

(E)-4-Oxo-2-hexenal Dimers in the Scent Glands of the Bark Bug *Phloea subquadrata* (Heteroptera, Phloeidae)

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Bark bugs belonging to the family Phloeidae are known for their camouflage on tree trunks. The nymphs store in dorsal abdominal glands defensive secretions with a pungent odor mainly constituted of (*E*)-2-hexenal, (*E*)-2-octenal, and (*E*)-4-oxo-2-hexenal. The metathoracic glands of adults (male and female) store (*E*)-2-hexen-1-ol and (*E*)-2-hexenyl acetate, which are less irritating than their corresponding aldehydes. Additional compounds of *m/z* 224 were detected in the scent glands of these insects and were previously suggested to be dimers of the (*E*)-4-oxo-2-hexenal. Thus, the aim of this study was to elucidate the details of the chemical structure of the dimers found in the scent glands of *Phloea subquadrata*. These dimers were obtained by synthesis and were compared with the natural products, confirming the dimeric structures of the latter. These (*E*)-4-oxo-2-hexenal dimeric compounds are novel and have not been reported before.

Keywords: synthetic product, (*E*)-4-oxo-2-hexenal dimers, oxoaldehyde, *Phloea subquadrata*

Introduction

Bark bugs belonging to the family Phloeidae are known for their camouflage on tree trunks.¹ This family comprises three genus: *Phloea*, with two species (*Phloea subquadrata* and *Phloea corticata*); *Phloeophana* (*Phloeophana longirostris*) and *Serbana* (*Serbana borneensis*), both with one species each.² *P. subquadrata* occurs exclusively in Brazil, from Southern Bahia to Rio Grande do Sul.³ All four species have cryptic coloration, with a flattened body with broad foliated expansions.² This insect is phytophagous, feeding on the sap of certain species of Myrtaceae trees, but also Combretaceae and Phyllanthaceae.⁴ It is subsocial and females with eggs or early nymphs display parental care.³ Both nymphs and adults release an odoriferous yellow liquid when disturbed.

The defensive secretion stored in the dorsal abdominal glands of nymphs has a pungent odor characteristic of aldehydes and the metathoracic glands of adults (male and female) store similar derivatives that are less aggressive.

Additional compounds were detected in this insect's scent glands, many of which are still unknown.

Among these compounds, our attention was particularly attracted to the (*E*)-4-oxo-2-hexenal dimers, which were detected in the metathoracic and the dorsal abdominal glands of *P. subquadrata* individuals. The occurrence of these dimers has been mentioned in the literature, but no structure or biosynthesis has ever been proposed for these molecules.⁵

Dimeric natural products are ubiquitous and arise from aldol reactions, Michael-type reactions, Mannich-type reactions, etherification, and so forth, consequently, the dimerization attribute does not give a clue on the structure.⁶ The aim of this study was, therefore, to elucidate the chemical structure of the dimers (*m/z* 224) found in the scent glands of *P. subquadrata*.

Experimental

Chemical analysis

P. subquadrata individuals (males, females, and second to fifth instar nymphs) and exuviae were collected from *Plinia cauliflora* (Myrtaceae) at the Serra do Japi

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Biological Reserve (Jundiá, São Paulo State, Brazil) and Itapetinga State Park (Atibaia, São Paulo State, Brazil) from 2010 to 2012. Fresh exuviae were collected wherever newly moulted nymphs were found. All adult bugs were dissected within 48 h of capture under a stereoscopic magnifying microscope. The contents of the metathoracic glands of adults were sampled by piercing the gland with a microsyringe and analyzed (10 μ L, see Figure 1). Exuviae and the insects (second, third, fourth and fifth instars) were macerated in bidistilled ethyl acetate (500 μ L) and filtrated. Solvent evaporation yielded exuviae and insect extracts. The nymphal dorsal abdominal glands were analyzed using the exuviae extracts or insect gland content. All samples were stored at -20 °C in the dark. The gas chromatography-mass spectrometry (GC-MS) analyses were performed with an Agilent 6890 chromatograph and a (70-eV) Hewlett Packard 5975 MSD equipped with a fused capillary column (HP-5MS, 30 m \times 0.25 mm \times 0.25 μ m). Helium (1 mL min $^{-1}$) was the carrier gas. The analysis conditions consisted of a splitless mode (1 μ L), mass range m/z 40-600, and temperatures of 250 and 280 °C for the injector and detector, respectively. The column temperature program was 40 °C (3 min) increasing to 290 °C at 10 °C min $^{-1}$. Samples of the glandular content of male and female individuals were injected (1 μ L) in the splitless mode, without solvent. The exuvial glandular content was extracted in ethyl acetate (1 mg mL $^{-1}$). The retention indices of all compounds were calculated using the retention times of a standard mixture of *n*-alkane mixture (C8-C32, Sigma, USA), at 20 ppm with a split ratio of 1:100.



Figure 1. (A) Metathoracic glands of *P. subquadrata*, male; (B) extraction of glandular content with a microsyringe.

NMR analysis

The nuclear magnetic resonance (NMR) analyses were conducted with either a Varian Inova 500 operating at 499.89 and at 125.71 MHz for ^1H and ^{13}C , respectively, a Bruker Avance III-400 operating at 400.13 and 100.61 MHz for ^1H and ^{13}C , respectively, or a Bruker Avance 250 operating at 250.13 and 62.90 MHz for ^1H and ^{13}C , respectively. Deuterated chloroform was used as the solvent

and the samples were analyzed in 5 mm diameter NMR tubes. The chemical shifts (δ) are measured in parts *per* million, with the tetramethylsilane (TMS) signal at 0.0 ppm or the residual solvent signal (7.26 ppm) as the internal reference. All spectra were processed using the VNMRJ or TopSpin 2.1 programs and ^{13}C NMR, with decoupling, distortionless enhancement by polarization transfer (DEPT) 135°, DEPT 90°, and 2D NMR (^1H , ^{13}C heteronuclear single quantum correlation (HSQC), ^1H , ^{13}C heteronuclear multiple-bond correlation (HMBC), ^1H - ^1H correlation spectroscopy (COSY), and nuclear Overhauser effect spectroscopy (NOESY)) were applied in the structural elucidation of the compounds.

Synthesis of standards

Anhydrous tetrahydrofuran (THF), acetone, and pyridine were obtained according to Purification of Laboratory Chemicals.⁷

(E)-4-Oxo-2-hexenal

2-Ethyl-furane (1.1 mL, 10 mmol), *N*-bromo-succinimide (NBS) (freshly recrystallized, 2.72 g, 15 mmol), and pyridine (1.6 mL, 20 mmol) were added to a round-bottom flask (50 mL) containing THF/acetone/water 10:8:2 (20 mL), using a magnetic stirrer to mix, and kept at -15 °C (ethanol/dry ice). The temperature was kept at -15 °C for 3 hours. A sample (700 μ L) of the reaction was taken to monitor the reaction kinetic products. The reaction was kept at room temperature for 12 hours. The reaction was quenched with HCl (0.5 mol L $^{-1}$, 20 mL) and extracted with diethyl ether (3 \times 20 mL). The organic phase was treated with saturated sodium chloride aqueous solution and dried over MgSO₄. The solvent was evaporated under reduced pressure, yielding a yellow oil as a residue that was purified by silica column chromatography eluted with pentane:diethyl ether 85:15 (v/v). Fractions of pentane:diethyl ether (85:15, R_f = 0.24) were combined (0.35 g, 35%) and analyzed by GC-MS, ^1H and ^{13}C NMR, revealing the presence of (E)-4-oxo-2-hexenal.⁸ A second purification by preparative layer chromatography eluted with pentane:diethyl ether (85:15, v/v) furnished a pure sample.

R_f = 0.24, silica eluted with pentane:diethyl ether 85:15; IR (neat) ν / cm $^{-1}$ 2981.81, 2936.71, 1696.23, 1123.33, 1059.42, 981.29, 768.23; ^1H NMR (250.13 MHz, CDCl₃) δ 9.78 (d, 1H, *J* 7.2 Hz, CH), 6.88 (d, 1H, *J* 16.4 Hz, CH), 6.79 (dd, 1H, *J* 16.4, 7.2 Hz, CH), 2.74 (q, 1H, *J* 7.2 Hz, CH₂), 1.17 (t, 3H, *J* 7.2 Hz, CH₃); ^{13}C NMR (62.9 MHz, CDCl₃) δ 200.4 (C-4), 193.4 (C-1), 144.8 (C-3), 137.2 (C-2), 34.5 (C-5), 7.5 (C-6); MS m/z 112 (M⁺, 16), 97 (2), 84 (15), 83(100), 57 (18), 55 (77), 53 (10).

(E)-4-Oxo-2-hexenal dimers

Dimerization of *(E)*-4-oxo-2-hexenal (4.7 g) occurred when the monomer was left overnight in a silica column in pentane:diethyl ether (8:2) (100 mL). The dimers were eluted with 100% diethyl ether. The GC-MS analysis of this fraction revealed the presence of four dimers of *m/z* 224 with the predominance of two dimers in a 3:1 ratio.

Spectral analysis used major peaks in the NMR (¹H, ¹³C, DEPT 135, DEPT 90, HSQC, HMBC, COSY, and NOESY).

$R_f=0.075$, silica thin layer chromatography (TLC) eluted with pentane:diethyl ether 50:50; ¹H NMR (499.9 MHz, CDCl₃) δ 9.69 (s, 1H, CH), 6.85 (dt, 1H, *J* 6, 15 Hz, CH), 6.42 and 6.39 (dd, 1H, *J* 15, 1.5 Hz, CH), 4.56 (ddd, 1H, *J* 9, 5.5, 1.5 Hz, CH), 4.44 (ddd, 1H, *J* 6, 5.5, 1.5 Hz, CH), 3.1 (qd,

1H, *J* 18.5, 5 Hz, CH), 2.90 and 3.10 (m, 2H, CH₂), 2.62 (m, 2H, CH₂), 1.29 (d, 3H, *J* 7 Hz, CH₃), 1.12 (t, 3H, *J* 7.5 Hz, CH₃); ¹³C NMR (125.7 MHz, CDCl₃) δ 215.5 (C-3), 198.0 (C-3'), 197.5 (C-7), 141.0 (C-1'), 130.1 (C-2'), 81.5 (C-5), 75.5 (C-2), 47.0 (C-4), 44.7 (C-6), 34.0 (C-4'), 10.6 (C-5'), 7.9 (C-8); MS *m/z* 224, 195(10), 180(8), 167(10), 151(5), 139(8), 125(35), 109(15), 95(100), 83(15), 67(30), 55(32).

Results and Discussion

The GC-MS analyses of the glandular contents of *P. subquadrata* individuals (glands of nymphs and adults and exuviae, see Figure 1) revealed the presence of low-molecular weight compounds and dimers of *(E)*-4-oxo-2-hexenal (**2**) (see Figure 2 and Table 1).

Table 1. Detected volatile compounds of *P. subquadrata*

No.	SS	MW	Name	Structure	RI ^{cal}	RI ^{lit}	Main fragment (<i>m/z</i>)
1	Sd	98	<i>(E)</i> -2-hexenal		878	853 ⁹	98(M ⁺ , 28), 83(81), 69(88), 57(49), 55(93), 42(57), 41(100)
2	MS, NMR, Sd	112	<i>(E)</i> -4-oxo-2-hexenal		957	–	112(M ⁺ , 17), 84(15), 83(100), 57(13), 55(49)
3	MS, Sd	142	decane		1000	1000	142(M ⁺ , 10), 85(35), 71(45), 57(100), 43(81), 41(38)
4	MS, NMR, Sd	142	<i>(E)</i> -2-hexenyl acetate		1015	1014 ¹⁰	142(M ⁺ , 1), 100(26), 82(40), 67(53), 55(20), 43(100), 41(22)
5	MS, Sd	126	<i>(E)</i> -2-octenal		1060	1060	84(20), 82(40), 70(100), 69(49), 67(25), 57(57), 55(92), 41(88)
6	MS, Sd	156	undecane		1100	1100	156(M ⁺ , 10), 85(41), 84(12), 85(41), 71(60), 57(100), 56(20), 43(73), 41(44)
7	MS	170	2-hexenyl butanoate		1193	–	170(2), 82(19), 71(100), 67(28), 55(24), 43(37), 41(24)
8	MS	170	dodecane		1200	1200	170(M ⁺ , 10), 85(37), 71(65), 70(14), 57(100), 56(16), 55(18), 43(82), 41(40)
9	MS	ND	<i>(E)</i> -2-octenyl acetate		1210	–	128(15), 110(18), 95(11), 82(19), 81(30), 67(26), 55(22), 54(40), 43(100), 41(28)
10	MS	184	tridecane		1300	1300	184(M ⁺ , 12), 85(52), 71(73), 57(100), 55(20), 43(67), 41(39)
11	MS	204	α -caryophyllene		1474	1454	204(M ⁺ , 10), 147(24), 121(33), 93(100), 91(19), 80(32), 79(16), 67(11)

SS = structural suggestion; MS = mass spectrometry; MW = molecular weight; ND = non-detected molecular ion; Sd = standard; RI^{cal} = calculated relative retention index; RI^{lit} = literature relative retention index; St = spectroscopic data.

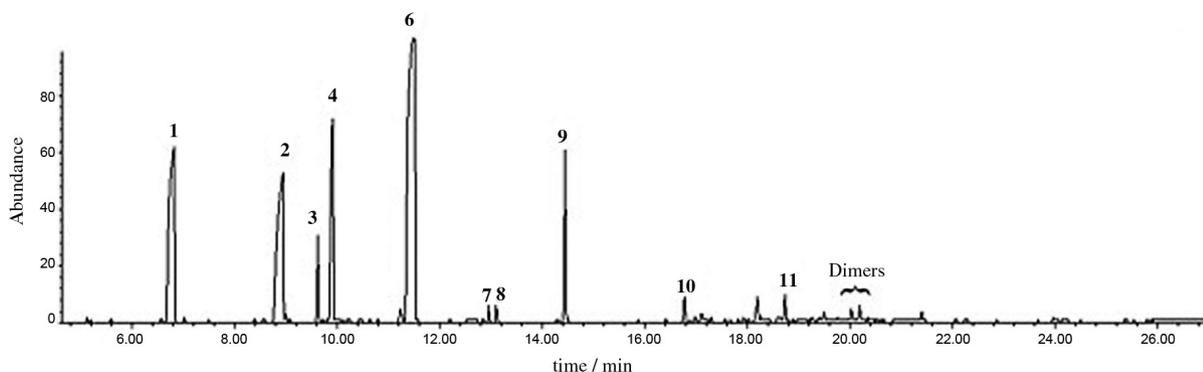


Figure 2. Ion chromatogram (GC-MS) of *P. subquadrata* female (two dimer isomers). Analytical conditions: 40 °C (3 min) increasing to 290 °C (10 °C min⁻¹). Peak numbers refer to the compounds listed in Table 1.

Most of the known glandular components were characterized by their relative retention indices, which were compared with data in the literature, and their respective mass spectra matched those in the Wiley spectral data.¹¹ *(E)*-4-Oxo-2-hexenal (**2**) was one of the most abundant constituents in these mixtures, characterized by ¹H NMR (*P. subquadrata* males), and possesses *E* stereochemistry, displaying the characteristic vicinal coupling constants between H-2 and H-3 (*J* 16.4 Hz). Joint analysis of the spectral data produced a more detailed data set of the major male metathoracic gland constituents (Figure 2).

(E)-4-Oxo-2-hexenal dimers

Minor constituents of *m/z* 224 were present in the glandular content of *P. subquadrata* nymphs and adults, which suggested the dimerization of two 4-oxo-2-hexenals (twice *m/z* 112). The synthetic dimers were obtained from 4-oxo-2-hexenal catalyzed by silica gel. The GC-MS analysis revealed the presence of four isomeric dimers. These dimers were compared to the natural compounds present in the glandular content of males, revealing perfect co-elution of all the constituents (see Figure 3).

The rationale of the 4-oxo-hexenal dimerization is

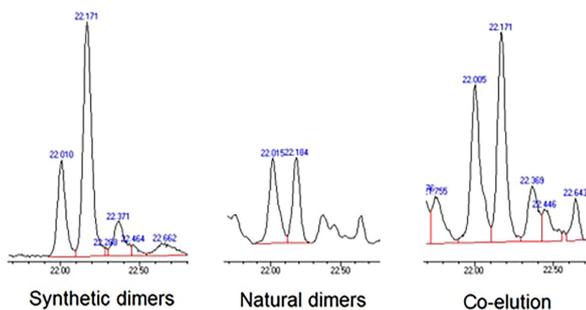


Figure 3. Chromatograms of a synthetic dimer mixture, natural dimers from *P. subquadrata* males, and co-elution of both natural synthetic dimers and natural dimers.

depicted in Figure 4 and implies an aldol dimerization as a first step followed by an intramolecular Michael addition leading to a cyclic derivative of 5 or 6 member. This could be explained applying the 5-exo-trig and 6-endo-trig pathways, both of which are favored by Baldwin's rules for ring closure (see Figure 4).¹²

The relative configurations of the three chiral centers was achieved by NMR employing a sample containing a major dimer, as shown in the chromatogram in Figure 3. The ¹³C NMR chemical shifts of the two isomers were discriminated by their relative signal intensities, taking care to compare carbons bonded to equal numbers of hydrogens (CH₃, CH₂ and CH) and with similar relaxation times.¹² Thus, the relative abundance and signal intensities were compatible.

Assignment of the ¹³C NMR signals was based on the comparison of the carbon chemical shifts of the monomer and dimer (see Table 2). The 2D NMR data (¹H, ¹³C HMBC and HSQC) were used to confirm the suggested structures.

This set of chemical shifts led to structures possessing either a five- or a six-member ring (Figure 4). However, the substantial limitation of these rules was supplanted by the careful mass fragmentation analysis that revealed the presence of fragment *m/z* 180, which could only arise from the five-membered ring (see Figure 5).

This analysis revealed that both dimers displayed similar hydrogen and carbon chemical shifts, with major differences in the chemical shifts of carbon 4 and 2 ($\Delta\delta$ ca. 2). The NOESY and selective NOE experiments with the most abundant isomer indicated that the saturation of methyl 8 (1.30 ppm) enhanced signals at 2.50 ppm (H-6, 0.2%) and 4.56 ppm (H-5, 0.7%), consistent with the five-membered ring substituent relative stereochemistry of 5,2-*trans*, 4,2-*cis*, and 4,5-*trans* (**13**).

Conclusions

These dimeric structures were never reported before,

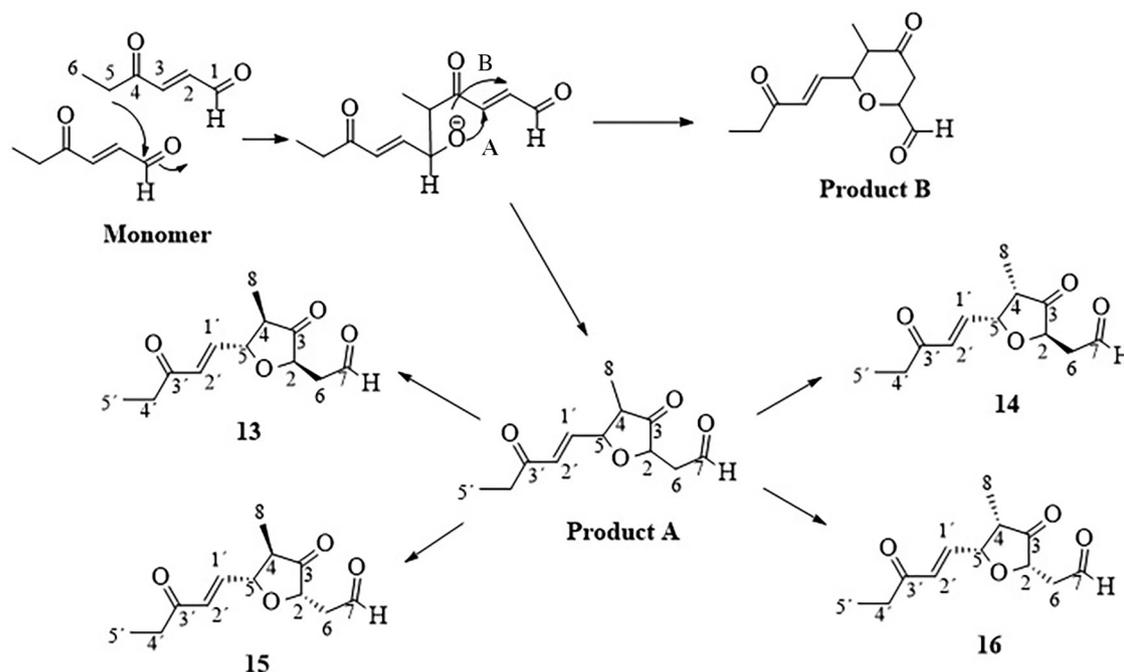


Figure 4. Dimerization of 4-oxo-hexenal. Pathways A and B are possible, but only the products produced by pathway A were observed, allowing for the appearance of four dimers (**13**, **14**, **15**, **16**).

Table 2. Major dimer (pathway B in Figure 4) ^1H and ^{13}C NMR chemical shifts supported by ^{13}C NMR (DEPT 135, DEPT 90), ^1H - ^1H COSY, ^1H , ^{13}C HMBC and ^1H , ^{13}C HSQC

Carbon	δ_{C} major / ppm	δ_{H} / ppm	δ_{C} minor / ppm
3	214.5; C_O	–	215.5
3'	200.5; C_O	–	198.0
7	198.2; CH	9.69 (s)	197.5
1'	142.5; CH	6.85 (dt, J 6, 15 Hz)	141.0
2'	129.5; CH	6.42/6.39 (dd, J 15, 1.5 Hz)	130.1
5	82.5; CH	4.56 (ddd, J 1.5, 5.5 and 9 Hz)	81.5
2	74.3; CH	4.44 (ddd, J 1.5, 5.5 and 6 Hz)	75.5
4	47.5; CH	3.1 (qd, J 5, 18.5 Hz)	47.0
6	46.7; CH_2	2.90 and 3.10 (m)	44.7
4'	34.3; CH_2	2.62 (m)	34.0
8	10.6; CH_3	1.29 (d, J 7 Hz)	10.6
5'	7.9; CH_3	1.12 (t, J 7.5 Hz)	7.9

therefore, the present report contributes novel data on the chemistry of bark bugs. Concerns about whether these dimers were naturally occurring or not were mitigated by the detection of these compounds in gland liquid that was directly injected in the GC-MS but not when the pure synthetic monomer was analyzed under the same conditions, ruling out dimerization during GC-MS analysis. Therefore, these dimeric structures do occur naturally in the bark bugs *P. subquadrata* and could be a storage strategy, since one molecule of dimer yields two monomers. Additionally, one cannot dismiss that the monomer is probably more toxic than the dimer, which is an advantage for the insects, since monomer toxicity is present when needed to fight enemies and produced from the dimer by retro-Michael and retro-aldol reactions.

Supplementary Information

Supplementary data (1D and 2D NMR, IR and MS spectra) are available free of charge at <http://jbcns.s bq.org.br> as PDF file.

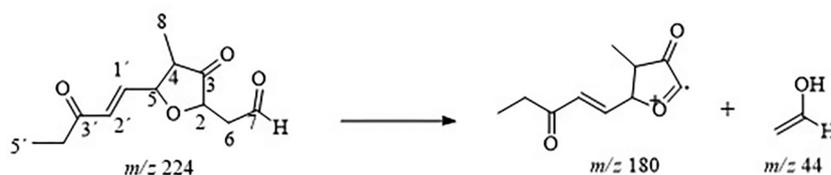


Figure 5. Diagnostic fragment of m/z 180 observed in the four dimers' mass spectra.

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