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2009

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# Determination by HPLC fluorescence analysis of the natural enantiomers of sex pheromones in the New World screwworm fly, *Cochliomyia hominivorax*

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**Abstract.** Bioassays of six racemic synthesized candidate sex pheromone compounds against male New World screwworm *Cochliomyia hominivorax* (Coquerel) flies showed that the most potent bioactivity was found with 6-acetoxy-19-methylnonacosane and 7-acetoxy-15-methylnonacosane compared with four other isomeric acetoxy nonacosanes and a larger aliphatic ketone. As all these methyl-branched compounds have two asymmetric carbons and four possible enantiomers, characterization of the natural enantiomers was essential. All four enantiomers for the two most bioactive isomers of the natural sex pheromone were synthesized for bioassay. Hydrolysis and derivatization of these enantiomers with different fluorescent reagents was followed by column-switched high-performance liquid chromatography. The use of two linked, reversed-phase columns of different polarity held at sub-ambient temperatures allowed good separation of each enantiomer. This analysis applied to natural material was successful, as (6*R*,19*R*)-6-acetoxy-19-methylnonacosane, and (7*R*,15*R*)- and (7*R*,15*S*)-7-acetoxy-15-methylnonacosane were detected in extracts of recently colonized female flies.

**Key words.** Pheromones, screwworm.

## Introduction

The identification of the sex pheromone of the New World screwworm (NWS), *Cochliomyia hominivorax* (Coquerel) (Diptera: Calliphoridae), has been a long-standing challenge. Mackley & Broce (1981) found that sexual stimuli could be removed from female adults by solvent washing and could be restored by application of the extracts onto clean decoys. Their efforts to identify this sex pheromone were continued with the separation of a polar bioactive fraction obtained from open-column liquid chromatography (LC) and high-performance LC (HPLC). It contained 16 components, of which 13 were 29-carbon secondary alcohol acetates with acetoxy positions from 5 to 8. Six of these had a methyl branch internal to the chain in selected positions. There was about 0.8 µg of the mixture present per female in

these isolates. Curiously, there was much less of this material on the cuticle of NWS females after some time in colony (Pomonis *et al.*, 1993). Despite repeated efforts, no natural pheromonal materials could be individually isolated for bioassay and the absolute activity of the individual isomers, as well as the stereochemistry of enantiomeric pairs, remained unknown. The sex pheromone of NWS was putatively defined by derivatization and gas chromatography-mass spectrometry (GC-MS) analysis as a mixture of acetate derivatives of 29-carbon secondary alcohols, seven of which had a single methyl branch at position 15 or 19, and six had no methyl branch (Pomonis *et al.*, 1993). Subsequent analysis by LC, HPLC and GC-MS of many different strains of NWS females showed that female flies from longterm colonies produced little of the presumed sex pheromone materials (G. Pomonis, personal communication, 2001).

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Old colony females from Chiapas and Jamaica produced much less pheromone than wild females from Belize and Libya (c. 200×). When too little of these pheromones were present, wild and colony males would not attempt to actively mate with these 'pheromone-depleted' females. No synthetic compounds were available at that time for further study.

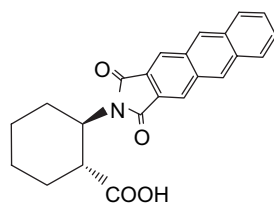
Six compounds were selected for synthesis as racemic mixtures (Furukawa *et al.*, 2002), and two were found to be highly bioactive, with 6-acetoxy-19-methylnonacosane showing full copulatory responses in 50% of the test male subjects at 1.1 µg. Similarly, 7-acetoxy-15-methylnonacosane yielded the same 50% response at 4-µg and 6-µg treatment on a decoy, although a few full copulatory responses were obtained with some other compounds (Carlson *et al.*, 2007). Four stereoisomers of these two compounds were then synthesized for dose-response studies. Bioassay results showed that 50% full copulatory attempts were calculated for a minimum dose of 0.2 µg of (6*R*)-acetoxy-(19*S*)-methylnonacosane, although the other three stereoisomers of this compound showed strong (50%) pheromone activity at higher treatment levels of 0.5 µg, and the bioactivity of treatments was indistinguishable at 1–6 µg (Mori *et al.*, 2004a, 2004b). All the methylnonacosane acetoxy compounds have two asymmetric carbons and four possible enantiomers. We describe the characterization of the natural enantiomers using column-switching HPLC of fluorescent derivatives and the full complement of available synthetic enantiomers.

## Materials and methods

### Chemicals

Both (1*R*,2*R*)- and (1*S*,2*S*)-2-(2,3-anthracenedicarboximido)-cyclohexanecarboxylic acids (**1**, Fig. 1) were prepared by the method previously reported (Mori, 2003). Methanol and acetonitrile were used as purchased (HPLC grade; Kanto Chemical Co., Tokyo, Japan), as was tetrahydrofuran (THF) (HPLC grade; Wako Pure Chemical Ind., Osaka, Japan). Other reagents and solvents used were purchased from Wako Pure Chemical Industries, Yokyo Kasei Organic Chemicals (Tokyo, Japan) and Sigma-Aldrich Japan (Tokyo, Japan).

Authentic samples including four enantiomerically active isomers of 19-methylnonacosane-6-ol, and four isomers of 15-methylnonacosane-7-ol were synthesized by Mori *et al.* (2004a, 2004b).



(1*R*,2*R*)-2-(2,3-anthracenedicarboximido)-cyclohexane carboxylic acid (**1**)

**Fig. 1.** Structure of reagent.

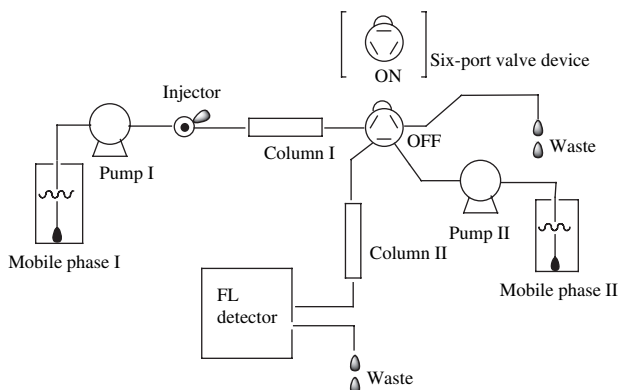
Wild NWS larvae were collected from wounds at seven unrecorded locations in Jamaica from 29 October 2002 to 4 September 2005 and transported to the U.S. Department of Agriculture Midwest Livestock Insect Unit (Lincoln, NE, U.S.A.) to initiate a colony. After 7 months, mature flies from this colony were collected in batches of 200 over several weeks, frozen and transported frozen to the Center for Medical, Agricultural and Veterinary Entomology (Gainesville, FL) in May 2004. A crude extract of 1400 females was prepared in ethyl acetate, the solvent was removed, and a large portion was sent to Japan for analysis.

### HPLC instruments

The column-switching HPLC system was composed of two pumps, a six-port valve device, two analytical columns and a fluorescence detector (Fig. 2). The HPLC pumps were two Jasco PU-980 models (Jasco Corp., Tokyo, Japan) equipped with a Rheodyne 7125 sample injector (Perkin Elmer, Inc., Waltham, MA, U.S.A.) and a 20-µL sample loop. The six-port valve device was a PT-8000 (Tosoh Corp., Tokyo, Japan). The fluorescence detector was a Jasco FP-920 (Jasco Corp.) and the integrator was a Chromatocorder 12 (System Instruments, Inc., Tokyo, Japan). Column temperatures were lowered to 0 °C in an ice bath for the first column and to –30 °C in an acetone bath, using Cryocool CC100-II (Neslab Instruments Inc., Portsmouth, NH, U.S.A.), for the second column.

### Derivatization procedure of a natural and synthetic sample with reagent **1**

An aliquot of ethyl acetate extract of female flies was dissolved in 5 mL methanol and a catalytic amount of sodium methoxide was added before the mixture was stirred overnight at room temperature to hydrolyze the acetate. After removing the solvent under a stream of dry N<sub>2</sub>, the residue was dissolved in 20 mL of ethyl acetate and washed three times with water. The solvent was removed under a stream of N<sub>2</sub> and the residue was dried under reduced pressure. The residue was then dissolved in 0.3 mL of toluene and acetonitrile (1:1 v/v). The solution was divided into two portions; one



**Fig. 2.** Liquid chromatography-liquid chromatography system diagram.

was derivatized with (1*R*,2*R*)-**1** and the other with (1*S*,2*S*)-**1**. About 10 mg of optical pure reagent **1**, a catalytic amount of 4-dimethylaminopyridine and about 20 mg of 1-ethyl-3-(3-dimethylamino) carbodiimide hydrochloride were added to the sample solution and the mixture was held overnight at room temperature. After derivatization, an aliquot was loaded onto a silica gel thin-layer chromatography plate (10 cm length, Silicagel 60 F<sub>254</sub>, Art-5744; Merck KGaA, Darmstadt, Germany) and developed with toluene/ethyl acetate (20:1 v/v). The target spot was collected and packed into a Pasteur pipette, which was eluted with 0.3 mL ethyl acetate. This eluate was used for HPLC analysis. The same procedure was used with all synthetic enantiomers of 6-acetoxy-19-methylnonacosane and 7-acetoxy-15-methylnonacosane to produce the derivatives.

### HPLC separation

The derivatives were first separated on a Develosil ODS-A-3 column (3 µm, 4.6 × 150 mm) (Nomura Chemical Co., Aichi, Japan) at 0 °C, eluted with acetonitrile/tetrahydrofuran (2:1, v/v) at 0.6 mL/min. Then, only the target fraction was introduced onto the second column through a six-port valve. The timing of column switching was recorded in minutes for proper repetition. The second column used was a Develosil C30 UG-3 (3 µm, 4.6 mm × 150 mm), which was eluted with acetonitrile/methanol/tetrahydrofuran/n-hexane (4:8:21:12, v/v/v/v) at 0.4 mL/min and –30 °C. A different mobile phase of acetonitrile/methanol/tetrahydrofuran/n-hexane (4:8:21:9, v/v/v/v) at 0.4 mL/min and –20 °C was used for (7*R*,15*S*)- and (7*R*,15*R*)-15-methylnonacosane-7-yl (1*R*,2*R*)-**1** derivatives and their enantiomers (Table 1). The fluorescence intensities of these derivatives were monitored using excitation at 298 nm and emission at 462 nm. Sensitivity was linear in the range of 100 femtomoles with synthetic derivatives, with natural derivatives detected in the range of 10–20 femtomoles.

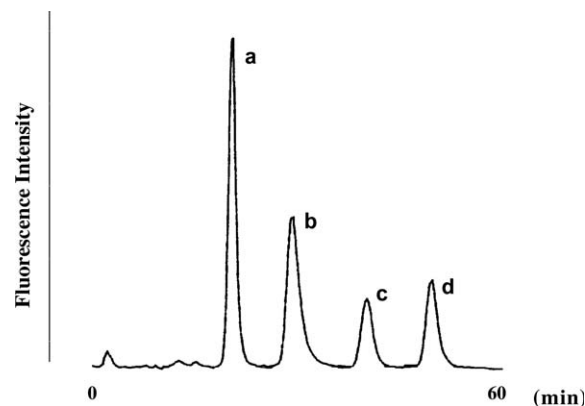
## Results

As pheromone components were acetates of branched alkyl alcohols, the ethyl acetate extract was first subjected to methanolysis. Then the sample was divided into two portions to be derivatized with both enantiomers of reagent **1**, respectively, to check both possibilities. If a component was detected by derivatization with (1*R*,2*R*)-**1**, the corresponding (1*S*,2*S*)-**1** derivative, which had a different retention time, should have been detected. If only one

of them was detected, the peak was not derived from the target compound.

It was possible to separate each isomer of the known C29 compounds on the C30 column (Mori *et al.*, 2004a). However, the sample extract contained too many other compounds to separate and detect the pheromones with a single-column system. As a result, a column-switching LC–LC system was applied to improve resolution. On the first column system, all eight possible isomers of 19-methylnonacosane-6-ol and 15-methylnonacosane-7-ol were separated into four peaks, depending on their positions of hydroxyl groups and their stereochemistry (Fig. 3). Here, the (1*R*,2*R*)-**1** derivatives of (6*S*,19*S*)- and (6*S*,19*R*)-alcohol and (1*S*,2*S*)-**1** derivatives of (6*R*,19*R*)- and (6*R*,19*S*)-alcohol were eluted for 34.6–37.0 min. The (1*R*,2*R*)-**1** derivatives of (6*R*,19*R*)- and (6*R*,19*S*)-alcohol and (1*S*,2*S*)-**1** derivatives of (6*S*,19*S*)- and (6*S*,19*R*)-alcohol were eluted for 40.0–44.0 min. The (1*R*,2*R*)-**1** derivatives of (7*S*,15*S*)- and (7*S*,15*R*)-alcohol and (1*S*,2*S*)-**1** derivatives of (7*R*,15*R*)- and (7*R*,15*S*)-alcohol were eluted for 52.4–55.6 min. The (1*R*,2*R*)-**1** derivatives of (7*R*,15*R*)- and (7*R*,15*S*)-alcohol and (1*S*,2*S*)-**1** derivatives of (7*S*,15*S*)- and (7*S*,15*R*)-alcohol were eluted for 46.4–49.6 min. Only the target fraction, which contained a pair of stereoisomers, was introduced into the second column by switching the six-port valve. By changing the timing of the valve switching, it was possible to detect each pair of stereoisomers.

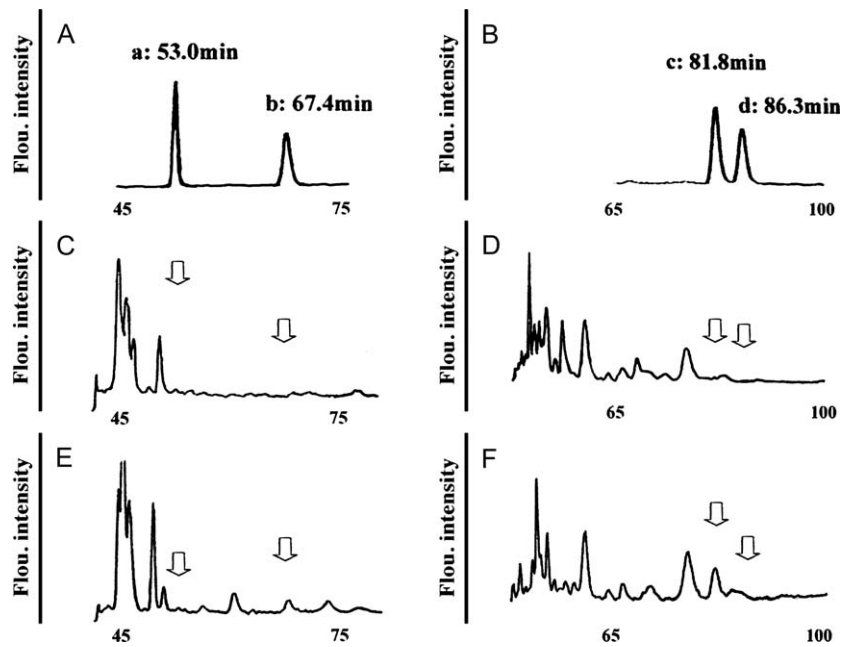
Figure 4 shows HPLC chromatograms of 19-methylnonacosane-6-ols. Authentic samples were detected at 53.0 min (peak a = [1*R*,2*R*]-**1** derivative of [6*S*,19*R*]-alcohol and its enantiomer), 67.4 min (peak b = [1*R*,2*R*]-**1** derivative of [6*S*,19*S*]-alcohol and its enantiomer), 81.8 min (peak c = [1*R*,2*R*]-**1** derivative of [6*R*,19*R*]-alcohol and its enantiomer) and 86.3 min (peak d = [1*R*,2*R*]-**1** derivative of [6*R*,19*S*]-alcohol and its enantiomer), respectively. On chromatograms C and D, both (6*S*,19*S*)- and (6*S*,19*R*)-alcohol derivatives were not detected. By



**Fig. 3.** High-performance liquid chromatography (HPLC) chromatogram of (1*R*,2*R*)-**1** derivatives of authentic samples on the first column. Derivatives were separated on a Develosil ODS-A-3 at 0 °C eluted with acetonitrile/tetrahydrofuran (2:1, v/v) at 0.6 mL/min. Peaks a, b, c and d represented a mixture of (6*S*,19*S*) and (6*S*,19*R*) derivatives, a mixture of (6*R*,19*R*) and (6*R*,19*S*) derivatives, a mixture of (7*R*,15*R*) and (7*R*,15*S*) derivatives, and a mixture of (7*S*,15*S*) and (7*S*,15*R*) derivatives, respectively. HPLC chromatograms of authentic samples and an ethyl acetate extract (1).

**Table 1.** Timing of valve device switching.

Timing of switching (valve device ON)	Alcohols	
	(1 <i>R</i> ,2 <i>R</i> )- <b>1</b> derivatives	(1 <i>S</i> ,2 <i>S</i> )- <b>1</b> derivatives
34.6–37.0 min	(6 <i>S</i> ,19 <i>S</i> ) and (6 <i>S</i> ,19 <i>R</i> )	(6 <i>R</i> ,19 <i>S</i> ) and (6 <i>R</i> ,19 <i>R</i> )
40.0–44.0 min	(6 <i>R</i> ,19 <i>S</i> ) and (6 <i>R</i> ,19 <i>R</i> )	(6 <i>S</i> ,19 <i>S</i> ) and (6 <i>S</i> ,19 <i>R</i> )
46.4–49.6 min	(7 <i>R</i> ,15 <i>S</i> ) and (7 <i>R</i> ,15 <i>R</i> )	(7 <i>S</i> ,15 <i>S</i> ) and (7 <i>S</i> ,15 <i>R</i> )
52.4–55.6 min	(7 <i>S</i> ,15 <i>S</i> ) and (7 <i>S</i> ,15 <i>R</i> )	(7 <i>R</i> ,15 <i>S</i> ) and (7 <i>R</i> ,15 <i>R</i> )



**Fig. 4.** The valve was switched at 34.6 min and 37.0 min on chromatograms A, C and E, and at 40.0 min and 44.0 min on B, D and F. Chromatograms A and B showed authentic samples, C and F showed (1*R*,2*R*)-1 derivatives, and D and E showed (1*S*,2*S*)-1 derivatives of the ethyl acetate extract. Peaks a, b, c and d represented (1*R*,2*R*)-1 derivative of (6*S*,19*R*)-alcohol and its enantiomer, (1*R*,2*R*)-1 derivative of (6*S*,19*S*)-alcohol and its enantiomer, (1*R*,2*R*)-1 derivative of (6*R*,19*R*)-alcohol and its enantiomer and (1*R*,2*R*)-1 derivative of (6*R*,19*S*)-alcohol and its enantiomer, respectively.

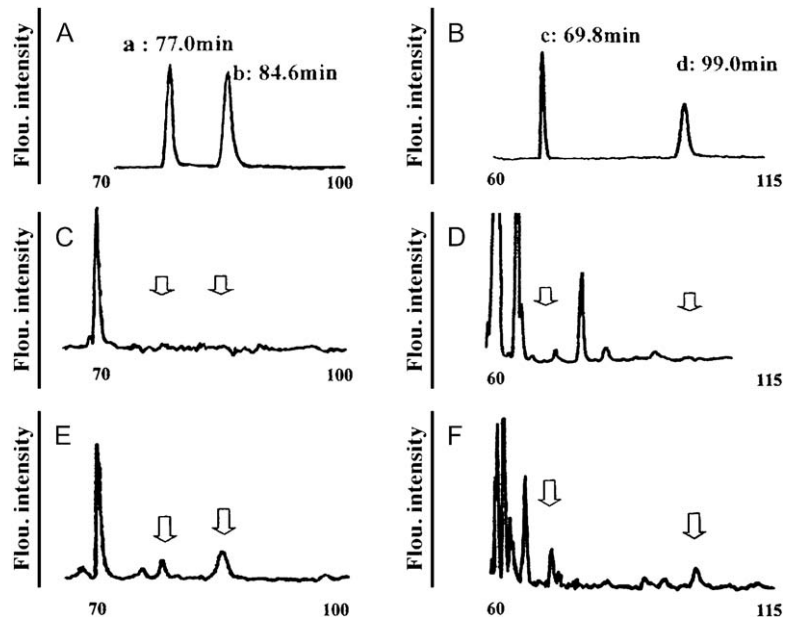
contrast, (6*R*,19*R*)-alcohol derivatives were detected on both chromatograms E (at 67.7 min) and F (at 81.9 min), whereas (6*R*,19*S*)-alcohol derivatives were not detected. Thus, these results showed unequivocally the existence of (6*R*,19*R*) isomer and the absence of other three isomers in the ethyl acetate extract.

Figure 5 shows HPLC chromatograms of 15-methylnonacosane-7-ols. As (7*R*,15*R*)-15-methylnonacosane-7-yl (1*R*,2*R*)-1 derivatives and its enantiomer had much longer retention times, the separation of (7*R*,15*S*)- and (7*R*,15*R*)-15-methylnonacosane-7-yl (1*R*,2*R*)-1 derivatives were performed at a higher column temperature ( $-20^{\circ}\text{C}$ ) to reduce retention times. Authentic samples were detected at 77.0 min (peak a = [1*R*,2*R*]-1 derivative of

[7*S*,15*S*]-alcohol and its enantiomer), 84.6 min (peak b = [1*R*,2*R*]-1 derivative of [7*S*,15*R*]-alcohol and its enantiomer), 69.8 min (peak c = [1*R*,2*R*]-1 derivative of [7*R*,19*S*]-alcohol and its enantiomer) and 99.0 min (peak d = [1*R*,2*R*]-1 derivative of [6*R*,19*R*]-alcohol and its enantiomer), respectively.

## Conclusions

On chromatograms C and D, (7*S*,15*S*)- and (7*S*,15*R*)-alcohol derivatives were not detected. By contrast, both (7*R*,15*R*)- and (7*R*,15*S*)-alcohol derivatives were detected on both chromatograms



**Fig. 5.** High-performance liquid chromatography (HPLC) chromatograms of authentic samples and an ethyl acetate extract (2). The valve device was switched at 52.4 min and 55.6 min on chromatograms A, C and E, and at 46.4 min and 49.6 min on B, D and F. Chromatograms A and B showed authentic samples, C and F showed (1*R*,2*R*)-1 derivatives, and D and E showed (1*S*,2*S*)-1 derivatives of the ethyl acetate extract. Peaks a, b, c and d were (1*R*,2*R*)-1 derivative of (7*S*,15*S*)-alcohol and its enantiomer, (1*R*,2*R*)-1 derivative of (7*S*,15*R*)-alcohol and its enantiomer, (1*R*,2*R*)-1 derivative of (7*R*,19*S*)-alcohol and its enantiomer, and (1*R*,2*R*)-1 derivative of (7*R*,15*R*)-alcohol and its enantiomer, respectively.



E (at 77.0 min and 84.5 min) and F (at 69.8 min and 98.6 min). Thus, these results showed unequivocally the existence of (7*R*,15*R*) and (7*R*,15*S*) isomers in the ethyl acetate extract, thus proving the existence of at least these enantiomers in female flies. Proof of the presence of other structures of the same molecular weight containing methyl branches or the acetoxyl function at other locations is not possible without the synthesis of all enantiomers of each, but this is an unlikely prospect at this time. The samples and authentic synthetic standards available were too small an amount to weigh and it was difficult to estimate the amount of sample derivatized. As the most acid derivatives of this reagent had  $10^{-15}$  to  $10^{-14}$  mole levels of detection limits (signal : noise ratio = 2) and the peaks detected here had a signal : noise of about  $6^{-10}$ , the amounts detected were about several  $10^{-14}$  mole levels.

The LC–LC method removed most interfering substances by switching them away in the first column and the resulting chromatograms were very simple. The synthetic samples were repeatedly injected to ensure that peak assignments were correct. Although each derivatized natural sample by each enantiomer of the reagents was analysed once, the results obtained by using both enantiomers of the reagents, respectively, did not contradict one another.

### Acknowledgements

We thank the International Atomic Energy Agency for partial support of this work.

### Conflicts of interest

All authors declare no conflicts of interests.

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Accepted 15 October 2008