea* were collected at the edge of an Atlantic Forest fragment near Itatiaia National Park (22°15′ S; 44°34′ W; 2100 m alt.), state of Minas Gerais, southeastern Brazil. Individuals of all studied species were taken to the laboratory and kept alive in plastic vials containing a piece of wet cotton to maintain moisture. The scent gland exudates were collected by pressing the gland openings with cotton wool previously cleaned with doubly distilled EtOAc. The liquid absorbed in the cotton wool was washed with $CDCl_3$ (2 mL) for the NMR analyses and eluted with EtOAc (2 mL) for the GC-MS analyses. Between 7 and 30 individuals of each species were used for each extraction.

Scent Glands. Five individuals of each species were dissected, and their gland sacs were removed. In many representatives of the suborder Laniatores, both glandular sacs remain firmly attached to the gland opening when the dorsal scute is carefully removed under a stereomicroscope. Thus, after removing the dorsal scute, the gland sacs were detached and macerated in a vial containing ethyl acetate. Different vials were used to store the scent gland exudates of each harvestman species.

General Experimental Procedures. The IR spectra were recorded with a Thermo Scientific Nicolet 380 FT-IR Smart Performer spectrophotometer. NMR spectra were acquired with either an 11 T Varian Inova spectrometer, operating at 499.88 MHz for ^{1}H NMR and 125.71 MHz for ^{13}C NMR, or a 5.87 T Bruker Avance DPX

spectrometer, operating at 250.13 MHz for ^{1}H NMR and 62.89 MHz for ^{13}C NMR. $CDCl_3$ was used as the solvent and TMS as an internal reference (0.0 ppm). Chemical shifts (δ) were recorded in ppm and coupling constants J in Hz. ESIMS analyses were performed using a LTQ-Orbitrap hybrid mass spectrometer (Thermo Fischer Scientific, Bremen, Germany). Nitrogen gas was used for the nebulization, desolvation, and collision-induced dissociation (CID). The sample was diluted in MeOH, infused at a flux of 10 μL min^{-1}, and detected in the positive ion mode. Sheath gas = 8, spray voltage = 3.5 kV, capillary voltage = 43 V, capillary temperature = 275 °C. Full scan experiments (range m/z 150−400) were performed in both a linear trap as well as the Orbitrap. Masses were acquired as profile data at a resolution of 30 000 at m/z 400. The automatic gain control (AGC) ion population target in full scan MS was 50 000 for LTQ-MS and 500 000 for Orbitrap-MS, and the ion population target for MS^n was set to 10 000 for LTQ-MS. Mass data were analyzed with Xcalibur software (Thermo Finnigan). GC-MS analyses were carried out using an Agilent 6890/5973 system equipped with an HP-5 fused silica capillary column (30 m × 0.25 mm × 0.25 mm). Oven temperatures were programmed from 50 to 200 °C at 10 °C min^{-1} and subsequently to 290 °C (16 °C min^{-1}) for general analysis. Samples were injected in the split mode (1/50). The injector temperature was 250 °C. Helium was used as the carrier gas at a flow rate of 1 mL min^{-1}. Determination of the retention index (RI) of compound **1** used a temperature program from 50 to 290 °C at a rate of 4 °C min^{-1} and held at 290 °C for 20 min; injection was carried out in the splitless mode. Retention indices were obtained by co-injecting the harvestmen exudates with a mixture of hydrocarbons (C_8, C_{11}, C_{17}, C_{25}, and C_{32}) (Aldrich). A plot was generated with the *n*-hydrocarbon retention times vs the hydrocarbon carbon number × 100, generating the equation RI = 38.496X + 586.88 by linear regression. Consequently, the retention index of each constituent was obtained by inserting the retention time (X) into the above equation. GC-FID analyses were conducted with an Agilent 6890 chromatograph using a stationary phase similar to Lipodex E [octakis (3-*O*-butyryl-2,6-di-*O*-pentyl) γ-cyclodextrin] (28 m × 0.25 mm × 0.25 μm) that was prepared by Prof. Ademir F. Morel from Santa Maria Federal University, RS, Brazil. Highly pure H_2 was employed as the carrier gas (1 mL min^{-1}). The injector and detector temperatures were 220 and 250 °C, respectively. Samples were injected in the split mode (1/50). The temperature program started at 85 °C, held at 85 °C for 160 min, increased from 85 to 180 °C (10 °C min^{-1}), and held for 10 min at 180 °C. EIMS were recorded at 70 eV at a scanning speed of 3.54 scans s^{-1} from m/z 40 to 450. TLC analyses were performed on Merck F_{254} Al sheets, and the spots were visualized by UV light (254 nm) or *p*-anisaldehyde/sulfuric acid. All solvents were of high analytical grade and doubly distilled before use. Cotton wool, used to isolate secretions, was successively extracted with EtOAc, and the solvent was evaporated under vacuum before use.

Synthesis of Compound 1. Hepten-3-one was obtained through oxidation of 1-hepten-3-ol (Aldrich) with Jones' reagent.[18] A glass ampule containing 1-hepten-3-one (112 mg, 1 mmol) and hydroquinone (2 mg) was flushed with nitrogen, sealed, and placed in an oil bath at 180 °C for 3 h.[19] The ampule was opened, taking care to cool the system with EtOH and dry ice. The unreacted 1-hepten-3-one was removed through evaporation at room temperature, while filtration removed crystals of hydroquinone, yielding **1** (63.9 mg, 57.1%).

*1-(6-Butyl-3,4-dihydro-2H-pyran-2-yl)pentanone (**1**):* yellow oil; IR (film) ν_{max} 2955, 1711, 1675, 736 cm^{-1}; ^{1}H NMR ($CDCl_3$, 499.88 MHz, TMS) δ 4.52 (1H, t, J = 3.2 Hz, H-5′), 4.25 (1H, dd, J = 8.8, 3.1 Hz, H-2′), 2.65 and 2.55 (each 1H, dt, J = 17.7, 7.4 Hz, H-2), 2.06 (2H, t, J = 7.7 Hz, H-1″), 2.00 and 1.98 (each 1H, m, H-4′), 1.98 and 1.82 (each 1H, m, H-3′), 1.56 (2H, m, H-3), 1.48 (2H, m, H-2″), 1.30 (4H, m, H-4, H-3″), 0.91 (6H, t, J = 7.3, H-5, H-4″); ^{13}C NMR ($CDCl_3$, 125.71 MHz) δ 211.8 (C, C-1), 153.5 (C, C6″), 95.6 (CH, C5′), 80.2 (CH-C2′), 37.8 (CH_2, C-2), 33.8 (CH_2-C1″), 29.1 (CH_2, C-2″), 25.2 (CH_2-C-3), 24.0 (CH_2-C-3′), 22.25 (CH_2-C-4 or C3′)′, 22.34 (CH_2-C-4 or C-3″), 19.3 (CH_2-C4′), 13.90 (CH_3-C-5 or C-4″), 13.89 (CH_3-C-5 or C-4″); other spectroscopic methods such as ^{13}C NMR (DEPT-135 and DEPT-90), 2D NMR (^{1}H,^{1}H homonuclear correlation, gCOSY, and ^{1}H,^{13}C heteronuclear correlation, ^1J HSQC) were also employed (Supporting Information); EIMS m/z 224 $[M]^+$ (25), 182 (1), 139 (100), 97(13), 95(17), 85(9), 79(13), 69(11), 55(24), 41(19); retention index 1691; HRESIMS m/z $[M + H]^+$ 225.1852 (calcd for $C_{14}H_{25}O_2$ 225.1849).

■ ASSOCIATED CONTENT

Supporting Information. NMR, IR, and MS spectroscopic characterization data of natural and synthetic compound **1** and 1-hepten-3-one. This material is available free of charge via the Internet at http://pubs.acs.org.

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