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Male-Specific Sesquiterpenes from *Phyllotreta* Flea Beetles

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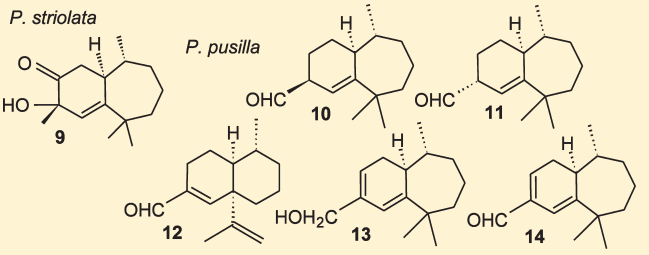
Supporting Information

ABSTRACT: Flea beetles in several genera are known to possess male-specific sesquiterpenes, at least some of which serve as aggregation pheromones that attract both sexes. In continuing research on the chemical ecology of *Phyllotreta* flea beetles, six new male-specific sesquiterpenes were identified, one from *P. striolata* (hydroxyketone **9**) and five from *P. pusilla* (aldehydes **10**−**12** and **14** and alcohol **13**); both species are crop pests. The minute amounts from beetles provided mass spectra and chromatographic data but were insufficient for complete structure determination. However, it was discovered that the new compounds could all be produced by applying organic reactions to previously identified flea beetle sesquiterpenes, and the resulting, larger amounts of material permitted definitive structure analysis by NMR. Molecular modeling was used in conjunction with NMR to define relative configurations of several newly created stereogenic centers. The absolute configurations of natural **9**−**14** were established by chiral gas chromatography/mass spectrometry. In electrophysiological tests (GC-EAD) conducted with *P. striolata*, compound **9** was detected with high sensitivity by the beetle antennae, which is consistent with a pheromonal function. The research opens new possibilities for using behavioral chemicals to monitor or manage these pest species.



Flea beetles (order Coleoptera, family Chrysomelidae, subfamily Alticinae) are a large, important, and interesting group of small insects (adults mostly <4 mm in length). The group contains significant agricultural pests such as the crucifer flea beetle, *Phyllotreta cruciferae* Goeze,[1] and the eggplant flea beetle, *Epitrix fuscula* Crotch.[2] Adults are defoliators, while larvae live in the soil and feed on roots. However, the group also contains beneficial species. Some *Aphthona* spp. are specialists on leafy spurge (*Euphorbia esula* L.) and have been introduced into the U.S. as biological control agents on this invasive, noxious weed.[3]

Pheromones, which are typically produced by just one sex, have become important scientific tools for monitoring and managing economic insects, and some information now exists about the chemistry of sex-specific compounds of flea beetles, including *P. cruciferae*, several *Aphthona* species, and *E. fuscula*. Males of *P. cruciferae* emit a group of six sesquiterpenes, **1**−**6**, that are lacking from females,[4] and the same compounds plus **7** and **8** occur in males of three *Aphthona* species.[4] Sesquiterpenes **1**, **3**, **5**, **7**, and **8** are also emitted by male *E. fuscula*, but (2*E*,4*E*,6*Z*)- and (2*E*,4*E*,6*E*)-2,4,6-nonatrienal are the major components in their blend.[5] Component ratios in the emissions from the beetles are generally species-specific.

Compounds **1**, **3**, and **5**−**8** were synthesized in racemic form,[6] and **1**, **3**, **5**, and **6** were synthesized as pure enantiomers,[7] which allowed the absolute configurations of the beetle compounds to be unambiguously assigned.[7,8] Sesquiterpene **4** is obtainable from a plant source, citronella oil.[4] Blends of the racemic compounds[9] and of pure enantiomers[10] were demonstrated to be attractive to *P. cruciferae* of both sexes in the field. Thus **1**−**6** or some subset of these constitutes a male-produced aggregation pheromone in this species. We have now investigated volatile emissions from additional *Phyllotreta* species, *P. striolata* (Fabricius) and *P. pusilla* Horn. *P. striolata* is an important pest of oilseed rape in Canada[11] and also attacks Brussels sprouts, cabbage, and other cruciferous vegetables.[2] *P. pusilla* is particularly damaging to mustard, radish, and turnip crops in the western regions of the United States, and losses can be especially serious for seedling plants in early spring.[2]

Six new male-specific sesquiterpenes (**9**−**14**) were discovered. The minute amounts from beetles made NMR of the natural compounds impracticable, but NMR became accessible when ad hoc methods were developed to synthesize **9**−**14** from related sesquiterpenes. Relative configurations at several stereogenic centers were established with the aid of molecular modeling. Subsequently, chiral GC was used to determine absolute configurations. Preliminary electrophysiological analysis was conducted by coupled gas chromatography/electroantennographic detection (GC-EAD).

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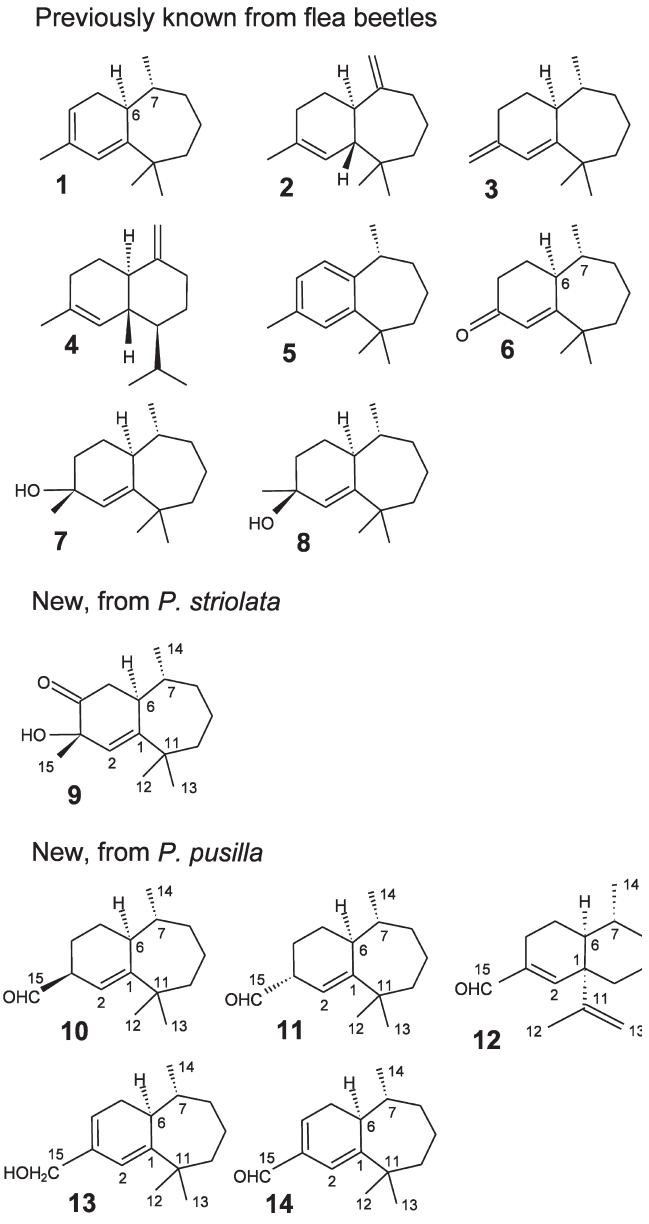

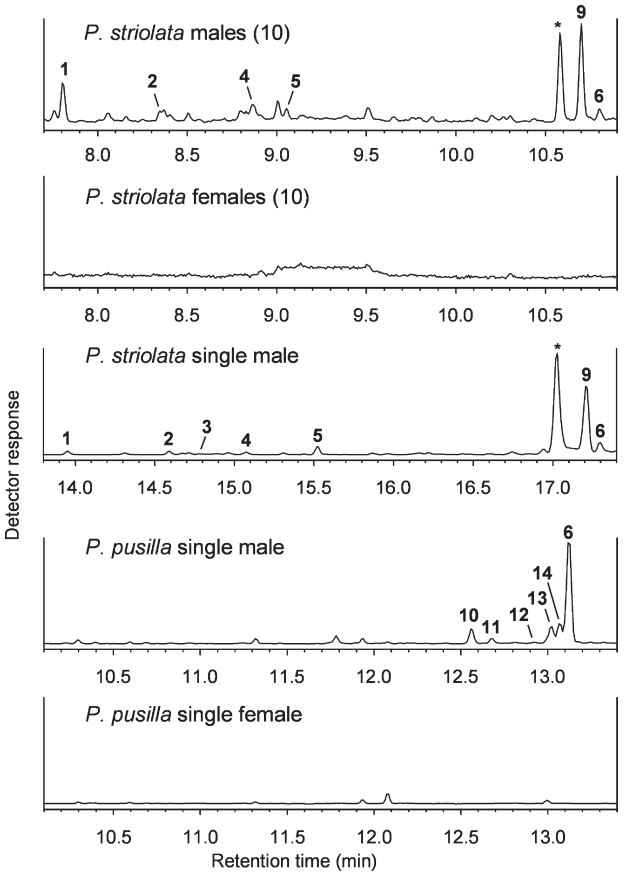

Figure 1. Example gas chromatograms of volatiles collected from *Phyllotreta* flea beetles feeding on cabbage. Compounds **1**−**6** were previously known, and **9**−**14** were new. Peak marked * is an artifact from rearrangement of **9** (see text). Three different sets of GC conditions were used: DB-1 with 0.1 μm film, 10 °C/min rate (*P. striolata* groups of 10); DB-1 with 0.25 μm film, 10 °C/min rate (*P. striolata* single male); DB-1 with 0.25 μm film, 15 °C/min rate (*P. pusilla*). See text for other GC parameters.

RESULTS AND DISCUSSION

Emission of Male-Specific Compounds. GC-MS comparison of volatiles collected from male and female *P. striolata* in the presence of host material (cabbage) revealed eight compounds in samples from males that were not present from females (Figure 1, peaks **1**−**6**, **9**, and that marked with *). Based on EIMS and GC retention times relative to synthetic standards, **1**−**6** from *P. striolata* were identical to compounds previously encountered from *P. cruciferae* and three *Aphthona* species; however, the mass spectrum of peak **9** was new (Figure 2). An earlier peak in the chromatogram, marked (*) in Figure 1, had a very similar spectrum but was subsequently found to be an artifact, presumably a thermal rearrangement of **9** from injection into a hot (250 °C) GC inlet. This peak was small (<10% the size of **9**) when the inlet temperature was 150 °C and was absent altogether with injection through a cool-on-column inlet at 53 °C. Thus, only **9** was investigated further.

Male-specific compounds were also found consistently from *P. pusilla* on cabbage (Figure 1). The most abundant of these was the previously identified ketone **6**, but there were five others (**10**−**14**) that were not encountered before, based on mass spectra (Figure 2). Hydrocarbons **1**−**5** were not found in *P. pusilla* samples.

Typical amounts of **9** emitted from *P. striolata* males in groups of 10 were 20−50 ng per male per day, and **1**−**6** were less abundant. Interestingly, amounts of **9** in collections from individuals (100−300 ng/male/day) were nearly an order of magnitude greater on a per capita basis than from groups. We focused primarily on collections from individuals rather than groups because the total amount of material obtained would be similar and there would be no ambiguity with respect to species composition (see Experimental Section). In 41 analyzed collections, the male-specific compounds were detected from six of the seven individual males; the seventh died during the first week. Emission began within 2 days of setup and usually continued until the beetles died (as long as 5 weeks).

For individual males of *P. pusilla*, the emission rate of **6** was as high as 400 ng per day. Nineteen of the 24 males produced a pattern of volatiles as in Figure 1 (**6** was detectable in 127 out of the 174 collections). Production usually began about 2 weeks after capture in the field, but the compounds were not detected from one male until 5 weeks. Emission continued for as long as

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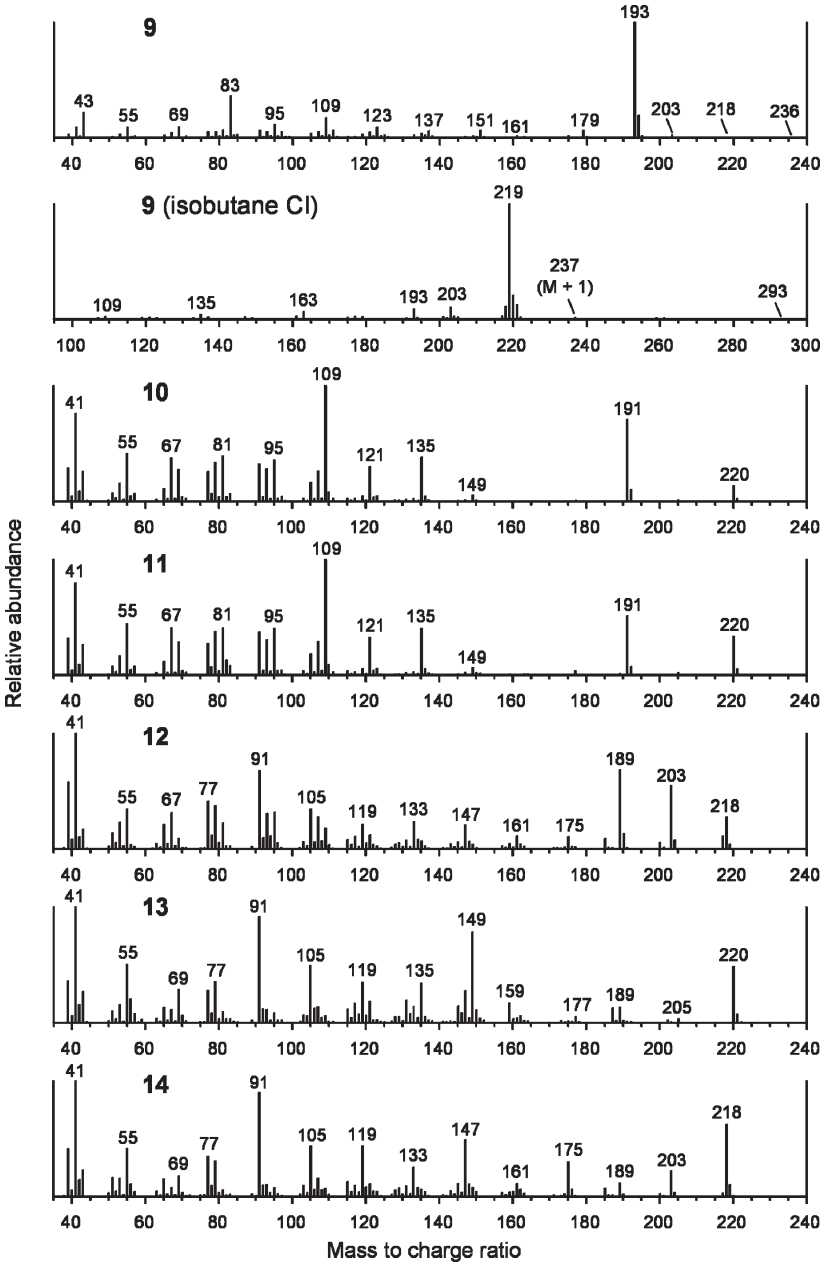

Figure 2. Mass spectra of compounds **9**−**14**. Unless otherwise noted, spectra are EI (70 eV ioization energy).

12 weeks once it began. The five beetles that did not produce died soon after setup.

Mass Spectra and Polarity. None of the EI mass spectra in Figure 2 were identifiable from the spectral library. The spectra of **10**−**14** showed prominent, likely molecular ions at *m*/*z* 218 or 220, but the molecular ion of **9** was not readily apparent. Single mass chromatograms of ions *m*/*z* 218, 236, and 193 of **9** (0.4% and 0.06% of the base peak and the base peak, respectively) all had peaks that coincided in retention time; thus 236 was believed the likely molecular weight of **9**, with 218 representing an ion formed by dehydration. With isobutane CI (Figure 2), *m*/*z* 219 was the base peak (M + 1 − H_2O), but *m*/*z* 237 (M + 1, 2% of base peak) and *m*/*z* 293 (M + 57, a typical ion in isobutane CI, 0.4%) were significant.

All of the compounds were recovered after passage through silica gel. Compounds **10**, **11**, **12**, and **14** eluted with 5% Et_2O in hexanes, consistent with aldehydes; **13** eluted with 25% Et_2O in hexanes, indicative of an alcohol; and **9** was the most polar, elution requiring 50% Et_2O in hexanes.

Mass spectra were not sufficient to define structures, and NMR was not feasible on the minute amounts of the compounds obtainable from the beetles. Serendipitously, an unintentional preparation of **9** and **14** led to a synthetic approach for solving the unknown structures. During an earlier project, compound **1** was aromatized to **5** prior to measurement of optical rotation,[4] and leftover **1** was removed by oxidation with potassium permanganate.[12] Examination of the oxidation products by GC-MS revealed traces of **9** and **14**, which were identical to the beetle compounds by MS and GC retention on an achiral column.

Conversion Precursors. Enantiomerically pure **1** and **6** (prepared as in Scheme 1) were precursors for reactions leading to **9**−**14**; the **1** and **6** fully matched prior authentic standards and

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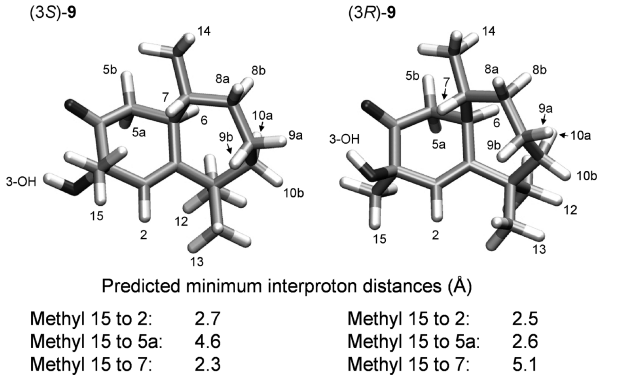
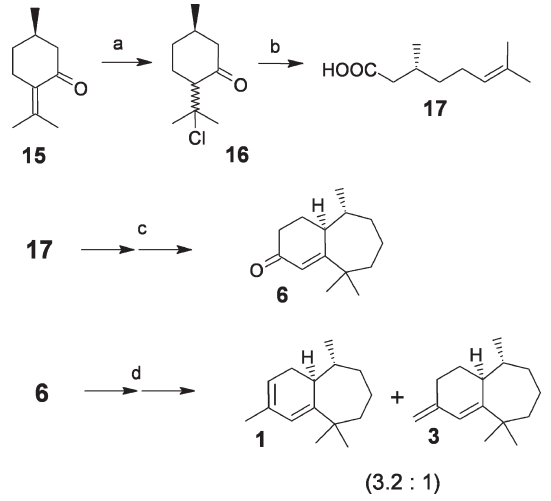


Figure 3. Modeled epimers of synthetic compound **9**. Numbering corresponds to Table 1. The predicted minimum interproton distances for the two epimers aided the interpretation of NOE results (see text).

Scheme 1. Synthesis of Compounds 1 and 6 from (*R*)-(+)-Pulegone (15) by Previously Described Methods[a]



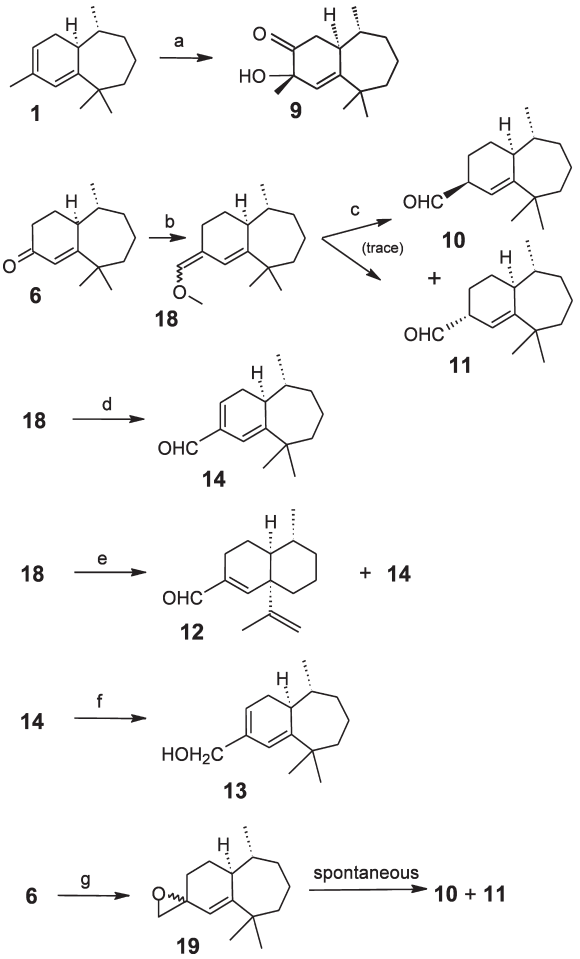
[a] Conditions: (a) HCl gas, 0 °C;[19] (b) NaOH(aq), RT, then H^+;[19] (c) 8 steps;[7] (d) 3 steps.[6] Compound **3**, a synthetic byproduct, was not removed.

were the specific enantiomers of **1** and **6** found in *P. cruciferae*. (The stock of **1** contained 24% of **3** as an impurity; these can interconvert under acidic conditions.[6]) Racemic **1** and **6** were also available and would afford racemic **9**−**14**, needed as standards for chiral GC.

Hydroxyketone 9. Excess 3-chloroperbenzoic acid[13] was superior to $KMnO_4$ for converting **1** to **9** (Scheme 2). The product was identical to natural **9** by MS, by GC retention on an achiral column, and by polarity on silica. Although the product of the reaction was not fully predictable, enough **9** was obtained so that the structure could be studied by NMR.

The NMR data for **9** (Table 1) indicated that 15 carbons and 24 hydrogens were present, and a molecular weight of 236 would require two additional oxygen atoms. From the proton and carbon spectra, there were four aliphatic methyls (three tertiary and one secondary), one trisubstituted double bond, one carbonyl group, and one tertiary hydroxy group. The HMBC spectrum established that the carbon skeleton of **1** was conserved and that the keto-hydroxy-olefin system was as shown, except that the configuration at C-3 was ambiguous. The 10 methylene and two

Scheme 2. Synthesis of 9−14 from 1 and 6[a]



[a] Conditions, results: (a) 3-chloroperbenzoic acid (2.2 mol), CH_2Cl_2, RT, 1 h, yield 44%, purity 99% after chromatography; (b) (methoxymethyl)triphenylphosphorane (2 mol), dry THF, 0 °C to RT, 1 h, yield 65%, purity 97% after chromatography; (c) conc HI in THF or hexanes, or Et_2O saturated with $HClO_4$, RT, ~1 h, monitor by GC; (d) Dess-Martin periodinane (1.4 mol), dry CH_2Cl_2, RT, 1 h, yield 30%, purity 81% after initial chromatography; (e) Et_2O saturated with $HClO_4$, 24 h, RT, **12**: yield 5%, purity 90% after chromatography, **14**: yield 18%, purity 96% after chromatography; (f) LAH (4 mol H^-), Et_2O, 0 °C to RT, 0.5 h, yield ~100%, purity 90% after workup; (g) dimethylsulfonium methylide (1.8 mol), dry THF, 0 °C then RT, 1.5 h, presumed intermediate **19** not isolated, yield of **10**, 14%, and of **11**, 12%, purity after chromatography ~80% (**10** + **11**).

methine protons had sufficient separation in the ^1H NMR spectrum so that all of the couplings could be measured and assigned to particular pairs of protons; simulation (not shown) supported the assignments.

The configuration at C-3 was established through molecular modeling and NOE experiments (the configurations at C-6 and C-7 were expected to remain as in starting material **1**). The (3*R*)-**9** and (3*S*)-**9** epimers were modeled separately (Figure 3). Observed NOESY correlations (especially that between H-6 and H-10a) assisted modeling of the flexible seven-membered ring. The generally good agreement between observed and predicted ^1H and ^{13}C NMR shifts and coupling constants (Table 1) indicated that the modeled ring conformation was realistic. NOESY correlations involving Me-15 protons were observed

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Table 1. ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR Data in C_6D_6 for Compound 9, Assigned *J* Couplings, and Predicted NMR Values for Epimers (3*S*)-9 and (3*R*)-9 (Figure 3)

	Observed NMR Data and Assignments					
position	δ_C, mult.	δ_H, (*J* in Hz)	HMBC[a]	NOESY[b]	assigned *J* (Hz)	
1	152.3, C				5a, 5b	−14.4
2	125.7, CH	5.71, s	1,4,6,11,15	13, 15	5a, 6	5.6
3	70.3, C	OH: 2.14 v br s			5b, 6	2.5
4	211.0, C				6, 7	10.1
5	41.1, CH_2	5a: 2.61, dd, (14.4, 5.6)	1, 4, 6, 7	5b, 6	7, 8a	4.7
		5b: 2.50, dd, (14.4, 2.5)	1, 4, 6, 7	5a, 6, 14	7, 8b	8.3
6	41.8, CH	2.12, ddd (10.1, 5.6, 2.5)	1, 2, 4	5a, 5b, 10a, 12, 14	7, 14	6.5
7	39.1, CH	1.24, dddq (10.1, 8.3, 4.7, 6.5)		14, 15 (weak)	8a, 8b	−13.6
8	37.3, CH_2	8a: 1.57, dddd (13.6, 7.6, 4.7, 2.9)	6, 7, 9, 14	8b, 9b	8a, 9a	7.6
		8b: 0.95, dddd (13.6, 9.4, 8.3, 3.4)		8a, 9a, 10a	8a, 9b	2.9
9	21.9, CH_2	9a: 1.44, ddddd (14.2, 7.6, 7.5, 3.4, 2.5)		8b, 9b	8b, 9a	3.4
		9b: 1.18, ddddd (14.2, 10.7, 9.4, 2.9, 2.4)	8, 10	8a, 9a	8b, 9b	9.4
10	40.8, CH_2	10a: 1.37, ddd (14.2, 10.7, 2.5)	1, 9	6, 8b, 12	9a, 9b	−14.2
		10b: 1.30, ddd (14.2, 7.5, 2.4)	1, 9	12, 13	9a, 10a	2.5
11	38.2, C				9a, 10b	7.5
12	30.7, CH_3	0.97, s	1, 10, 11, 13	6, 10a, 10b	9b, 10a	10.7
13	27.1, CH_3	1.10, s	1, 10, 11, 12	2, 10b	9b, 10b	2.4
14	19.4, CH_3	0.85, d (6.5)	6, 7, 8	5b, 6, 7	10a, 10b	−14.2
15	24.9, CH_3	1.44, s	2, 3, 4	2, 7 (weak)		

	Predicted NMR Values for (3*S*)-9 and (3*R*)-9						
	predicted δ_C		predicted δ_H		predicted *J* (Hz)		
position	(3*S*)-9	(3*R*)-9	(3*S*)-9	(3*R*)-9	description	(3*S*)-9	(3*R*)-9
1	150.9	150.0			5a, 5b	−17.6	−14.1
2	122.0	122.3	5.66	5.61	5a, 6	6.2	6.1
3	69.5	72.6	OH: 1.51	OH: 2.99	5b, 6	2.5	3.1
4	213.4	213.0			6, 7	10.6	10.6
5	40.6	40.0	5a: 2.58	5a: 2.68	7, 8a	5.3	5.3
			5b: 2.36	5b: 2.36	7, 8b	11.2	11.4
6	42.3	45.3	2.32	2.38	7, 14	6.7	6.6
7	41.0	40.1	1.32	1.40	8a, 8b	−15.8	−15.8
8	40.7	39.6	8a: 1.65	8a: 1.63	8a, 9a	5.5	5.5
			8b: 1.08	8b: 1.05	8a, 9b	2.1	2.2
9	24.2	22.9	9a: 1.35	9a: 1.30	8b, 9a	2.9	3.0
			9b: 1.28	9b: 1.31	8b, 9b	12.9	12.9
10	40.8	40.1	10a: 1.45	10a: 1.37	9a, 9b	−16.5	−16.4
			10b: 1.47	10b: 1.46	9a, 10a	0.3	0.4
11	40.5	39.7			9a, 10b	9.6	9.5
12	28.5	28.1	0.95	0.93	9b, 10a	10.8	11.0
13	25.1	24.1	1.02	1.02	9b, 10b	0.3	0.3
14	18.4	16.9	0.69	0.75	10a, 10b	−16.4	−16.4
15	23.7	27.0	1.17	1.11			

[a] HMBC correlations are from proton(s) stated to the indicated carbon. [b] NOESY correlations are between proton(s) stated and the other indicated protons.

only to H-2 and H-7 (weak). The latter result was crucial and was verified with the NOE difference experiment: irradiation at H-7 led to a slight (0.3%) but significant enhancement of Me-15. From the predicted distances in Figure 3 for the (3*S*) epimer, Overhauser enhancements could be expected between Me-15 and H-2 and between Me-15 and H-7 but not between Me-15 and H-5a. However for the (3*R*) epimer, enhancements would be expected between Me-15 and H-2 and between Me-15 and H-5a but not between Me-15 and H-7. Thus, we conclude that synthetic **9** was the (3*S*) epimer.

Aldehyde 14. NMR analysis of **14** obtained during the synthesis of **12** led to the data in Table 2. In the proton and carbon spectra there was evidence for three aliphatic methyl groups (two tertiary and one secondary), two trisubstituted double bonds,

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Table 2. ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR Data in C_6D_6 for Compounds 13 and 14 and Assignments of Shifts to Position Numbers in the Structures

	compound 13			compound 14		
position	δ_C, mult.	δ_H, (J in Hz)	HMBC[a]	δ_C, mult.	δ_H, (J in Hz)	HMBC[a]
1	154.1, C			155.8, C		
2	116.73[b], CH	5.95, br s	1, 3, 4, 6, 11, 15	111.4, CH	6.64, s	3, 4, 6, 15
3	137.7, C			139.8, C		
4	116.70[b], CH	5.52, br d (6.2)		141.9, CH	5.96, ddt (6.4, 3.0, 1.0)	2, 5, 6, 15
5	27.8, CH_2	2.35, ddd (17.0, 6.2, 1.3)	1, 3, 4, 6, 7	28.9, CH_2	2.29, ddd (18.0, 6.4, 1.7)	1, 3, 4, 6, 7
		2.24, ddddd (17.0, 6.0, 2.5, 2.5, 2.5)	7		2.06, ddd (18.0, 7.0, 2.8)	1, 3, 4, 6, 7
6	37.8, CH	1.80, m	1, 2, and/or 4	37.3, CH	1.74, br dd (9.8, 7.0)	1, 2, 4, 5, 7, 8
7	34.4, CH	1.74, m		34.5, CH	1.40, m	
8	39.9, CH_2	1.79, m		39.3, CH_2	1.64, m	6, 7, 9, 10, 14
		1.03, m			0.90, m	6, 7, 9, 10, 14
9	23.2, CH_2	1.54, m		22.8, CH_2	1.43, m	
		1.25, m			1.11, m	10, 11
10	40.8, CH_2	1.51, m		40.6, CH_2	1.41, m	
		1.51, m			1.41, m	
11	38.7, C			38.9, C		
12	32.5, CH_3	1.16, s	1, 10, 11, 13	32.2, CH_3	1.03, s	1, 10, 11, 13
13	26.6, CH_3	1.22, s	1, 10, 11, 12	26.3, CH_3	1.16, s	1, 10, 11, 12
14	22.3, CH_3	0.95, d (6.4)	8	22.0, CH_3	0.78, d (6.8)	6, 7, 8
15	65.2, CH_2	4.07, d (12.3)		189.9, CH	9.42, s	2, 3, 4
		4.04, d (12.3)				
		OH: 0.78, s				

[a] HMBC correlations are from proton(s) stated to the indicated carbon. [b] Assignments may be reversed.

and one formyl group. The HMBC data supported that the carbon skeleton and diene system of **1** were present, but in **14**, branch carbon 15 was a formyl group rather than a methyl. The proton and carbon shifts for **14** were very similar to those reported for **1**,[6,7] except in the region of the formyl group, supporting that the relative configuration at C-6 and C-7 remained as in starting material **1**, as would be expected. Synthetic **14** matched the beetle compound exactly with respect to MS and GC retention on an achiral column. Periodinane[14] oxidation of enol ether **18** was later found to be a more efficient means of preparing **14** (Scheme 2).

Alcohol 13. The mass spectrum of **13** was similar to that of **14** except that many of the peaks, including the molecular ion, were shifted two mass units higher (Figure 2). This, and the higher polarity, suggested that **13** could be obtained by reducing aldehyde **14** to the alcohol. Thus, treatment with LAH cleanly afforded a product that was identical to the beetle compound in MS and GC retention on an achiral column. The reaction was not expected to affect the ring system of **14**.

The NMR data for **13** (Table 2) supported the structure. Main features were three aliphatic methyl groups (two tertiary and one secondary), one primary alcohol group, and two trisubstituted double bonds. Except near the alcohol group, the carbon and proton shifts were similar to those observed previously for compounds **1**[6,7] and **14**, and HMBC results supported the ring system as presented.

Aldehydes 10 and 11. The polarity of beetle compounds **10** and **11** (elution from silica with 5% Et_2O in hexanes) and mass spectra with intense M − 29 fragments (at m/z 191) were consistent with aldehydes. The mass spectra of **10** and **11** were similar, suggesting the compounds could be epimers. These results prompted the attempt to synthesize **10** and **11** from ketone **6** by conversion to the enol ether (**18**), followed by hydrolysis.[15] The enol ether formed readily, but hydrolysis proved difficult. By GC-MS, **18** was consumed over time following treatment with either HI or $HClO_4$ (within several hours or overnight, depending on conditions). Traces of **10** and **11** formed initially, but disappeared subsequently (presumably due to an acid-catalyzed isomerization such as double-bond migration). These results were encouraging with respect to the proposed structures for **10** and **11**, but the yield was too low for NMR analysis.

Useful amounts of **10** and **11** were obtained by treating ketone **6** with dimethylsulfonium methylide. The expected product was epoxide **19**,[16] which could give the aldehydes after subsequent hydrolysis, dehydration, and tautomerization. In fact, the epoxide was not isolated, and **10** and **11** were the major reaction products upon workup. These products matched the corresponding beetle compounds exactly by MS and GC retention on an achiral column. Passage through an open column of silica gel removed impurities from **10** and **11** but did not separate them from each other. HPLC on silica separated them with difficulty, but each pass through the column led to unacceptable losses of these labile compounds. Thus, NMR analysis was based on a 55:45 mixture of **10** and **11** from column chromatography, where the more abundant compound, **10**, corresponded to the earlier GC peak from the beetles.

Thirty resonances were observed in the ^{13}C NMR spectrum (Table 3). The spectrum appeared as 15 pairs of peaks, where the members of each pair had the expected peak-height ratio of ca. 55:45, allowing assignment of signals to the proper compound. The proton spectrum confirmed that both compounds were

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Table 3. ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR Data in C_6D_6 for Compounds 10−12 and Assignments of Shifts to Position Numbers in the Structures

	compound **10**			compound **11**			compound **12**		
position	δ_C, mult.	δ_H, (J in Hz)	HMBC[a]	δ_C, mult.	δ_H, (J in Hz)	HMBC[a]	δ_C, mult.	δ_H, (J in Hz)	HMBC[a]
1	154.8, C			154.9, C			47.0, C		
2	113.33, CH	5.48, d (3.4)	3, 4, 6, 11, 15	113.28, CH	5.54, d (4.1)	3, 4, 6, 11, 15	156.3, CH	6.19, q (∼1.7)	1, 4, 6, 10, 15
3	50.3, CH	2.81, dddd (11.5, 7.7, 3.4, 2.1)		47.4, CH	2.58, dd (7.7, 4.1)		142.4, C		
4	16.6, CH_2	1.54, m (4a)[b]		16.0, CH_2	2.02, br dt (13.8, ∼3.5) (4a)[b]		17.7, CH_2	2.33, ddt (18.8, 6.5, ∼1.7)	2, 3, 5, 6
		1.69, m (4b)			1.50, m (4b)			2.08, dddd (18.8, 12.2, 6.9, 2.3)	2, 3, 5, 15
5	26.55, CH_2	1.39, m (5a)		25.0, CH_2	1.57, m (5a)		19.1, CH_2	1.60, m	
		1.28, m (5b)			1.29, m (5b)			1.55, m	
6	38.6, CH	1.88, m		38.5, CH	∼1.88, m		41.4, CH	1.31, dddd (10.8, 3.5, 3.5, 1.7)	
7	37.2, CH	1.37, m		36.3, CH	1.39, m		29.3, CH	1.02, m	
8	35.4, CH_2	1.68, m		35.5, CH_2	1.68, m		34.9, CH_2	1.47, m	
		1.11, m			1.11, m			0.83, qd (∼12.8, 3.1)	
9	21.83, CH_2	1.55, m		21.93, CH_2	1.55, m		23.2, CH_2	1.45, m	
		1.33, m			1.33, m			1.03, m	
10	39.7, CH_2	1.55, m		39.6, CH_2	1.55, m		37.0, CH_2	1.57, m	
		1.30, m			1.30, m			1.43, m	
11	38.7, C			38.8, C			150.8, C		
12	32.3, CH_3	0.99, s	1, 10, 11, 13	32.7, CH_3	0.98, s	1, 10, 11, 13	19.1, CH_3	1.63, d (1)	1, 11, 13
13	26.48, CH_3	1.14, s	1, 10, 11, 12	26.62, CH_3	1.18, s	1, 10, 11, 12	113.6, CH_2	4.88, appar. pen (∼1.2)	1, 11, 12
								4.59, br d (0.7)	1, 11, 12
14	19.33, CH_3	0.91, d (6.8)	6, 7, 8	19.39, CH_3	0.90, d (6.8)	6, 7, 8	20.6, CH_3	0.80, d (6.5)	6, 7, 8
15	200.2, CH	9.40, d (2.1)		199.5, CH	9.44, br s		192.7, CH	9.52, s	2, 3, 4

[a] HMBC correlations are from proton(s) stated to the indicated carbon. [b] Assignment of ^{1}H shifts to particular protons (Figure 4) based on modeling results.

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aldehydes (δ 9.40 and 9.44 for H-15, Table 3). The NMR data were consistent with a Δ^1 trisubstituted double bond in each. A ring system as in **6** was supported, including the three methyl groups on the seven-membered ring. There was no reason to expect that the rings or the relative configurations at C-6 and C-7 would be affected by the reaction.

Determination of the configuration at C-3 relative to C-6 relied on both molecular modeling and the ^{1}H NMR spectrum. The resonances for H-4 to H-6 were too complex to establish the conformation of the six-membered ring. However, modeling conclusions, based on the low-energy structures calculated for **10** and **11**, were that H-6 is *gauche* to the C-5 methylene group and that the six-membered ring has a nearly chair conformation (Figure 4). The H-3 resonances were unobstructed for both compounds, and these established the spatial relationship between H-3 and the C-4 methylene group. In compound **10**, H-3 gave a multiplet at δ 2.81, with $J_{2,3}$ = 3.4 Hz, $J_{3,4a}$ = 7.7 Hz, $J_{3,4b}$ = 11.5 Hz, and $J_{3,15}$ = 2.1 Hz. A simulation using these J values (Figure 4) agreed closely with the actual signal. The relatively large values of both $J_{3,4a}$ and $J_{3,4b}$ indicate that H-3 must be *anti* to one of the C-4 methylene protons and *syn* to the other (Figure 4). Given the chair conformation of the ring, H-3 must be α-oriented and the formyl group, β-oriented, as drawn in Figure 4. Thus, synthetic **10** must have the (3*S*) configuration.

In the ^{1}H NMR spectrum of compound **11**, H-3 appeared as a doublet of doublets at δ 2.58 ($J_{2,3}$ = 4.1 Hz and $J_{3,4b}$ = 7.7 Hz); the lack of further splitting indicated that $J_{3,4a}$ and $J_{3,15}$ were <1 Hz. The relatively small $J_{3,4a}$ and $J_{3,4b}$ values indicated a *gauche* relationship between H-3 and the C-4 methylene protons. Thus, H-3 must be β-oriented and the formyl group, α-oriented (Figure 4), and synthetic **11** has the (3*R*) configuration.

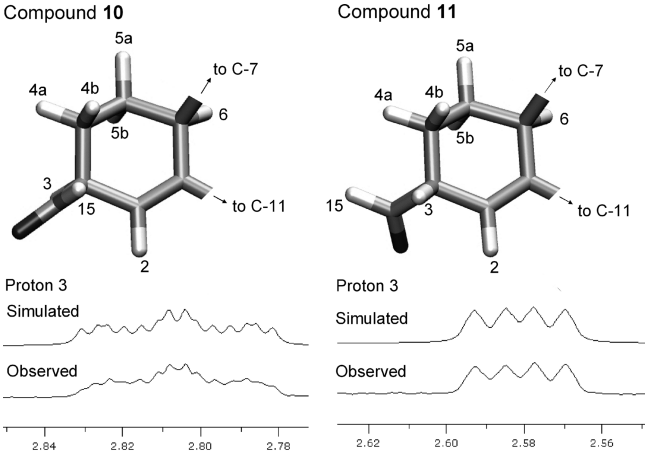

Figure 4. ^{1}H NMR and modeling results for compounds **10** and **11**. Conformation of the six-membered ring was established by modeling for both compounds. The observed ^{1}H resonances for H-3 are shown, along with simulations based on the observed coupling constants. The J values were consistent between the models and the observed NMR data. The results established that the earlier-eluting beetle compound is structure **10**.

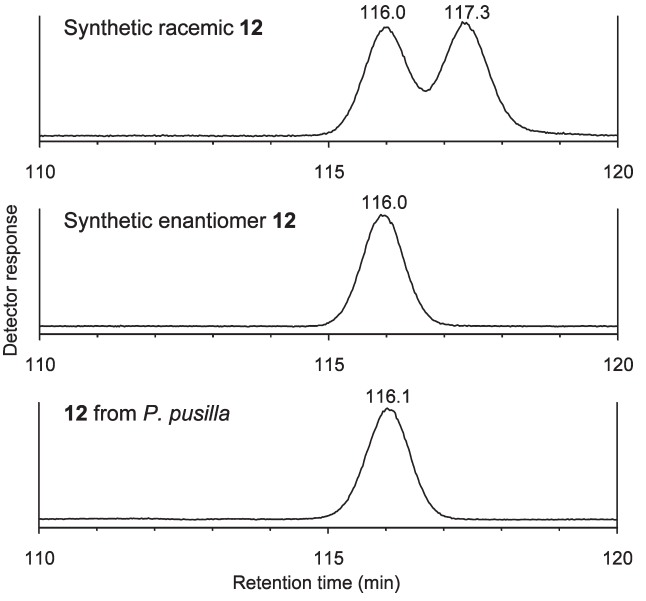

Figure 5. Gas chromatograms of compound **12** on a BDEX-325 chiral column. Mass spectra for all peaks as for **12** in Figure 2. Oven program: 50 °C for 1 min, followed by 30 °C/min increase to the final temperature of 120 °C.

Table 4. GC Retention Times of Synthetic and Beetle-Derived Compounds on Chiral Columns

			GC retention time (min)			
			synthetic racemic			
compound	column	final temp (°C)[a]	first peak	second peak	synthetic enantiomer	beetle
9	GDEX-225	150	45.3	46.8[b]	46.7	46.7
10	BDEX-325	140	41.6[b]	42.4	41.7	41.7
11	BDEX-325	140	46.2[b]	48.0	46.3	46.3
12	BDEX-325	120	116.0[b]	117.3	116.0	116.1
13	BDEX-325	140	62.1[b]	62. 7	62.2	62.2
14	BDEX-325	140	58.7[b]	59.4	58.8	58.7
6	BDEX-325	140	68.0[b]	68.5	67.9	68.0

[a] GC temperature program: 50 °C for 1 min, then 30 °C/min to final temperature. [b] Peak that matched synthetic enantiomer and beetle-produced compound.

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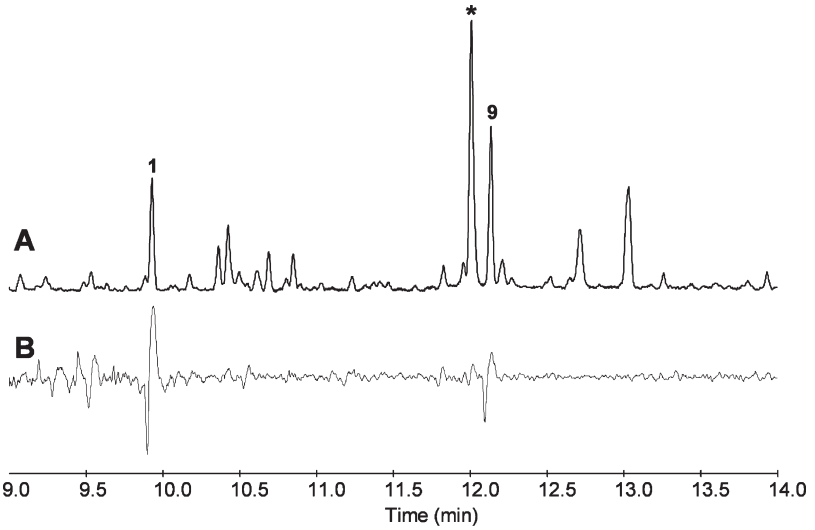

Figure 6. Simultaneously recorded gas chromatogram of collected volatiles from male *P. striolata*, containing **1** and **9** (A), and antennal activity of female *P. striolata* (B). Peak marked * is a GC artifact due to injection of **9** into a hot inlet.

Aldehyde 12. This compound unexpectedly resulted from the treatment of enol ether **18** with perchloric acid and had the polarity of an aldehyde, eluting from silica gel with 5% Et_2O in hexanes. The apparent molecular weight was 218 (Figure 2). Compound **12** had 15 ^{13}C NMR resonances, including one carbonyl and four olefinic signals (Table 3). There was NMR evidence (^{13}C and ^{1}H shifts and HMBC correlations) for an isopropenyl group (C-11, C-12, and C-13), a secondary aliphatic methyl group (C-14), and a formyl group (C-15). These groups accounted for five carbon atoms, thus leaving no more than 10 for a ring system. A decalin-type ring system with the substitution pattern shown was supported by the HMBC data and the ^{13}C and ^{1}H NMR shifts. The rearrangement that converted the himachalane ring system to the decalin presumably involved C-1, C-10, and C-11. It was expected that the configurations at C-6 and C-7 would be unaffected, but it was not clear whether the ring junction would be *cis* or *trans* after the rearrangement at C-1.

The ^{1}H NMR spectrum and, in particular, the resonance for H-6 (δ 1.31) gave information about the ring junction. Couplings of 10.8, 3.5, 3.5, and 1.7 Hz were observed for H-6. By the ^{1}H and COSY spectra, $J_{2,6}$ = 1.7 Hz ("*W*-coupling"). The other couplings for H-6 were to H-5a, H-5b, and H-7, and $J_{6,7}$ was concluded to be the largest of these. The width of the multiplet for H-7 was ~49 Hz, from the ^{1}H and HSQC spectra. Most of this width (~36 Hz) was due to the three protons of Me-14 ($J_{7,14}$ = 6.5 Hz) to H-8b ($J_{7,8b} \approx$ 12.8 Hz, axial−axial) and to H-8a ($J_{7,8a} \approx$ 4 Hz, typical for an axial−equatorial relationship, not directly observable). By difference, $J_{6,7}$ would be ~13 Hz, which was close to only one observed value, 10.8 Hz (this magnitude indicates that H-6 and H-7 have an axial−axial relationship and supports that the configurations at C-6 and C-7 are as in starting material **6**). Thus, both $J_{5a,6}$ and $J_{5b,6}$ were concluded to be 3.5 Hz, indicating a *gauche* relationship between H-6 and the C-5 methylene protons. Molecular modeling and structural models showed that a *cis* ring junction would accommodate the *gauche* conformation between H-6 and C-5 methylene protons, but this relationship was impossible with a *trans* ring junction. We conclude that the ring junction must be *cis*.

Chiral GC Analysis. Racemic **9**−**14** resulted when the reactions (Scheme 2) were applied to racemic **6** or **1**. These matched the chiral products exactly by GC on an achiral column and by MS. As shown in Table 4, GC conditions were found so that the enantiomers of **9**−**14** could be separated. Only one enantiomer of each was detected from the beetles, and this matched the enantiomer from the chiral synthesis in each case. The example in Figure 5 shows the GC traces for **12**. Since the chiral precursors to **9**−**14** (synthetic **1** and **6**) were identical to natural **1** and **6** from *P. cruciferae* and since the synthetic enantiomers of **9**−**14** were identical to the natural compounds from *P. striolata* and *P. pusilla*, the absolute configurations at C-6 and C-7 are conserved in the compounds from these beetle species, despite other structural differences.

Preliminary GC-EAD Analysis. Female *P. striolata* antennae (N = 5) readily sensed the presence of the two most abundant compounds, **1** and **9**, in volatile collections from male *P. striolata* (Figure 6). The other apparent antennal responses seen in Figure 6 were random noise signals. As noted above, the peak marked * was probably due to thermal rearrangement of **9** in the hot (250 °C) GC inlet port. This artifact would not occur from *P. striolata* in nature; correspondingly, the beetle antennae did not detect it as it emerged from the GC column (Figure 6). Female *P. striolata* antennae responded equally well to natural **9** and to synthetic **9** (results not shown). Male antennae were not tested. GC-EAD analyses for *P. pusilla* were not successful due to the small number of individuals available for study and the rapid deterioration of the antennal preparations in this species.

Chemistry, Chemical Ecology, and Practical Implications. The current research revealed six new male-specific compounds that likely play a role in *Phyllotreta* chemical ecology, doubling the number of male-specific compounds known from this large genus. Five of the new compounds have the himachalane carbon skeleton that is prevalent in the volatiles from studied flea beetle species, but there are also structural features not encountered previously in flea beetle sesquiterpenes. These include formyl groups (in **10**−**12** and **14**), an unsaturated hydroxyketone substructure (**9**), a primary hydroxy group (in **13**), and a decalin-based ring system with unusual branching (**12**). *Phyllotreta pusilla* is the first studied *Phyllotreta* species in which none of **1**−**5** have been detected.

Preliminary electrophysiological analysis revealed that one of the new compounds, **9**, has biological activity in *P. striolata* that is

consistent with a pheromonal function, although the precise behavioral effect of the compound has yet to be determined. The lesser per capita laboratory emission of **9** from *P. striolata* in groups than from individuals may be related to a natural mechanism for regulating the size of beetle aggregations. Field trapping experiments and other behavioral studies are still needed for *P. striolata* and *P. pusilla*. Nevertheless, the compounds described here open new possibilities for detecting and managing pest species of flea beetles and for bringing a new level of understanding to the chemical ecology of flea beetles.

EXPERIMENTAL SECTION

General Experimental Procedures. NMR spectra of synthetic compounds were obtained on a Bruker Avance 500 spectrometer. Samples were dissolved in C_6D_6, and spectra were acquired at 300 K. Experiments included ^{1}H, ^{13}C, COSY, HSQC, and HMBC. The DEPT-135 experiment was conducted for the sample containing **10** and **11**, and the NOESY and 1D NOE difference experiments were conducted for **9**. For compounds **12** and **14**, the sample amounts were too small for ^{13}C NMR, and ^{13}C resonances were read from the HSQC and HMBC spectra. ^{1}H and ^{13}C NMR shifts were assigned to the proposed structures, using the previous assignments for compounds **1** and **6**[4,6,7] as the starting point. Some data processing and simulations were performed with Spinworks 3.1 software.[18]

Most EIMS (70 eV) was done on a Hewlett-Packard (HP) 5973 mass selective detector, interfaced to an HP 6890 gas chromatograph. GC columns included DB-1 (30 m, 0.25 mm i.d., and 0.25 μm film thickness, or 15 m, 0.25 mm i.d., and 0.1 μm film thickness, J&W Scientific, Folsom, CA). Helium was the carrier gas (34 cm/s), and injection was through the splitless inlet. The usual oven temperature program was 50 °C for 1 min, then increasing at 10 or 15 °C/min to 250 °C. Inlet temperature was usually 250 °C. The Wiley mass spectral library was installed on the data system. An HP 5971 mass selective detector, coupled to an HP 5890 GC, was also used, primarily for EIMS of synthetic products. CIMS (isobutane reagent gas) was done for one compound from *P. striolata* on a Finnigan 4535 instrument with GC inlet. Chiral GC-MS analysis was done on the HP 5973, using BDEX-325 or GDEX-225 columns (both 30 m, 0.25 mm i.d., 0.25 μm film thickness, Supelco, Bellefonte, PA). The initial oven temperature was 50 °C; after a 1 min hold, the oven temperature was increased at 30 °C/min to one of the final values (given with results). HREIMS was done at the Department of Chemistry, University of Minnesota, on a VG 70SE instrument with GC inlet.

Some volatile collections were analyzed on an HP 5890 GC with flame ionization detector (GC-FID) for quantitative purposes (external standard method, relative to heptadecane). Injection was through a splitless or cool-on-column inlet.

High-performance liquid chromatography (HPLC) of synthetic products employed a Waters 515 pump and a Waters R-401 differential refractometer detector. A Rainin Dynamax silica column (Si-80-125-C5, 4.6 mm i.d. by 25 cm) was used with a flow rate of 1 mL/min. Separations were monitored on the refractometer, but collected fractions were also analyzed by GC-MS. Solvents and separation details are given in the Supporting Information.

Insects. The beetles were collected from cabbage plots located at NCAUR, Peoria, IL. *P. striolata* were collected during July−September of 1999, 2001, and 2002, and *P. pusilla*, during October and November of 2002, 2003, and 2009. Beetle sex was determined under a microscope by examining the ventral surface of the abdomen tip.[17] Species were tentatively determined[17] prior to volatile collections, and identifications were checked by dissection and examination of the genitalia, once volatile collections were completed. Example specimens were submitted to the USDA-ARS Systematic Entomology Laboratory, Beltsville, MD, to confirm identity, and voucher specimens were retained at the Smithsonian Institution, Washington, DC. Also collected in Peoria were *P. conjuncta* (Gentner) and *P. zimmermanni* (Crotch), both of which had yellow and black elytral markings similar to *P. striolata* and required careful examination under a microscope for certain identification.

Volatile Collections. Volatiles from male and female beetles feeding on chunks of cabbage were trapped in filters containing Super-Q porous polymer as described previously.[4] For *P. striolata*, 87 collections were made from groups of 5−10 males, 10 were made from groups of females, and nine were made from cabbage only, as controls. The collection period was usually 2−10 days. Subsequently, 41 collections were made from individual males on cabbage. The study of *P. pusilla* volatiles was based on 174 collections from 24 individual males and 27 from 3 individual females, for comparison.

The volatile collections (400 μL, in hexanes) were usually concentrated about 10-fold under a gentle stream of nitrogen and submitted to GC-MS. Sex-specific compounds that occurred consistently in collections were of particular interest. Representative samples with such compounds were submitted to column chromatography on silica gel to gain information about compound polarity. A typical column was 0.5 by 3 cm (in a Pasteur pipet) and was eluted with hexanes, followed by 5, 10, and 25% Et_2O in hexanes, Et_2O, and 10% MeOH in DCM.

Synthetic Compounds. Single enantiomers **1** and **6** were prepared as synthetic precursors for **9**−**14** and for other projects (Scheme 1). The synthesis of **6** was mostly by the published method,[7] but (*R*)-(+)-pulegone (**15**) was used as the starting material rather than citronellal for reasons of cost and availability. By a known method,[19] **15** was converted with HCl gas to hydrochloride **16**, which was then hydrolyzed to citronellic acid **17**, an early intermediate in the previous synthesis of **6**. The yield of **6** was 5 g from 100 g of **15**. A portion of the **6** was used to produce a 3.2:1 mixture of **1** and **3** (1.4 g) via alcohols **7** and **8**.[6]

Synthetic **1** and **6** were subjected to a variety of small-scale reactions, attempting to generate compounds identical to those from the beetles and in amounts large enough for NMR analyses. The syntheses of **9**−**14** are summarized in Scheme 2. Details are given in the Supporting Information. The successful reactions were repeated on racemic **1** and **6**, prepared by the achiral route,[6] to provide standards for chiral GC.

NMR data for the synthetic compounds are presented in Tables 1−3. EIMS for **9**−**14** were as in Figure 2. HREIMS: **9**, 236.1759, calc for $C_{15}H_{24}O_2$, 236.1776. **10**, 220.1842; **11**, 220.1833; and **13**, 220.1822, calc for $C_{15}H_{24}O$, 220.1827. **12**, 218.1657, and **14**, 218.1650, calc for $C_{15}H_{22}O$, 218.1671. Nomenclature: **9**, (3*S*,9*R*,9a*S*)-3-hydroxy-3,5,5,9-tetramethyl-1,3,5,6,7,8,9,9a-octahydro-2*H*-benzo[7]annulen-2-one; **10**, (3*S*,9*R*,9a*S*)-5,5,9-trimethyl-2,3,5,6,7,8,9,9a-octahydro-1*H*-benzo[7]-annulene-3-carbaldehyde; **11**, (3*R*,9*R*,9a*S*)-5,5,9-trimethyl-2,3,5,6,7,8,9,9a-octahydro-1*H*-benzo[7]annulene-3-carbaldehyde; **12**, (4a*S*,5*R*,8a*S*)-5-methyl-8a-(prop-1- en-2-yl)-3,4,4a,5,6,7,8,8a-octahydronaphthalene-2-carbaldehyde; **13**, (9*R*,9a*S*)-5,5,9-trimethyl-5,6,7,8,9,9a-hexahydro-1*H*-benzo[7]annulen-3-yl]methanol; **14**, (9*R*,9a*S*)-5,5,9-trimethyl-5,6,7,8,9,9a-hexahydro-1*H*-benzo[7]annulene-3-carbaldehyde.

Chiral GC-MS. Conditions (column type and temperature) were sought that would give separation of enantiomers for synthetic racemic **9**−**14** and **6**. Under the successful conditions, the synthetic enantiomers of **9**−**14** and **6** were compared to compounds derived from *P. striolata* and *P. pusilla* to determine whether these had the same or opposite absolute configuration. Mass spectra were acquired to confirm that the GC peaks corresponded to the expected compounds.

Computational Procedure. Calculation of ^{13}C and ^{1}H NMR chemical shifts was performed within the GIAO (gauge-independent atomic orbital)[20,21] framework using the GAUSSIAN03 suite of ab initio programs[22] and the PQS (Parallel Quantum Systems) density functional theory (DFT) optimization programs,[23] implemented on PQS 64-bit 16 processor computers. Empirical potentials (AMBER)[24,25] were initially

used with Insight/Discover modeling software (version 2000.3 L, Accelrys Corp.) to derive trial structures, which were then optimized at the B3LYP/6-31+G* level of theory. Due to the flexibility of the seven-membered ring, many different stable conformations had to be calculated via the DFT optimization procedure. The numbers of unique DFT-optimized conformations considered in this study were 11 for **9** (including both possible epimers), 12 for **10**, 13 for **11**, and 18 for **12** (including the *cis* and *trans* ring junctions). Relative energies of formation and ^{1}H and ^{13}C NMR shifts (relative to TMS) were calculated for all structures at the B3LYP/6-31+G* level of theory. The predicted chemical shifts were further adjusted with linear regression so that the rmsd relative to observed values was minimized (notably, the predicted ^{13}C NMR shifts were systematically lower than the observed shifts by about 4 ppm). At the same level of theory, *J* values were calculated for all relevant structures.

GC-EAD Analysis. Coupled gas chromatographic−electroantennographic detection (GC-EAD) analyses were made by methods and equipment generally described earlier.[26] GC-EAD connections were made by inserting a glass-pipet silver-grounding electrode into the back of an excised beetle head. A second glass-pipet silver-recording probe was placed in contact with the distal end of one antenna. Both pipettes were filled with Beadle-Ephrussi saline.[27]

■ ASSOCIATED CONTENT

Supporting Information. Synthetic details for **9**−**14**. This information is available free of charge via the Internet at http://pubs.acs.org.

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Notes

[§]Retired.

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