J|A|C|S
COMMUNICATIONS

Published on Web 06/05/2002

[^{18}O]-Oxygen Incorporation Reveals Novel Pathways in Spiroacetal Biosynthesis by *Bactrocera cacuminata* and *B. cucumis*

Mary T. Fletcher,[†] Barry J. Wood,[†] Ian M. Brereton,[‡] Jeanette E. Stok,[†] James J. De Voss,*[,†] and William Kitching*[,†]

Department of Chemistry and Centre for Magnetic Resonance, The University of Queensland, Brisbane 4072, Australia

Received March 17, 2002

Although spiroacetals represent a growing and important class of insect-derived semiochemicals,[1,2] the very limited information on their biosynthesis[3−5] hinders development of control strategies based on suppression of spiroacetal formation. Monooxygenase-mediated pathways have previously been implicated in the biosynthesis of *Bactrocera* fruit fly spiroacetals,[4,5] and in this context we now reveal disparate patterns of [^{18}O]-oxygen incorporation into the spiroacetals of two Australian *Bactrocera* species. These unanticipated incorporation patterns demonstrated surprisingly divergent origins for spiroacetals **1**−**4** and require novel pathways in their construction.

A general paradigm devised[4,5] for *Bactrocera* spiroacetal biosynthesis involves monooxygenase-mediated hydroxylation of an intermediate alkyltetrahydropyranol (or a biological equivalent) in the penultimate step. Figure 1 illustrates the postulated route to 1,7-dioxaspiro[5.5]undecane (**1**) in *B. cacuminata* and 2,8-dimethyl-1,7-dioxaspiro[5.5]undecane (**3**) in *B. cucumis*, with further hydroxylation yielding the accompanying hydroxyspiroacetals,[2,6] **2** and **4**, respectively. Monooxygenase involvement in these pathways was investigated by using [^{18}O]-oxygen incorporation from both dioxygen and water.

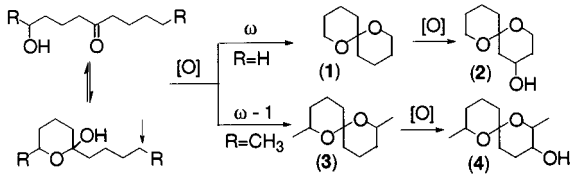
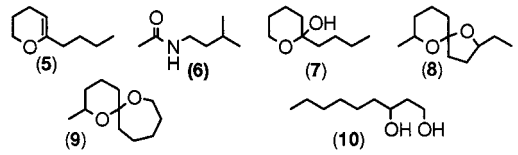


Figure 1. Proposed biosynthesis of *Bactrocera* spiroacetals.

Specimens of *B. cacuminata*[7] were placed in an atmosphere of 20% $^{18}O_2$ (99.8 atom % ^{18}O) and 80% N_2[8] with a sugar and water diet. Headspace volatiles were regularly sampled by the Solid-Phase Micro-Extraction technique[8] (SPME) and ^{18}O-incorporation was monitored by GC-MS analyses. This sampling demonstrated a smooth dilution of endogenous metabolites with material generated in the [$^{18}O_2$] atmosphere. After ca. 5 days, the flies were sacrificed and the rectal glandular components examined by combined GC-MS methods that had been employed previously[2,9,10] for the unambiguous identification of all the components. In addition, mass spectral analysis allowed quantitative assessment of ^{18}O incorporation into these molecules and, in many cases, identification of the site(s) of incorporation. Complementary labeling experiments were carried out with normal air in the presence of [^{18}O]-water (20% ^{18}O enrichment). Similar experiments with both [$^{18}O_2$]-dioxygen and ^{18}O-labeled water were conducted with *B. cucumis*. The major glandular component (>90%) from *B. cacuminata* is **1**,[9] along with low levels of the dihydropyran (**5**), amide (**6**), tetrahydropyranol (**7**), and hydroxyspiroacetal (**2**) and minor positional isomers.[6,9] In *B. cucumis*, the major compound in the released volatiles, and also in the glandular secretion, is spiroacetal (*E*,*E*)-(**3**), along with its (*E*,*Z*) and (*Z*,*Z*) diasteromers.[10] The spiroacetals **4**, **8**, and **9** are also present along with amide (**6**) and 1,3-nonanediol (**10**).[2,10]



The presence of amide **6** in each of the species provided an internal monitor for these experiments. Formation of this amide by established routes from the corresponding acid would require the amide carbonyl to be derived ultimately from water. In agreement with this, amide **6** from flies exposed to 99.8% [$^{18}O_2$]-dioxygen incorporated ^{18}O at a low level (∼5%) compared with incorporation into the accompanying spiroacetals **1** (∼45%) or **3** (∼75%). In flies exposed to 20% [^{18}O]-water, a significantly higher level of [^{18}O] incorporation into **6** was seen (11% incorporation or >50% uptake of available [^{18}O]). Besides supporting our hypothesis of the unexceptional origin of **6**, these results clearly demonstrate minimal metabolic mixing of the dioxygen- and water-derived pools of oxygen atoms in these flies.

All spiroacetals from both species of flies had significant incorporation of ^{18}O from [$^{18}O_2$]-dioxygen. In addition, the hydroxyl moiety of the hydroxyspiroacetals (**2** and **4**) from both species was extensively labeled by [$^{18}O_2$]-dioxygen and derived no label from [^{18}O]-H_2O. These observations are consistent with the postulate[4,5] that alkyltetrahydropyranols are the penultimate precursors to spiroacetals in these Dipteran species, with side chain oxidation by a monooxygenase being followed by cyclization to the spiroacetal. Monooxygenation of the parent compound then produces the observed hydroxy derivatives. e.g. **2** and **4** (Figure 1).

However, the patterns of oxygen labeling in the spiroacetals from the two fly species were strikingly different. GC-MS comparisons of spiroacetal (**1**) generated normally with that produced in an [$^{18}O_2$]-atmosphere **demonstrated that both oxygen atoms of 1 were labeled and hence dioxygen derived**. This follows because the molecular ion of **1** ($M^+ = 156$) is now displaced to $M^+ = 160$ and identifiable fragment ions also require the incorporation of two [^{18}O] atoms. The extremely low level of material with $M^+ = 158$ confirms the near absence of monolabeled **1**. **In contrast**, the

* Corresponding author. E-mail: devoss@chemistry.uq.edu.au.
[†] Department of Chemistry.
[‡] Centre for Magnetic Resonance.

10.1021/ja026215l CCC: $22.00

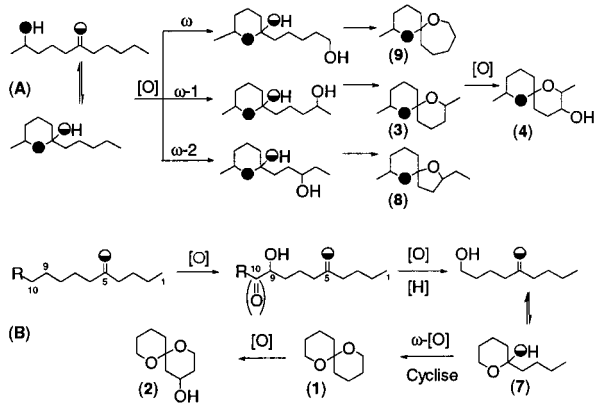


Figure 2. Incorporation of water (H_2●) and dioxygen (O_2) into spiroacetals of (A) *B. cucumis* and (B) *B. cacuminata*. The symbol ◒ indicates oxygen of undetermined origin.

spiroacetals **3**, **8**, and **9** isolated from *B. cucumis* incorporated only **one oxygen atom from [$^{18}O_2$]-dioxygen**. When ^{18}O-labeled water was employed, there was no incorporation into **1**, but one labeled oxygen atom appeared in each of **3**, **8**, and **9**. Consistent with the unexpected results for **1**, the dihydropyran (**5**) from *B. cacuminata* was also found to incorporate one ^{18}O-label when produced in an [$^{18}O_2$]-dioxygen-enriched atmosphere, but no label from [^{18}O]-water. Strikingly, in 1,3-nonanediol (**10**) from *B. cucumis*, **both** oxygen atoms derive from [$^{18}O_2$]-dioxygen.

Evidence for the remarkable double incorporation from [$^{18}O_2$]-dioxygen into **1** is also provided by 187 MHz ^{13}C NMR measurements of the glandular components of 15 flies, directly extracted into $CDCl_3$. With the high spectral resolution obtained at this frequency, ^{18}O-isotope effects (Δ ppm, all to higher field) on shifts of four of the five different carbon atoms in the molecule (**1**) were measurable. The one- and two-bond ^{18}O-induced effects on the ^{13}C shifts of C_2 (0.028 ppm) and (C_3 and C_5) (0.005 and 0.006 ppm) are in the normal range for oxygen atoms singly bonded to carbon.[11] However, the substantial upfield shift in the spirocarbon resonance (C_6, $\Delta = 0.053$ ppm) requires C_6 to be flanked by **two** ^{18}O atoms, as deduced from the mass spectral data.

These incorporation results reveal not only the generality of monooxygenase mediation of spiroacetal formation, but also an unexpected complexity in their biosynthesis. We propose that two distinct but convergent pathways operate in these *Bactrocera* species.

With respect to the components from *B. cucumis*,[2,10] a modified fatty acid/polyketide construction of the tetrahydropyranol or equivalent system (Figure 2A) is proposed, resulting in the ether oxygen being water derived. Such a specialized pathway has been implicated previously in insect pheromone biosyntheses.[12] Monooxygenase-mediated ($\omega - 1$) oxidation would introduce an oxygen atom from dioxygen, so that, upon dehydrative cyclization, the singly ^{18}O-labeled 2,8-dimethyl-1,7-dioxaspiro[5.5.]undecane system (**3**) would result. GC-MS analyses confirmed that the [$^{18}O_2$]-derived label was located only in the ethyl-bearing ring in each of the (*E*,*E*) and (*E*,*Z*) isomers of **8**, and almost certainly in the seven-membered ring of **9**. We propose that the less favored ($\omega - 2$) or ω hydroxylation of the side chain of the tetrahydropyranol would lead to the 2-ethyl-1,6-dioxaspiro[4.5]decanes (**8**) and 2-methyl-1,7-dioxaspiro[5.6]dodecane (**9**), respectively. The co-occurring, hydroxy derivatives, e.g. **4**, would then result from monooxygenase-mediated oxidation of the initially formed **3**. These proposals, and the predicted oxygenation patterns for the use of ^{18}O-labeled dioxygen and water, are summarized in Figure 2A and are in harmony with the experimental outcomes described here.[13]

The incorporation of two dioxygen-derived oxygen atoms into both nonane-1,3-diol (**10**) in *B. cucumis* and spiroacetal **1** in *B. cacuminata* was totally unexpected. Without this knowledge, precedent would suggest a fatty acid/polyketide origin[12] for both **1** and **10** in which one (in **1**) or both (in **10**) oxygen atoms were derived from water. The presence of these two C_9 metabolites, with unexpected oxygen incorporation from [$^{18}O_2$]-dioxygen, implies that insect biosynthesis may utilize unique biosynthetic processes. Among several possibilities, an oxidative cascade achieving C−C bond cleavage, perhaps via 1,2-diol creation, to furnish a functionalized C_9 system, appears economical.[14,15] Other possibilities such as oxidative fission of an α-ketoacid are also possible (Figure 2B).

The present results, while confirming the salience of alkytetrahydropyranols in spiroacetal formation, demonstrate divergent biosynthesis of these precursors in two Dipteran species. Ongoing studies of spiroacetal biosynthesis in other insect orders will define the generality of monooxygenase activity in the production of such metabolites.

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

Supporting Information Available: Table of ^{18}O incorporations in both species, mass spectra analysis for [$^{18}O_2$]-oxygen uptake into **1**, **3**, **8**, and **9**, and ^{13}C NMR spectra for [$^{18}O_2$]-(**1**) (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Francke, W.; Kitching, W. *Curr. Org. Chem.*, **2001**, *5*, 233.

(2) (a) Fletcher, M. T.; Kitching, W. *Chem. Rev.*, **1995**, *95*, 789. (b) Tu, Y. Q.; Hübener, A.; Zhang, H.; Moore, C. J.; Fletcher, M. T.; Hayes, P.; Dettner, K.; Francke, W.; McErlean, C. S.; Kitching, W. *Synthesis* **2000**, *13*, 1956. Hayes, P.; Fletcher, M. T.; Moore, C. J.; Kitching, W. *J. Org. Chem.* **2001**, *61*, 2530.

(3) Reference 2a, pp 798−799, contains a summary of the situation as of 1995.

(4) Hungerford, N. L.; Mazomenos, B. E.; Konstanopolou, M. A.; Krokos, F. D.; Haniotakis, G. E.; Hübener, A.; Fletcher, M. T.; Moore, C. J.; De Voss, J. J.; Kitching, W. *Chem. Commun.* **1998**, 863.

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

(6) Fletcher, M. T.; Jacobs, M. F.; Kitching, W.; Krohn, S.; Drew, R. A. I.; Haniotakis, G. E.; Francke, W. *Chem. Commun.* **1992**, 1457.

(7) These specimens were obtained from a culture maintained by the Department of Primary Industries, Queensland. We are grateful to Dr. Annice Lloyd for her cooperation in the provision of fruit flies.

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

(9) Krohn, S.; Fletcher, M. T.; Kitching, W.; Drew, R. A. I.; Moore, C. J.; Franke, W. *J. Chem. Ecol.* **1991**, *17*, 485.

(10) Kitching, W.; Lewis, J. A.; Perkins, M. V.; Drew, R. A. I.; Moore, C. J.; Schurig, V.; König, W. A.; Francke, W. *J. Org. Chem.* **1989**, *54*, 3893.

(11) Vederas, J. C. *Natural Prod. Rep.* **1987**, 277−357 (Section 5.2.2). Also: Hansen, P. E. *Ann. Rep. NMR Spectrosc.* **1983**, *15*, 105.

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

(13) Experiments with ^{2}H-labeled precursors demonstrate that the dioxygen-derived oxygen atom is the second of the two oxygen atoms to be installed in the spiroacetals **8** and **9**.

(14) Analogous oxidative cleavage pathways have been proposed for the monooxygenase-catalysed formation of pregnenolone (Ortiz de Montellano, P. R. *Cytochrome P450 Structure, Mechanism and Biochemistry*, 2nd ed.; Plenum Press: New York, 1995) and pimelic acid (Stok, J. E.; De Voss J. J. *Arch. Biochem. Biophys.* **2000**, *384*, 351−60).

(15) For a proposal of enantioselective hydroxylation of a fatty acid in pheromone biosynthesis see: Leal, W. S.; Zarbin, P. H. G.; Wojtasek, H.; Ferreira, J. T. *Eur. J. Biochem.* **1999**, 259, 175.

JA026215L