EurJOC European Journal of Organic Chemistry

FULL PAPER

DOI: 10.1002/ejoc.201500196

Pheromone Bouquet of the Dried Bean Beetle, *Acanthoscelides obtectus* (Col.: Chrysomelidae), Now Complete

József Vuts,[a] Wittko Francke,[b] Kenji Mori,[c] Paulo H. G. Zarbin,[d] Antony M. Hooper,[a] Jocelyn G. Millar,[e] John A. Pickett,[a] Miklós Tóth,[f] Keith Chamberlain,[a] John C. Caulfield,[a] Christine M. Woodcock,[a] Armin G. Tröger,[b] Éva Bálintné Csonka,[f] and Michael A. Birkett*[a]

Dedicated to the late Anna-Rose and Hermann Z. Levinson; their scientific achievements keep them among us

Keywords: Pheromones / Bioassay / Coleoptera / *Acanthoscelides* / Bruchinae

Male-specific volatile components, released by the dried bean beetle, *Acanthoscelides obtectus*, were identified as methyl (*E*,*R*)-2,4,5-tetradecatrienoate, methyl (2*E*,4*Z*,7*Z*)-2,4,7-decatrienoate, methyl (2*E*,4*Z*)-2,4-decadienoate, octadecanal and the sesquiterpenes (3*Z*,6*E*)- and (3*E*,6*E*)-α-farnesene. In olfactometer bioassays, pure methyl (*E*,*R*)-2,4,5-tetradecatrienoate was only weakly attractive to unmated females. However, a blend of the six identified compounds released in physiologically relevant ratios and doses proved to be as active as headspace odours collected from live males.

Introduction

The dried bean beetle, *Acanthoscelides obtectus* Say (Coleoptera: Chrysomelidae, Bruchinae), is an economically important pest of stored dried beans, *Phaseolus vulgaris* L. (Fabaceae), worldwide.[1,2] Feeding larvae are well protected within the seeds, and so management strategies target adult beetles.[3] Because semiochemicals constitute valuable tools for the detection and monitoring of *A. obtectus* populations, attempts to isolate volatile attractants were started more than 45 years ago. Hope et al. first reported a single sex-specific compound isolated from hexane surface extracts of males and proposed it to either stimulate the emergence of females or to be a sex attractant.[4] This compound was later identified as the allenic methyl (2*E*)-2,4,5-tetradecatrienoate by Horler,[5] and it was shown to have the (4*R*)-configuration.[6] However, no successful bioassays using the synthetic compound have been published. Horler[5] and Halstead[7] had noted that sections of thin layer chromatograms containing the allenic ester did not consistently evoke behavioural responses from females. Horler proposed that "this could mean that the attractant is a similar material which is usually present in these fractions" and that "there are probably at least two closely related compounds". Recently, octadecanal was identified in solvent extracts of males and found to synergize the activity of the ester as an attractant for females.[8] In addition, unspecified C16 and C18 methyl and ethyl esters were reported from solvent extracts of both sexes, along with a stereochemically undefined α-farnesene.[9]

In view of these fragmentary data, we reinvestigated the composition of volatiles released by male beetles to set the stage for the development of pheromone-based management strategies. Here, we report on the structure elucidation and biological activity of volatile compounds from air entrainment collections of *A. obtectus* males, in addition to the known allenic methyl (*E*,*R*)-2,4,5-tetradecatrienoate.

Results and Discussion

Because initial studies suggested dynamic headspace collection to be more efficient than solvent extraction to obtain sex-specific volatiles from male *A. obtectus*, we applied this technique to collect samples from unmated males, using

[a] Department of Biological Chemistry and Crop Protection, Rothamsted Research,
AL5 2JQ Harpenden, United Kingdom
E-mail: mike.birkett@rothamsted.ac.uk
http://www.rothamsted.ac.uk/
[b] Institute of Organic Chemistry, University of Hamburg, Martin-Luther-King-Platz 6, 20146 Hamburg, Germany
[c] Photosensitive Materials Research Center, Toyo Gosei Co., Ltd.,
4-2-1 Wakahagi, Inzai City, Chiba 270-1609, Japan
[d] Department of Chemistry, Federal University of Parana, 81531-990, Curitiba-PR, Brazil
[e] Department of Entomology, University of California, Riverside, CA 92521, United States of America
[f] Centre for Agricultural Research, Plant Protection Institute HAS,
15 Herman O. u., 1022 Budapest, Hungary

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/ejoc.201500196.

Porapak Q as the adsorbent. Gas chromatographic (GC) analysis of diethyl ether extracts of the adsorbent on a non-polar HP-1 column revealed six major components to be consistently present in a ratio of 4.5:10:3.5:4.5:14:2, respectively (see Figure 1). No peaks of beetle origin were detected in headspace extracts of virgin sexually mature females. Structural assignments of the target compounds were based on comparison of their analytical data with those of authentic reference substances. The last eluting component (compound **6**) proved to be octadecanal,[8] whereas the main component (compound **5**) was the previously known methyl (*E*,*R*)-2,4,5-tetradecatrienoate.[5] A reinvestigation of the enantiomeric composition of the natural product by enantioselective gas chromatography confirmed Pirkle's general assignment[6] and showed an *R*/*S* ratio of 93–94:7–6. Minor constituents, volatiles **3** and **4**, proved to be the common sesquiterpenes (3*Z*,6*E*)- and (3*E*,6*E*)-α-farnesene.[10] The second most abundant *A. obtectus* volatile, component **2** in Figure 1, was conclusively identified as methyl (2*E*,4*Z*)-2,4-decadienoate.[10] This ester had earlier been identified as a component of the aggregation pheromone of the bark beetle *Pityogenes chalcographus* L. (Coleoptera: Scolytidae) [11] and several species of *Euschistus* stink bugs (Heteroptera: Pentatomidae).[12]

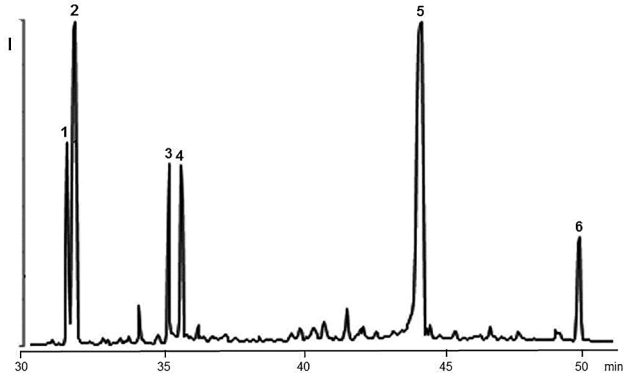


Figure 1. Gas chromatogram of a diethyl ether extract of Porapak Q trapped volatiles released by males of the dried bean beetle.

The structure of component **1** was unknown. Its 70 eV EI mass spectrum (Figure 2) showed a base peak at *m*/*z* 79 amu and a molecular ion at *m*/*z* 180 amu. Diagnostic fragments at *m*/*z* 59 (representing a methoxycarbonyl group), *m*/*z* 121 ($M^+ - 59$, loss of a methoxycarbonyl group), and *m*/*z* 149 ($M^+ - 31$, loss of a methoxy group) indicated the compound to be the methyl ester of a C_{10}-carboxylic acid with three double bond equivalents.

After catalytic hydrogenation[13] of a crude extract of *A. obtectus* males, coupled GC-mass spectrometry (GC/MS) analysis showed the presence of methyl tetradecanoate [hydrogenation product of methyl (*E*,*R*)-2,4,5-tetradecatrienoate], 2,6,10-trimethyldodecane (hydrogenation product of the farnesenes), and methyl decanoate [hydrogenation product of methyl (2*E*,4*Z*)-2,4-decadienoate and of the unknown methyl ester], revealing the unknown compound to be the methyl ester of a straight-chain decatrienoic acid. The sub-structure of a conjugated methyl 2,4-dienoate was

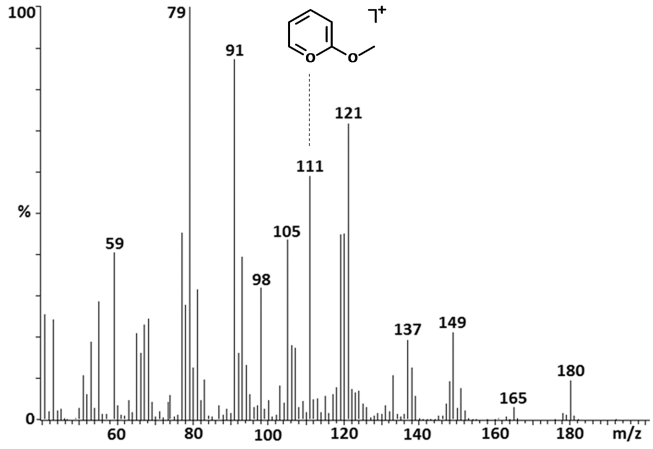


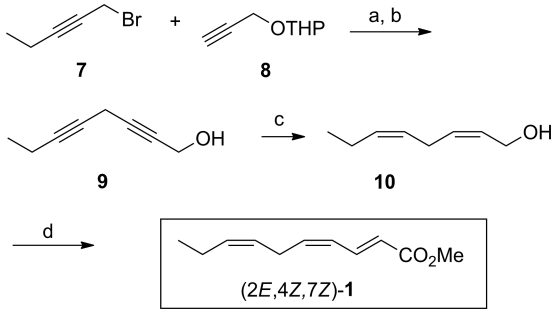
Figure 2. 70 eV EI mass spectrum of compound **1** in Figure 1, including a hypothetical structure for *m*/*z* 111.

indicated by a pronounced signal at *m*/*z* 111 in the mass spectrum (Figure 2), which was also present in those of methyl (2*E*,4*Z*)-2,4-decadienoate (**2**)[10] and methyl (2*E*,4*E*,7*Z*)-2,4,7-decatrienoate.[14] This fragment, $C_6H_7O_2$, may represent the methoxycarbonyl group and the subsequent four carbons of the chain as suggested in Figure 2. GC coupled with Fourier-transform infrared spectroscopy showed a strong absorption between 1715–1730 cm^{-1} as expected for an α,β-unsaturated ester. However, no absorption in the 1930–1950 cm^{-1} region could be observed, which excluded the possibility of an allenic system.

As a result, the determination of the position and geometry of the third double bond in the target compound needed further efforts. Preparative GC, using a DB-WAX column, resulted in the isolation of 12 μg of the methyl decatrienoate from headspace collections of males. ^{1}H 1D NMR spectroscopy of the sample revealed the presence of a doublet of doublets at δ = 7.66 ppm, a triplet at δ = 6.17 ppm and a doublet at δ = 5.92 ppm, showing almost identical multiplicity and chemical shift to H-3, H-4 and H-2 of methyl (2*E*,4*Z*)-2,4-decadienoate,[18] which implied the presence of the same moiety in the unknown ester. Other salient resonances included a triplet at δ = 3.09 ppm that was shown to be coupled to resonances at δ = 5.86 and 5.35 ppm by 2D correlation spectroscopy. The chemical shift and adjacent downfield protons identified this to be a bisallylic methylene group at C-6, indicating a double bond at C-7. To avoid any traces of acid that might originate from chloroform and could endanger the tiny amounts of the unknown natural product, we used d_2-dichloromethane as the NMR solvent, which unfortunately obscured H7. However, the H-8 multiplet revealed a coupling constant of 10.3 Hz, suggesting the (*Z*)-configuration for the double bond. The alkenyl proton at H-8 was coupled to a quintet at δ = 2.13 ppm, which coupled to a triplet at δ = 1.01 ppm, indicating a distal ethyl group. In sum, these data suggested the unknown compound to be methyl (2*E*,4*Z*,7*Z*)-2,4,7-decatrienoate.

For reference and bioassays, methyl (2*E*,4*Z*,7*Z*)-2,4,7-decatrienoate was prepared according to Scheme 1, starting from commercially available 1-bromo-2-pentyne (**7**). Alkyl-

ation of the tetrahydropyranyl derivative **8** of propargyl alcohol with **7** was executed as reported by Kobayashi et al.[15] to give 2,5-octadiyn-1-ol (**9**) in 90% yield after deprotection. Reduction of **9** to **10** was best carried out by Brandsma's method with Zn/Cu and ethanol yielding (2*Z*,5*Z*)-2,5-octadien-1-ol (**10**) in 99% purity and 70% yield after distillation.[16] The final step was the tandem oxidation-olefination to convert **10** to methyl (2*E*,4*Z*,7*Z*)-2,4,7-decatrienoate using Barrett's method.[17] Treatment of **10** with Dess–Martin periodinane and (methoxycarbonylmethylene)triphenyl phosphorane in the presence of benzoic acid in dichloromethane/dimethyl sulfoxide gave crude methyl 2,4,7-decatrienoate (**1**) in 71% yield. GC/MS analysis revealed the product to contain 85% of the desired (2*E*,4*Z*,7*Z*)-isomer, 6% of the (2*Z*,4*Z*,7*Z*)-isomer, and 2% of the (2*E*,4*E*,7*Z*)-isomer. Purification on silica gel impregnated with silver nitrate (elution with hexane/ethyl acetate, 100:1, v/v) yielded methyl (2*E*,4*Z*,7*Z*)-2,4,7-decatrienoate as a colourless oil of 99% purity. A synthetic sample co-eluted with the natural product on nonpolar HP-1 and polar DB-WAX GC columns and showed the same mass spectrum and the same NMR spectroscopic data. A detailed description of the synthesis of methyl (2*E*,4*Z*,7*Z*)-2,4,7-decatrienoate and a new route to methyl (*E*)-2,4,5-tetradecatrienoate will be published separately.[19]



Scheme 1. Synthesis of methyl (2*E*,4*Z*,7*Z*)-2,4,7-decatrieonate. Reagents: (a) EtMgBr, CuI, THF; (b) aq. HCl, MeOH (90%, two steps); (c) Zn, $Br(CH_2)_2Br$, CuBr, LiBr, EtOH, 100 °C (6 h), 80 °C (16 h) [70% (99% purity)]; (d) Dess–Martin periodinane, $Ph_3P{=}CHCO_2Me$, PhC_2H, DMSO, CH_2Cl_2 (60%).

The biosynthesis of (2*E*,4*Z*,7*Z*)-2,4,7-decatrienoic acid, i.e. the acid part of the novel ester, can be rationalized either via lipoxygenase-mediated cleavage of (9*Z*,12*Z*,15*Z*)-9,12,15-octadecatrienoic acid (α-linolenic acid) or by a sequence of four β-oxidation steps and rearrangement of the same precursor. The 12*Z*- and 15*Z*-double bonds of linolenic acid would become the 4*Z*- and 7*Z*-unsaturations, whereas the original 9*Z*-double bond would be transformed to the 2*E*-double bond in the chain-shortened product. Starting from (9*Z*,12*Z*)-9,12-octadecadienoic acid (linoleic acid), a corresponding biosynthetic pathway would yield (2*E*,4*Z*)-2,4-decadienoic acid, the precursor of methyl decadienoate **2**.

To the best of our knowledge, methyl (2*E*,4*Z*,7*Z*)-2,4,7-decatrienoate is a new natural product. However, (2*E*,4*Z*,7*Z*)-2,4,7-decatrienol and the corresponding aldehyde have been described as components of the scent of a South American orchid.[20]

In olfactometer assays, the test compounds were applied onto filter paper strips in proportions and doses in such a way that the amounts released per hour were similar to those emitted by one male beetle over 1 h. In test 1, methyl (*E*,*R*)-2,4,5-tetradecatrienoate was significantly more attractive to virgin female *A. obtectus* than a hexane control (Table 1). Addition of the two C_{10} methyl esters or octadecanal to the allenic methyl ester did not enhance beetle attraction. The two farnesenes, however, synergized the activity of the allenic methyl ester, indicating that the farnesenes may play a more important role than the other three compounds. A complete blend containing all six male-specific compounds proved to be much more attractive than the allenic ester alone (Table 1). Comparison of a blend containing the allenic methyl ester with one lacking it (test 6, Table 1) revealed that all 6 male-specific components were necessary for full activity. A blend of the 6 compounds

Table 1. Behavioural responses of virgin *Acanthoscelides obtectus* females in 4-arm olfactometer bioassays to synthetic blends. Composition of the complete male blend (ng/μL in hexane) used in the bioassays (10 μL offered on filter paper): methyl (2*E*,4*Z*,7*Z*)-decatrienoate (5), methyl (2*E*,4*Z*)-2,4-decadienoate (40), (3*Z*,6*E*)-α-farnesene (43), (3*E*,6*E*)-α-farnesene (50), methyl (*E*,*R*)-2,4,5-tetradecatrienoate (390), octadecanal (5). Results shown in test 7 were obtained with 7.2 μg of a mixture of compounds 1–6 in the ratio of 55:110:55:55:390:55 in hexane. Relative proportions in this mixture were found to be closer to the natural composition of the bouquet than the "complete blend" used in tests 2–6 (see Figure 1). Means with the same letters are not significantly different at $\alpha = 0.05$ (ANOVA), followed by Fisher's LSD-test (see also Supporting Information).

Test	Treatment	Mean time spent [min] ± SE	Significance
1	allenic ester	7.25 ± 0.58	$P < 0.001$
	solvent (control)	1.44 ± 0.33	
2	complete male blend	6.01 ± 1.43	b
	allenic ester	2.02 ± 0.65	a
	solvent (control)	2.67 ± 0.72	a
3	complete male blend	6.86 ± 1.22	b
	allenic ester + other methyl esters	2.81 ± 0.89	a
	solvent (control)	0.98 ± 0.25	a
4	complete male blend	5.13 ± 0.93	b
	allenic ester + α-farnesenes	3.58 ± 1.12	b
	solvent (control)	0.43 ± 0.12	a
5	complete male blend	5.53 ± 1.29	b
	allenic ester + octadecanal	2.56 ± 0.57	a
	solvent (control)	1.16 ± 0.26	a
6	complete male blend	4.37 ± 0.57	b
	allenic ester	1.93 ± 0.44	a
	complete male blend without allenic ester (control)	2.06 ± 0.36	a
7	complete male blend	3.94 ± 0.60	b
	male extract (1 male equivalent)	3.36 ± 0.75	b
	solvent (control)	0.92 ± 0.16	a

proved to be as attractive to virgin female *A. obtectus* as the corresponding equivalent of a headspace extract obtained from live males (test 7, Table 1).

Our data suggest that the newly identified compounds are Horler's missing pheromone components. We believe that after 45 years, our results will finally form the basis for development of effective semiochemical-based pest management tools for use against *A. obtectus*.

Experimental Section

General: Beetles were obtained from a laboratory population and reared on dry *Ph. vulgaris* "Cannellini" beans under artificial lighting at 20 °C/60% RH with a 16:8 h L:D photoperiod. In order to obtain virgin insects, seeds were kept individually in wells of a plastic rack until emergence of adults, when sexes were separated immediately. To obtain volatile compounds from 15 eight-day old male *A. obtectus*, dynamic headspace collection (air entrainment) was used.[21]

Methyl (*E*,*R*)-2,4,5-tetradecatrienoate and methyl (2*E*,4*Z*)-2,4-decadienoate were synthesized as described by Mori.[18] The sesquiterpenes (3*Z*,6*E*)- and (3*E*,6*E*)-α-farnesene were prepared according to known methods.[22,23] Octadecanal was obtained by TPAP-mediated oxidation of octadecanol.[24]

To investigate the behavioural responses of virgin female *A. obtectus* to the male-derived compounds, a Perspex four-arm olfactometer was used.[25,26] Two arms were used to test synthetic compounds (except in Test 1), two were used for the control, hexane (except in Test 1 and Test 6). The time spent in each arm by a single beetle over a 16-min period was recorded using custom-made software (OLFA, Udine, Italy). Data were analyzed statistically by ANOVA at $\alpha = 0.05$, followed by Fisher's LSD test (GenStat, 11th edition, VSN International Ltd.). Ten replicates per test were done.

For detailed experimental procedures such as GC, GC/MS or GC/FT-IR, NMR, the isolation of natural methyl (2*E*,4*Z*,7*Z*)-2,4,7-decatrienoate, and its synthesis and bioassays, see Supporting information.

Acknowledgments

This work was in part supported by the Hungarian Scientific Research Fund (OTKA) (grant number K81494), by the Hungarian Research and Technology Innovation Fund (grant number OMFB-00609/2010) and by the Royal Society, United Kingdom (grant IE111142 to J. V.). Rothamsted receives grant-aided support from the Biotechnology and Biological Sciences Research Council (BBSRC), United Kingdom.

[1] O. Imura, in: *Bruchids and legumes: economics, ecology and coevolution* (Eds.: K. Fujii, A. M. R. Gatehouse), Kluwer, Dordrecht, The Netherlands, **1990**, p. 257–269.
[2] T. Abate, J. K. O. Ampofo, *Annu. Rev. Entomol.* **1996**, *41*, 45–73.
[3] B. J. Southgate, *Annu. Rev. Entomol.* **1979**, *24*, 449–473.
[4] J. A. Hope, D. F. Horler, D. G. Rowlands, *J. Stored Prod. Res.* **1967**, *3*, 387–388.
[5] D. F. Horler, *J. Chem. Soc. C* **1970**, 859–862.
[6] W. H. Pirkle, C. W. Boeder, *J. Org. Chem.* **1978**, *43*, 2091–2093.
[7] D. G. H. Halstead, *J. Stored Prod. Res.* **1973**, *9*, 109–117.
[8] D. Annoscia, T. Bruce, T. Hooper, F. Nazzi, J. Pickett, International Society of Chemical Ecology, 26th Annual Meeting, Tours, France, **2010**, P029, p. 212.
[9] M. Gołębiowski, E. Maliński, J. Nawrot, P. Stepnowski, *J. Stored Prod. Res.* **2008**, *44*, 386–388.
[10] The National Institute of Standards and Technology, NIST/EPA/NIH Mass spectral Library combined with Wiley MS 9th edition, **2008**.
[11] J. A. Byers, G. Birgersson, J. Löfqvist, G. Bergström, *Naturwissenschaften* **1988**, *75*, 153–155.
[12] J. R. Aldrich, M. P. Hoffmann, J. P. Kochansky, W. R. Lusby, J. E. Eger, J. A. Payne, *Environ. Entomol.* **1991**, *20*, 477–483.
[13] A. B. Attygalle, K. D. McCormick, C. L. Blankespoor, T. Eisner, J. Meinwald, *Proc. Natl. Acad. Sci. USA* **1993**, *90*, 5204–5208.
[14] W. H. Gerwick, P. J. Proteau, D. G. Nagle, M. L. Wise, Z. D. Jiang, M. W. Bernart, M. Hamberg, *Hydrobiologia* **1993**, *260/261*, 653–665.
[15] A. Kobayashi, K. Kubota, M. Iwamoto, H. Tamura, *J. Agric. Food Chem.* **1989**, *37*, 151–154.
[16] M. H. P. J. Aerssens, R. van der Heiden, M. Heus, L. Brandsma, *Synth. Commun.* **1990**, *20*, 3421–3425.
[17] A. G. M. Barrett, D. Hamprecht, M. Ohkubo, *J. Org. Chem.* **1997**, *62*, 9376–9378.
[18] K. Mori, *Tetrahedron* **2012**, *68*, 1936–1946.
[19] K. Mori, *Tetrahedron* in press.
[20] R. Kaiser, *Chimia* **2000**, 346–363.
[21] N. Agelopoulos, J. A. Pickett, *J. Chem. Ecol.* **1998**, *24*, 1161–1172.
[22] P. Ramaiah, J. J. Pegram, J. G. Millar, *J. Org. Chem.* **1995**, *60*, 6211–6213.
[23] A. Svatõs, A. B. Attygalle, *Anal. Chem.* **1997**, *69*, 1827–1836.
[24] W. P. Griffith, S. V. Ley, G. P. Whitombe, A. D. White, *J. Chem. Soc., Chem. Commun.* **1987**, 1625–1627.
[25] J. Pettersson, *Insect Syst. Evol.* **1970**, *1*, 63–73.
[26] S. Y. Dewhirst, M. A. Birkett, J. D. Fitzgerald, A. Stewart-Jones, L. J. Wadhams, C. M. Woodcock, J. Hardie, J. A. Pickett, *J. Chem. Ecol.* **2008**, *34*, 1575–1583.

Received: February 10, 2015
Published Online: May 27, 2015

