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Sex pheromone biosynthesis in the female olive fruit-fly. Double labelling from [$^{18}O_2$]-dioxygen into 1,7-dioxaspiro[5.5]undecane

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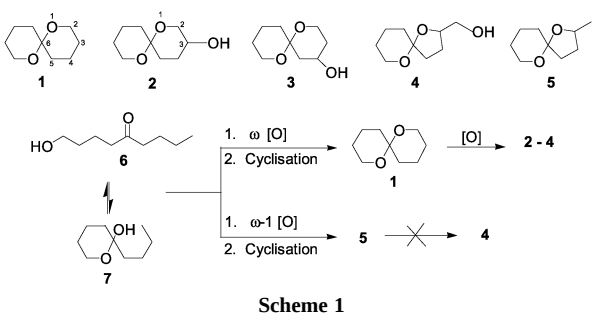
The demonstration that both oxygen atoms of 1,7-dioxaspiro[5.5]undecane (1), the sex-pheromone of the female olive fly, originate from dioxygen, strongly implicates monooxygenase mediated processes in assembly of (1), and reveals unexpected complexity in the formation of its nine-carbon precursor.

The olive fruit-fly (*Bactrocera oleae*) is a serious pest in Southern Europe and North Africa, and there has been considerable interest in its biology and semio-chemistry.[1–3] The major component of the female-generated pheromone is the racemic spiroacetal, 1,7-dioxaspiro[5.5]undecane, **1**, which is accompanied by low levels (1–4%, ~10% in total) of hydroxy derivatives, **2**–**4**.[3,4] In the current investigation, very low levels (~0.4 and 0.1%) of two isomers of 2-methyl-1,6-dioxaspiro[4.5]decane **5**, were detected for the first time in this species.[5,6]

Control of pestilent fruit-fly species by disruption of pheromone production requires an understanding of pheromone biosynthesis and enzymology. In this connection, we deduced that the likely penultimate step in the biosynthesis of **1** in *B. oleae*, and *B. cacuminata* was the ω-hydroxylation of an alkyltetrahydropyranol (see **6** ⇋ **7**) or an equivalent system, by a monooxygenase that strongly preferred oxidation at the fourth carbon outward from the hemiketal centre[7,8] (Scheme 1). ^{2}H-labelling studies[7,8] also indicated that **2–4** resulted from direct oxidation of **1**. The proposed monooxygenase involvement requires that at least one oxygen atom, derived from dioxygen, would be incorporated into **1**, and at least two into **2–4** (the likely route to **6** ⇋ **7**, and therefore the origin of the remaining oxygen atom in **1** and **2–4**, has not previously been addressed). The formation of isomers of 2,8-dimethyl-1,7-dioxaspiro-[5.5]undecane by *B. cucumis* was also accommodated in this general framework.[8]

We now describe the patterns of [^{18}O]-oxygen incorporation from both labelled dioxygen and water[9] into **1–5**, that provide confirmatory evidence for monooxygenase mediated pathways, but also reveal a surprising complexity in that all oxygen atoms of **1**–**5** are dioxygen derived. Female *B. oleae* (*ca*. 8 individuals) were introduced into a small air inlet tube (*ca*. 4 ml), atop a modified conical flask with a side-arm. This inlet tube was isolated from the general atmosphere with a suba-seal serum cap. Upon opening a 10 mm stopcock on the underside of the inlet tube, the flies migrated downwards into the larger chamber (~350 ml) which had previously been evacuated and then charged with an atmosphere of 20% [$^{18}O_2$]-dioxygen (99.8 atom% ^{18}O) and 80% N_2, and which contained a small amount of a 10% sugar solution (wettex dispenser). Head space volatiles were sampled by the Solid Phase Micro-Extraction technique[9] (SPME), with the needle penetrating the suba seal. Levels of ^{18}O incorporation into spiroacetal metabolites were monitored by direct GC–MS analyses of the SPME fibre. This sampling revealed a progressive dilution of the initial endogenous metabolites with material generated in the $^{18}O_2$-enriched atmosphere. After 3 days, the flies were sacrificed and the rectal glandular components examined by GC-MS methods that had been utilised previously for the unambiguous identification of the components.[4b] These analyses also provided semi-quantitative estimates of ^{18}O-incorporation, and generally, the identification of the site(s) of incorporation.[10] Complementary labelling experiments were conducted with flies in a sealed, normal atmosphere in the presence of ^{18}O-labelled water (20% ^{18}O-enrichment).

The spiroacetal **1** exhibited a high level of ^{18}O-incorporation from dioxygen, plainly evidenced by the displacement of the molecular ion from *m*/*z* 156 to *m*/*z* 160, with a low level of *m*/*z* 158 (*m*/*z* 156:158:160 = 5:8:87, see Fig. 1). Identifiable fragment ions also required the presence of two ^{18}O atoms, and demonstrated that both oxygen atoms in **1** were dioxygen derived, with a low level of monolabelled **1** (with *m*/*z* 158).[11] When ^{18}O water was employed, no incorporation into **1** was measurable. In the isomers of **5**, both oxygen atoms were also dioxygen derived. The low levels of **5** indicate that (ω-1) oxidation of **6** ⇋ **7** (giving **5**) competes poorly with ω-oxidation, that provides the heavily predominating **1**. Previously, we had revealed[7,8] that the putative monooxygenases involved in the formation of **1**, were also capable of (ω-1) oxidation and hydroxylation at the third carbon from the hemiketal centre in alkyltetrahydropyranols as required for the formation of **5**. Further oxidation of **1** provides **2**–**4**, now incorporating three labelled oxygens, although the ultimately



Scheme 1

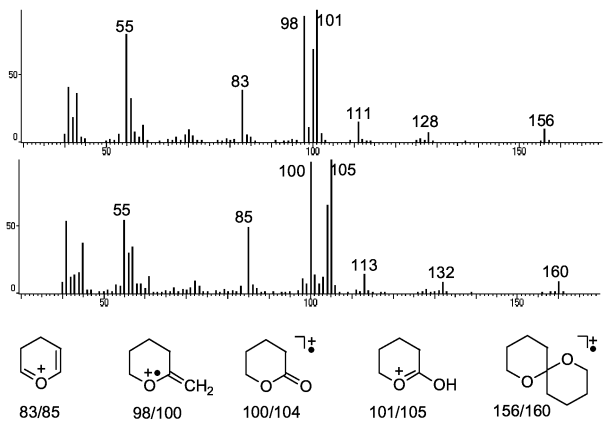


Fig. 1 Mass spectra of spiroacetal **1** from *B. oleae* in (A) normal air and (B) [$^{18}O_2$]-dioxygen atmosphere. Selected fragment ion structures[10] and predicted *m*/*z* for ions with ^{16}O/^{18}O are shown.

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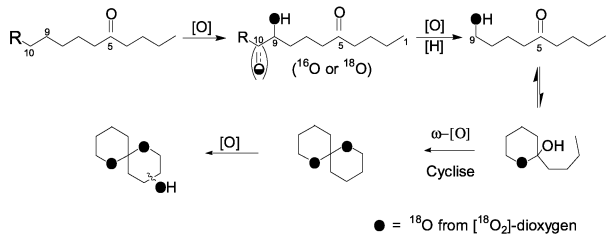
incorporated oxygen has a higher ^{18}O level, consistent with our hypothesis that hydroxylation occurs after spiroacetal formation. The hydroxymethyl derivative **4** does not arise predominantly by oxidation of the methyl group of **5**, but rather by equilibration with **2**, presumably by an *in vivo* ring opening–closing sequence after initial ring hydroxylation of **1** at C-3. This follows because the ^{18}O level in the CH_2OH group of **4** is slightly lower than that of the exocyclic hydroxy groups in **2** and **3**, which are in turn somewhat higher than those for the endocyclic oxygen atoms. Ethyl esters of long chain fatty acids are also present in the gland extract,[2] and these show ^{18}O uptake from [^{18}O]-H_2O, but negligibly from [$^{18}O_2$]-air, confirming minimal metabolic mixing of the dioxygen and water based pools of oxygen atoms.

Precedent[12] would suggest a fatty acid/polyketide origin for **1** in which one of the oxygens atoms was derived from water. Previously, Mazomenos had reported that ^{14}C-labelled polyketide precursors (*e.g.* acetate) were incorporated into **1** from female olive flies and proposed fatty acid/polyketide assembly to produce a nine-carbon unit.[13] Such a sequence, however, would not result in the observed double incorporation from dioxygen into **1**. This labelling pattern in **1** requires that the (formal) primary alcohol in **6** ⇋ **7** also be labelled and therefore be the result of an oxidative process. Likely precursors to the oxygenated nine carbon unit are those incorporating the oxidatively susceptible O–C–C–O moiety, representing 1,2-diols, α-hydroxy and α-ketoacids.[14] Although the order of oxidative events in the production of the difunctionalised nine-carbon unit is not yet clear, a reasonable generalised pathway is shown below in Scheme 2, and incorporates later steps for which the evidence is strong.[7,8]

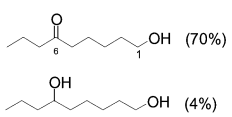
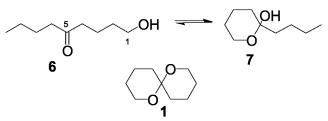
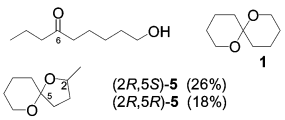
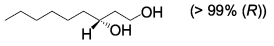


Scheme 2

This pathway for the production of nine-carbon units may be of general importance because, as we have noted previously[3] some *Bactrocera* species are adept at providing such metabolites, and some examples are summarised below in Table 1.

Assuming similar biosynthetic pathways operate within *Bactrocera* species, the data for *B. umbrosa*[3] are particularly instructive. These show regio-variability in the mid-chain oxidation by providing both 5-oxononanol (then processed to spiroacetal **1**) and 6-oxononanol, as well as in side-chain hydroxylation of **6** ⇋ **7** to afford either **1** (ω-oxidation) or **5** ((ω-1) oxidation). Nonane-1,3-diol from *B. cucumis* further demonstrates the regio-diversity of oxygen incorporation.

Table 1 Hydroxylated nine-carbon units from *Bactrocera* species

Species	Nine-carbon compounds
B. carambolae	 (70%)
	(4%)
B. cacuminata	
B. umbrosa	 (2*R*,5*S*)-**5** (26%) (2*R*,5*R*)-**5** (18%)
B. tau, *B. cucumis*	 (> 99% (*R*))

The above findings are consistent with the general paradigm previously presented,[7,8] for spiroacetal biosynthesis in *Bactrocera sp.* but reveal novel pathways with respect to construction of the oxygenated nine-carbon precursor, *e.g.* **6** ⇋ **7**. Nevertheless, our observations support the proposal[15] that, rather than utilising a complete set of unique enzymes for semiochemical production, insects adapt the products of "normal" biosynthetic construction with a species-specific set of auxiliary enzymes. Thus, specific monooxygeneses might divert fatty acids into a nine-carbon biosynthetic manifold. Subsequently, species specific suites of monooxygenases with varying regio- and stereospecificities would produce the observed range of metabolites (Table 1 and Scheme 1). Further studies will address this matter and the generality of monooxygenase activity in the biosynthesis of spiroacetals in other insect orders.

The authors are grateful to the Australian Research Council for support of this work.

Notes and references

1 H. Kuba, *Proc. Internat. Symp. Biological Control of Fruit Flies*, ed. K. Kawasaki, O. Iwahashi and K. Y. Kaneshiro, Okinawa, Japan, 1991.

2 B. E. Mazomenos, *World Crop Pests: Fruit Flies Their Biology, Natural Enemies and Control*, ed. A. S. Robinson and G. H. S. Cooper, Elsevier, Amsterdam, 1989.

3 M. T. Fletcher and W. Kitching, *Chem. Rev.*, 1995, **95**, 789.

4 (*a*) R. Baker and R. H. Herbert, *J. Chem. Soc. Perkins Trans. 2*, 1987, 1123; (*b*) S. Krohn, M. T. Fletcher, W. Kitching, R. A. I. Drew, C. J. Moore and W. Franke, *J. Chem. Ecol.*, 1991, **17**, 485; (*c*) M. T. Fletcher, M. F. Jacobs, W. Kitching, S. Krohn, R. A. I. Drew, G. E. Hanniotakis and W. Francke, *Chem. Commun.*, 1992, 1457.

5 This spiroacetal has been identified from *B. umbrosa* (See Table 1 and ref. 3) and also in several wasp species (see W. Francke, G. Hindorf and W. Reith, *Naturwissenschaften*, 1979, **66**, 618).

6 For a general discussion of the occurrence of spiroacetals in insects, see W. Francke and W. Kitching, *Curr. Org. Chem.*, 2001, **5**, 233.

7 N. L. Hungerford, B. E. Mazomenos, M. A. Konstantopolou, F. D. Krokos, G. E. Haniotakis, A. Hübener, M. T. Fletcher, C. J. Moore, J. J. De Voss and W. Kitching, *Chem. Commun.*, 1998, 863.

8 J. E. Stok, C.-S. Lang, B. D. Schwartz, M. T. Fletcher, W. Kitching and J. J. De Voss, *Org. Lett.*, 2001, **3**, 397.

9 Dioxygen ($^{18}O_2$) of >99 atom% was obtained from Isotech Inc., Miamisburg, Ohio. ^{18}O enriched H_2O (20 atom% ^{18}O) was from Yeda R & D Co. Ltd., Israel. A Carboxen PDMS fibre (Supelco) was employed for SPME monitoring.

10 For a brief discussion of mass spectral fragmentations in spiroacetals, see ref. 6 and references therein.

11 The question arises as to whether both oxygen atoms in **1** are incorporated from a single dioxygen molecule. However, the demonstrated incorporation[7] of **6** ⇋ **7** makes this very unlikely.

12 For example: (*a*) N. Islam, R. Bacala, A. Moore and D. Vanderwal, *Insect Biochem. Mol. Biol.*, 1999, **29**, 201; (*b*) P. Juarez, J. Chase and G. J. Blomquist, *Arch. Biochem. Biophys.*, 1992, **293**, 333.

13 J. G. Pomonis and B. E. Mazomenos, *Int. J. Invert. Reprod. Devel.*, 1986, **10**, 169; diethyl 5-oxononanedioate has been described from wild male olive flies and suggested as a possible precuror for **1** (see B. E. Mazomenos and J. G. Pomonis, in *Fruit Flies of Economic Importance*, ed. R. Cavalloro, Proceedings of the CEC/IOBC Internat. Symp., Athens, Greece, November (1982). p. 96), but this minor metabolite now appears not to be enroute to **1**.

14 Analagous oxidative cleavage pathways have been proposed for the monooxygenase catalysed formation of pregnenolone (P. R. Ortiz de Montellano, *Cytochrome P450 Structure, Mechanism and Biodiversity*, Plenum Press, 2nd edn., New York, 1995) and pimelic acid (J. E. Stok and J. J. De Voss, *Arch. Biochem. Biophys.*, 2000, **384**, 351).

15 (*a*) "*Pheromone Biochemistry*", ed. G. D. Prestwick and G. J. Blomquist, Academic Press, Orlando, 1987; (*b*) J. A. Tillman, S. J. Seybold, R. A. Jurenka and G. J. Blomquist, *Insect Biochemistry and Molecular Biology*, 1999, **29**, 481; (*c*) W. S. Leal, P. H. G. Zarbin, H. Wojtasek and J. T. Ferreira, *Eur. J. Biochem.*, 1999, **259**, 175.