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Monooxygenase Stereoselectivity in the Biosynthesis of Stereoisomeric Spiroacetals in the Cucumber Fly, *Bactrocera cucumis*

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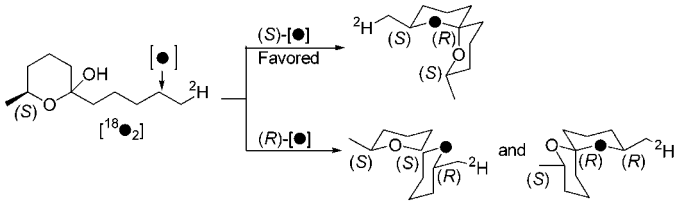
ABSTRACT



The stereoselectivity of hydroxylation of alkyltetrahydropyran-2-ols (or their biological equivalents) in the formation of stereoisomers of 2,8-dimethyl-1,7-dioxaspiro[5.5]undecanes in male *Bactrocera cucumis* has been investigated. Racemic, (6*R*)-, and (6*S*)-6-methyl-2-[5-$^2H_1$]-*n*-pentyltetrahydropyran-2-ol was administered under an [$^{18}O_2$]-enriched atmosphere. The stereochemistry and isotopic composition of generated spiroacetals were monitored by combined enantioselective GC-MS. The monooxygenase(s) strongly prefers the (6*S*)-substrate and furnishes predominantly the (*S*)-alcohol and then the (2*S*,6*R*,8*S*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane. The (2*S*,6*S*,8*R*) and (2*R*,6*S*,8*S*) (*E*,*Z*)-isomers appear to be derived in vivo predominantly from (*R*)-hydroxylation of the (6*S*)-tetrahydropyranol.

Recently we disclosed a general paradigm[1,2] for spiroacetal biosynthesis in *Bactrocera* species that involved monooxygenase mediated hydroxylation of an intermediate alkyltetrahydropyranol (or a biological equivalent) in the penultimate step. Patterns of [^{18}O]-oxygen incorporation from both dioxygen and water into the spiroacetals from *B. oleae*, *B. cacuminata*, and *B. cucumis* strongly support this proposal.[3] These spiroacetals are present in volatiles released by these fruit flies, and are known to have a pheromonal function in at least one species.[4] The [^{18}O]-oxygen incorporations seen in *B. cucumis* are summarized in Scheme 1 in the context of our general paradigm.

Extracts of the rectal glands, and the released volatiles from *B. cucumis*, contained as the major spiroacetals stereoisomers **2**−**4** of 2,8-dimethyl-1,7-dioxaspiro[5.5]-undecane (**1**), with isomer **2** predominating.[5] The stereodiversity of naturally occurring **1** can be explained either by (a) a corresponding diversity in the precursor or (b) a lack of selectivity in the monooxygenation reaction which mediates spiroacetal formation. We now report studies

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

(2) Hungerford, N. L.; Mazomenos, B. E.; Konstantopolou, 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.

(3) (a) Fletcher, M. T.; Wood, B. J.; Brereton, I. M.; Stok, J. E.; De Voss, J. J.; Kitching, W. *J. Am. Chem. Soc.* **2002**, *124*, 7666. (b) Fletcher, M. T.; Mazomenos, B. E.; Georgakopoulos, J. H.; Konstantopoulou, M. A.; Wood, B. J.; De Voss, J. J.; Kitching, W. *Chem. Commun.* **2002**, 1302.

(4) Fletcher, T.; Kitching, W. *Chem. Rev.* **1995**, *95*, 789.

(5) 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. The ratio of spiroacetals **2**:**3**:**4** in glandular extracts was 12:1.0:1.6.

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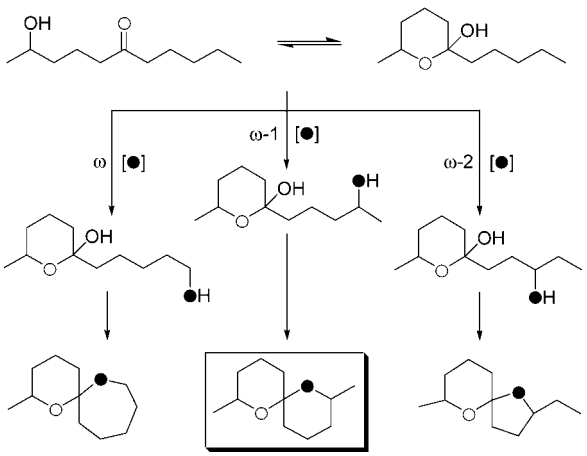
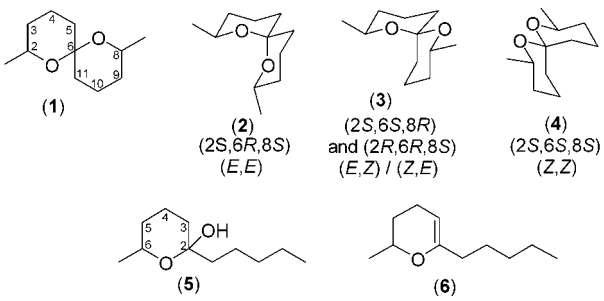
Scheme 1. Incorporation of Water (H_2O) and Dioxygen ($\bullet_2$) into Spiroacetals of *B. cucumis*

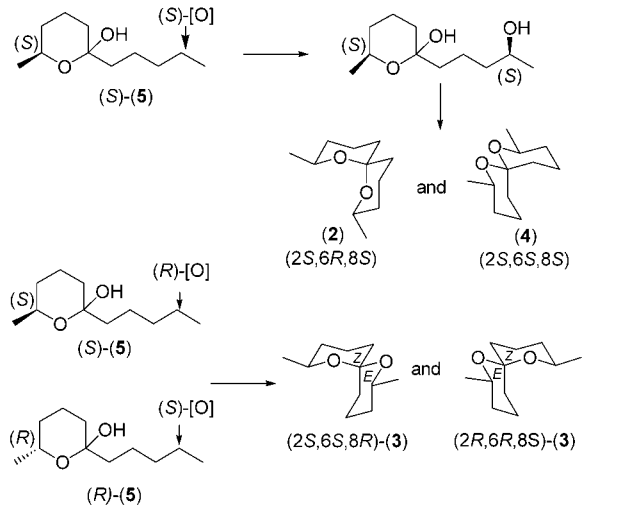


designed to distinguish these possibilities and to establish the specificity of the monooxygenase involved in the formation of **1**.



The naturally occurring stereoisomers of **1** can be divided into two groups. In the (*E*,*E*) and (*Z*,*Z*) isomers **2** and **4**, both methyl bearing methine carbons have the *S* configuration while the (*E*,*Z*) isomers **3** have one *S* and one *R* configured methine. Potential biosynthetic precursors, **5** and **6** although not identified from *B. cucumis*, do co-occur with **2**−**4** in other *Bactrocera* species,[5] and are (*S*)-configured at the methyl-bearing center.[6] Thus, (*S*)-hydroxylation of (*S*)-**5** followed by cyclization would yield (*E*,*E*) and (*Z*,*Z*) diastereomers **2** and **4**, that differ only in the configuration at the spiro center.[5,7] However, the origin of the (*E*,*Z*) diastereomer **3** is less clear. It may arise either from (*R*)-hydroxylation of (*S*)-**5** or via (*S*)-hydroxylation of (*R*)-**5**. These possibilities are shown in Scheme 2.

Scheme 2



The determination of the likely biosynthetic route to **3** in *B. cucumis* was made problematical by our previous finding[1] that (*R*)-**5** could serve as a precursor of **3** when administered exogenously, and that the monooxygenase involved in the formation of **1** was not stereospecific. Thus, both pathways (Scheme 2) were possible and we sought to determine which predominated in vivo. The proposal was that analysis of the incorporation of racemic and (*S*)- and (*R*)-configured **5** containing a ^{2}H-label, in the presence of [$^{18}O_2$]-dioxygen, would expose important details of the in vivo pathways. Thus the [$^2H_1$]-isotopomer of (*R*)-**5**, (6*R*)-6-methyl-2-[5-$^2H_1$]-pentyltetrahydropyranol, (*R*)-**7** was synthesized by sequential alkylation of acetone−dimethylhydrazone[6] as were the (*S*)-isomer, (*S*)-**7** and the racemate, *rac*-**7**.

The feeding experiments, in which the precursors were supplied ad libitum to male *B. cucumis*, utilized an apparatus in which the atmosphere was 20% [$^{18}O_2$]-dioxygen (>99 atom % of ^{18}O) and 80% N_2.[8] Any monooxygenase catalyzed formation of spiroacetal **1** would result in ^{18}O-incorporation at the carbon adjacent to the ^{2}H-labeled carbon. Consequently, one ring of the spiroacetal system would be 3 amu heavier than the other.

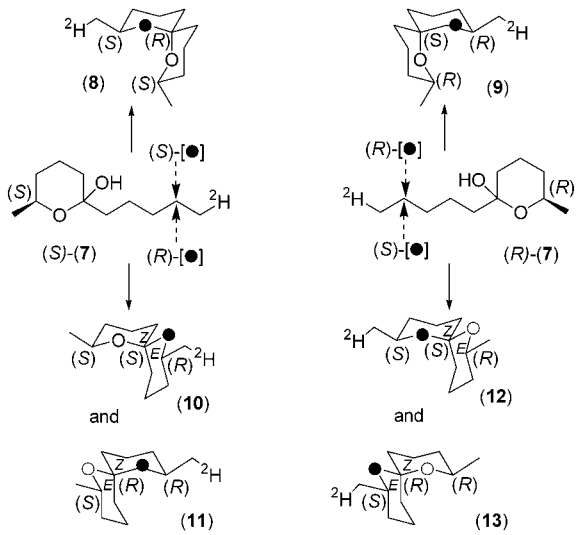
Administration of racemate, *rac*-7: Solid-phase microextraction (SPME) and GC-MS analyses of the (*E*,*E*)-spiroacetal demonstrated that ^{18}O was being incorporated, and over time the relative intensity of the ion at *m*/*z* 184 (M^+ for endogenous spiroacetal) decreased and those at *m*/*z* 186 and 187 increased. The *m*/*z* 186 ion corresponds to incorporation of one ^{18}O atom into unlabeled endogenous precursor, whereas *m*/*z* 187 arises from ^{18}O-oxygenation of the administered *rac*-**7** and confirms its in vivo transformation to spiroacetal system **1**. The location of the label was clearly shown to be *vicinal* to the deuterium atom by the mass

(6) Fletcher, M. T.; Wells, J. A.; Jacobs, M. F.; Krohn, S.; Kitching, W.; Drew, R. A. I.; Moore, C. J.; Franke, W. *J. Chem. Soc., Perkin Trans. 1* **1992**, 2827. Dihydropyran **6** was of 95% ee in *B. kraussi*, with the major component,being (2*S*,6*R*,8*S*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane (**2**), of >98% ee.

(7) Chen, J.; Fletcher, M. T.; Kitching, W. *Tetrahedron Asymmetry* **1995**, *6*, 967. Substituted spiroacetals may occur as (*E*)- and (*Z*)-diastereomers. When the substituted (alkyl) group and (spiro) oxygen atom of the alternate ring are on the same side of the reference plane, the isomer is (*Z*), and if not it is (*E*). (The reference plane is the substituted ring.) See: Blackwood, J. E.; Gladys, C. L.; Leonig, K. L.; Petrarca, A. E.; Rush, J. E. *J. Am. Chem. Soc.* **1968**, *90*, 509.

(8) A brief description of the apparatus is contained in ref 4 and a fuller description elsewhere. McErlean, C. S. P. Ph.D. Thesis, 2002, The University of Queensland. Repeated SPME and GCMS analyses were carried out over a 3−4 day period and demonstrated smooth incorporation of both isotopic labels, as confirmed by GCMS analysis of glandular extracts at the completion of the experiment (Figure 1).

Scheme 3



spectral fragmentation pattern and in particular, the ions at *m*/*z* 140 (M − CH_3CHO) and 143 (plus 3 amu). This constitutes the first unequivocal demonstration that the ^{18}O incorporation from labeled dioxygen we had previously observed[3] in **1** corresponds to the second oxygen atom introduced. Although predicted by our general paradigm, this observation clearly excludes other more complicated biosynthetic schemes, which could be proposed in view of the unusual double ^{18}O incorporation into the spiroacetals of *B. cacuminata* and *B. oleae*.[3]

Given our previous results with racemic **5** in *B. cucumis*,[1,3] we expected a complex manifold of products to result from incorporation of *rac*-**7** (Scheme 3). The naturally occurring (*E*,*E*) spiroacetal **8** and its enantiomer **9** would be expected from (*S*)-hydroxylation of (*S*)-**7** and (*R*)-hydroxylation of (*R*)-**7**, respectively. In addition, four different isomers of the (*E*,*Z*) spiroacetal would be produced. The relationship between these is complex. (*R*)-Hydroxylation of (*S*)-**7** gives rise to **10** and **11**, which are essentially enantiomers, except for the positioning of the isotopic labels. Similarly (*S*)-hydroxylation of (*R*)-**7** gives **12** and **13** which are again pseudoenantiomeric. The pairs **10**/**12** and **11**/**13** are identical except for the location of the isotopic labels, and the pairs **10**/**13** and **11**/**12** are true enantiomers.

A number of conclusions can be drawn from these results. Clearly, the racemic precursor *rac*-**7** was transformed (Figure 1a) into the enantiomers of the (*E*,*E*) spiroacetal with widely differing efficiencies. The only plausible route to **8** is via (*S*)-hydroxylation of the (*S*)-precursor. Similarly, the enantiomeric **9** must result from (*R*)-hydroxylation of the (*R*)-precursor. By comparing the absolute intensities of the *m*/*z* 187 ions for enantiomers **8** and **9**, (*S*)-hydroxylation of the (*S*)-precursor was deduced to show a 97:3 preference over (*R*)-hydroxylation of the (*R*)-precursor, i.e., the (2*S*,6*R*,8*S*) **8** had an ee of 94%.

Enantioselective GC-MS analysis of the (*E*,*Z*)/(*Z*,*E*) isomers **10**−**13** is more complex. The base peak in the mass

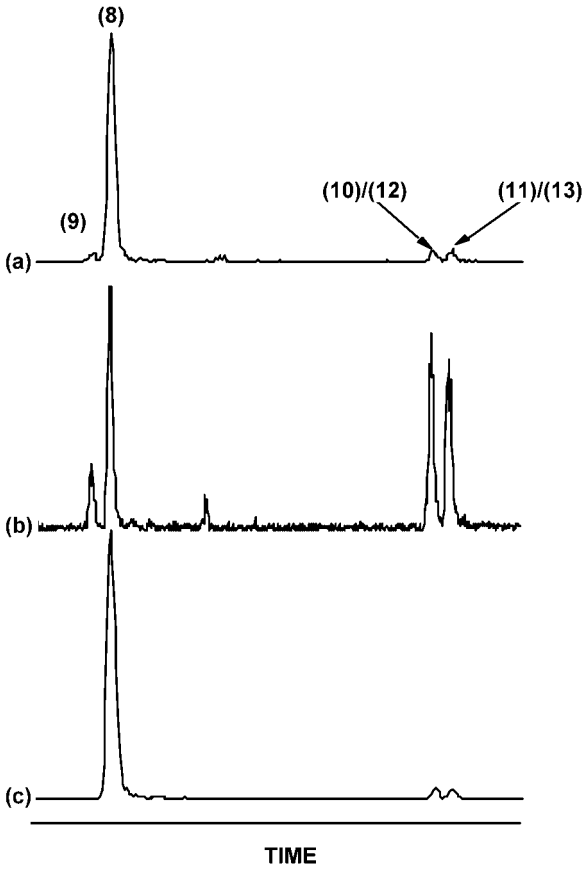


Figure 1. Enantioselective gas chromatograms[9] of the glandular extract from 10 male *B. cucumis* after administering (a) *rac*-**7**, (b) (*R*)-**7**, and (c) (*S*)-**7**. Traces are ion chromatograms for *m*/*z* 187.

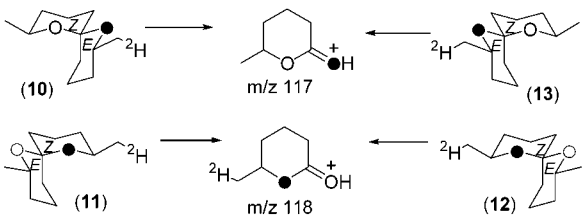
spectra of (*E*,*Z*)/(*Z*,*E*) spiroacetals is a protonated lactone fragment,[10] which arises from preferential retrocleavage of the *E* ring.[11] Thus (*E*,*Z*) stereoisomers with the labels located in the *E* ring (i.e. **10**/**13**) will exhibit mass spectra different from those for stereoisomers with the labels in the *Z* ring (i.e. **11**/**12**), with base peaks *m*/*z* 117 and 118 being particularly diagnostic (Scheme 4). Enantiomers **10** and **13** will have identical mass spectra, as will **11** and **12**. Enantioselective GC separated the four (*E*,*Z*) isomers into two peaks, one for the **10**/**12** pair and one for the **11**/**13** pair. In each of these peaks, one compound (**10**/**11**) is derived from the (*S*) precursor and one (**12**/**13**) from the (*R*) precursor. If (*S*)-**7** and (*R*)-**7** are processed equally well to the (*E*,*Z*) isomers, the ratios **10**:**12** and **11**:**13** should be 1:1, and mass spectra of the two (*E*,*Z*) peaks would be identical.

(9) A 50-m permethylated β-cyclodextrin column (Cydex(B)-0.25 supplied by SGE) was employed for enantioselective GC analysis. Spiroacetals **9**, **8**, **10**/**12**, and **11**/**13** had retention times of 15.3, 15.4, 17.1, and 17.2 min with a GC program of 40 °C for 2 min then 10°/min to 180 °C.

(10) (a) For a brief discussion of mass spectral fragmentations in spiroacetals see: Francke, W.; Kitching, W. *Curr. Org. Chem.* **2001**, *5*, 233 and references therein. (b) For mass spectral fragmentation of stereoisomers **10**−**13** see the Supporting Information.

(11) Unpublished observations. See Supporting Information, and for example (*E*,*Z*)-2,4,8-trimethylspiroacetals in: Tu, Y. Q.; Hübener, A.; Zhang, H.; Moore, C. J.; Fletcher, M. T.; Hayes, P. Y.; Dettner, K.; Franke, W.; McErlean, C. S. P.; Kitching, W. *Synthesis* **2000**, 1956.

Scheme 4



However, if the (*R*) and (*S*) precursors are processed differently the two (*E*,*Z*) peaks from the enantioselective GCMS experiments would be composed predominantly of either the **10**/**11** or the **12**/**13** pair, and exhibit different mass spectra. This was indeed the case and these observations imply that the enzymatic hydroxylation to form the (*E*,*Z*) spiroacetals proceeds more efficiently on one of (*R*)- or (*S*)-**7** than on the other.

More definitive interpretations required separate administration of the tetrahydropyranol enantiomers, (*R*)-**7** and (*S*)-**7**.

Administration of (6*R*)-6-Methyl-2-[5-$^2H_1$]-pentyltetrahydropyran-2-ol, (*R*)-7: The most striking aspect of the *m*/*z* 187 monitored enantioselective gas chromatogram (Figure 1b) is the relative abundance of the (2*S*,6*R*,8*S*) (*E*,*E*) isomer **8**. This isomer must result from (*S*)-hydroxylation of the labeled (*S*)-precursor, despite the latter's presence at low level (ee >98% for (*R*) precursor). This highlights the great preference of the monooxygenase for the (*S*)-substrate. However, as we have shown previously, (*R*)-**5** *can* act as a precursor of the (*E*,*Z*) isomers,[1] and this is reflected in the relatively larger amounts of (*E*,*Z*) isomers **12** and **13**. These arise from (*R*)-**7** that is (*S*)-hydroxylated. Enantioselective GC-MS analyses enabled the further deduction that the (2*S*,6*S*,8*R*) isomer **12** (with base peak *m*/*z* 118, Scheme 4) elutes before the (2*S*,6*R*,8*R*) isomer **13** (base peak *m*/*z* 117) under our GC conditions.[9] A significant portion of the (*R*)-substrate is also (*R*)-hydroxylated and leads to the (2*R*,6*S*,8*R*) (*E*,*E*) isomer **9**. This (2*R*,6*S*,8*R*) (*E*,*E*) isomer has never been observed naturally, and its formation in these feeding experiments strongly suggests that even though (*R*)-**5** is an exogenous precursor of the (*E*,*Z*) isomers, it cannot be the major precursor in vivo.

Administration of (6*S*)-6-Methyl-2-[5-$^2H_1$]-pentyltetrahydropyran-2-ol, (*S*)-7: Analysis of the spiroacetals produced after feeding (*S*)-**7** confirmed the formation of the labeled (2*S*,6*R*,8*S*) isomer **8**, as expected (Figure 1c). This requires the predicted (*S*)-hydroxylation of the administered (*S*)-precursor. Importantly, the high ee ((*S*) > 96%) of the substrate did not prevent significant formation of the two labeled (*E*,*Z*) isomers **10** and **11** with the (6*S*) isomer **10** again preceding the (6*R*) isomer **11**.[9] The (*E*,*E*):(*E*,*Z*) ratio from this experiment was comparable with that observed for wild-type extracts.[5] These results clearly indicate that (*R*)-hydroxylation of the (*S*)-substrate can occur and at a rate consistent with the level of the formation of the (*E*,*Z*) isomers in vivo.

In summary, detailed analyses of the results of feeding experiments of racemic, (6*R*)- and (6*S*)-6-methyl-2-[5-$^2H_1$]-pentyltetrahydropyran-2-ols (*rac*-**7**, (*R*)-**7**, and (*S*)-**7**) to *B. cucumis* in an [$^{18}O_2$]-dioxygen atmosphere reveal that the monooxygenation step is strongly favored for the (*S*)-substrate and furnishes predominantly the (*S*)-alcohol at the new secondary center. Cyclization of this product furnishes both the naturally observed (*E*,*E*) and (*Z*,*Z*) isomers of **1**. The (*E*,*Z*) isomers appear to derive in vivo predominantly from (*R*)-hydroxylation of the (*S*)-tetrahydropyranol (*S*)-**5** or a biological equivalent. This is in agreement with the high ee of naturally observed (*S*)-**5** in other flies. However, *B. cucumis* does accept exogenous (*R*)-**5** and produces both the (*E*,*Z*) isomers **3** via (*S*)-hydroxylation and the unnatural (2*R*,6*S*,8*R*) (*E*,*E*) isomer (*ent*-**2**) via (*R*)-hydroxylation. These investigations also provide insight into the selectivity of the monooxygenase(s) involved in spiroacetal biosynthesis in *Bactrocera* species. The oxidative enzyme system accepts both (*R*)- and (*S*)-configured **5**, and produces both the (*R*) and (*S*) alcohol at the new stereocenter, albeit with a strong preference for (*S*)-configured substrate and for (*S*)-oxidation. These results are in line with our previous investigations with structural analogues of **5** that indicated the involvement of a monooxygenase which accepted a range of substrates and produced alcohols with predominantly, but not exclusively, the (*S*) configuration.[1]

Acknowledgment. The authors are grateful to the ARC for support of this work and Dr. Annice Lloyd for her assistance in the provision of fruit flies.

Supporting Information Available: Preparative scheme for the synthesis of (*R*)-**7**, (*S*)-**7**, and *rac*-**7** and mass spectral fragmentation of **10**/**13** and **11**/**12**. This material is available free of charge via the Internet at http://pubs.acs.org.

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