Evidence of Pheromone Catabolism Via β-Oxidation in the European Corn Borer (Lepidoptera: Crambidae)[1]

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Abstract Experiments were conducted using tritiated European corn borer, *Ostrinia nubilalis* (Hübner), pheromone, (Z)-[11,12-$^3H_2$]-11-tetradecen-1-ol acetate, a tritiated fluorinated analog of the European corn borer pheromone, 2-fluoro-(Z)-[11,12-$^3H_2$]-11-tetradecen-1-ol acetate, and methyl-4-bromocrotonate (MBC) to determine if pheromone catabolism proceeds on the moth's antennae via the β-oxidation pathway of fatty acid degradation. When antennae were treated with tritiated natural pheromone plus MBC (a precursor of the known β-oxidation inhibitor, 4-bromocrotonic acid), catabolism of the pheromone was significantly inhibited. When the 2-fluoro pheromone analog was applied alone to antennae, it was hydrolyzed to the corresponding alcohol but was not degraded. MBC had no effect on catabolism of the 2-fluoro analog, and 2-fluoro substitution inhibited entrance of the compound into β-oxidation. These results demonstrate that β-oxidation is the primary oxidative pathway by which pheromone is degraded on the antennae of European corn borer moths.

Key Words Methyl-4-bromocrotonate, methyl-4-bromo-*E*-2-butenoate, (Z)-[11,12-$^3H_2$]-11-tetradecen-1-ol acetate, 2-fluoro-(Z)-[11,12-$^3H_2$]-11-tetradecen-1-ol acetate, *Ostrinia nubilalis*

The European corn borer, *Ostrinia nubilalis* (Hübner), uses geometric isomer mixtures of 11-tetradecen-1-ol acetate as pheromone (Klun and Huettel 1988). Its sex pheromone is a long-range chemical cue for mate finding and for stimulation of close-range male precopulatory behavior. Klun et al. (1991) postulated that male moths maintain a high level of chemoreceptive sensitivity to pheromone by rapid catabolic clearing of the pheromonal stimulus from the chemosensory structures on their antennae. Those results also showed that when the labeled pheromone was applied to male antennae, it was converted to the alcohol by a non-specific esterase. Detection of a trace radiolabeled catabolic pool of the 11-tetradecenoic acid and the formation of tritiated water circumstantially suggested that the β-oxidation pathway was operative on the antenna (Klun et al. 1992). β-oxidation is a well-known catabolic pathway (Schulz 1987) in which fatty acids are degraded. In this pathway, carbon 2 (β-position) of the acid is oxidized to yield a β-ketoacyl that cleaves to form acetic acid and a fatty acid shorter than the starting acid by two carbon atoms. The process repetitively shortens the carbon chain until the starting acid is completely degraded to acetic acid. The acetic acid enters the tricarboyxlic acid cycle where further oxidation produces carbon dioxide and water.

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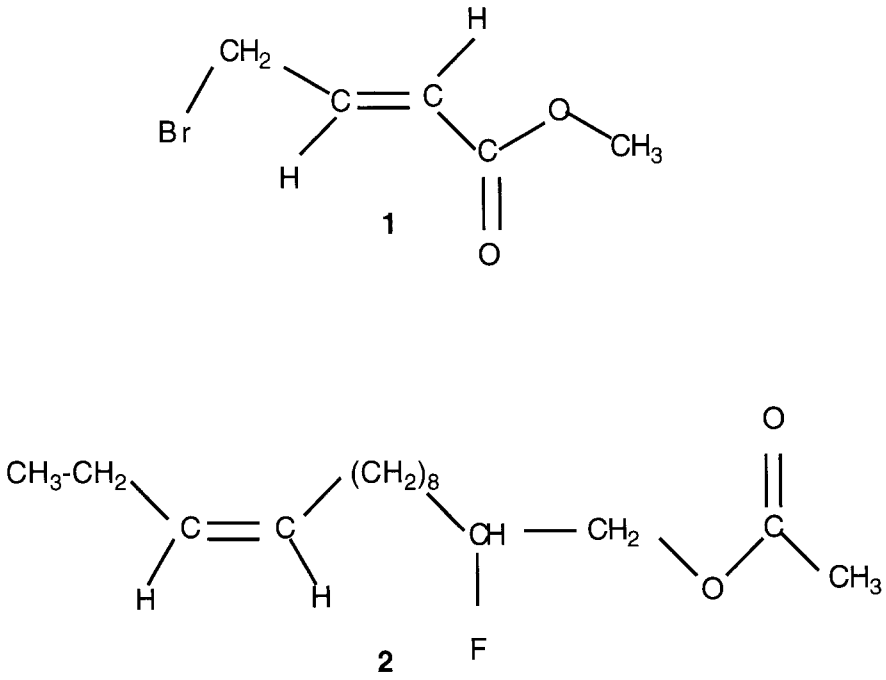

Fig. 1. Structures of methyl-4-bromocrotonate (1) and 2-fluoro-(Z)-11-tetradecen-1-ol acetate (2) used to evaluate the existence of the β-oxidation pathway in European corn borer sex pheromone antennal catabolism.

A racemic monofluorinated analog of the pheromone, 2-fluoro-Z-11-tetradecen-1-ol acetate, mimics the natural pheromone (Klun et al. 1997, Oliver et al. 1994). Because 2-fluoro substitution in alkanoic acids interferes with β-oxidation (Pattison et al. 1965, Plettner et al. 1996), the 2-fluoro pheromone analog was an ideal substrate for this study. 4-Bromocrotonic acid has been reported to inhibit β-oxidation irreversibly by active site inactivation at thiolysis (Schulz 1987). Thus, by using methyl-4-bromocrotonate (MBC) [1] (a pro-β-oxidation inhibitor) and the 2-fluoro substituted pheromone analog [2] (Fig. 1), we tested the hypothesis that β-oxidation is the pathway by which sex pheromone is cleared from the European corn borer antenna.

Materials and Methods

At the National Tritium Labeling Facility (Berkeley, CA), [11,12-$^3H_2$]-Z-11-tetradecen-1-ol acetate (T-Z-11-14:OAc) and racemic 2-fluoro-[11,12-$^3H_2$]-Z-11-tetradecen-1-ol acetate (T-2F-Z-11-14:OAc) were prepared by semireduction of the corresponding acetylenic precursors in a tritium atmosphere using 5% Pd on $CaCO_3$-Pb poisoned as catalyst (Strem Chemicals Inc., Newburyport, MA) with cyclohexene as solvent. Each product had a specific activity of 42 Ci/mmol. Corresponding alcohols, T-Z-11-14:OH and T-2F-Z-11-14:OH, and acids were prepared by standard conversions of the radiolabeled acetates.

European corn borer adults used in the study were from a colony maintained at Beltsville, MD. The moths used 97:3 (Z:E)-11-tetradecen-1-ol acetate (Z-11-14:OAc) as sex pheromone (Klun and Huettel 1988). The right antennae of equal numbers of 2 to 4-day-old European corn borer males, 3 to 6 h into scotophase, were pretreated with either 0.25 µL heptane containing 3.7 nmol MBC or 0.25 µL heptane alone (control) using a micrometer driven 50-µL bevel-tipped Hamilton syringe. The 3.7-nmol MBC dose was selected for the treatment after we determined that doses <3.7 nmol did not inhibit oxidation (unpubl. data). Also, rather than using 4-bromocrotonic acid directly, we applied the MBC (97% pure, Sigma Chemical Co., St. Louis, MO) because previous experience showed that carboxylic acids applied topically do not penetrate the antennal cuticle. We reasoned that MBC would penetrate the cuticle and that upon penetration, esterase on the antenna (Klun et al. 1991) would hydrolyze the methyl ester to 4-bromocrotonic acid which, in turn, would inhibit β-oxidation. Insects were pre-incubated 20 to 30 min in the dark after treatment with the ester or heptane, and then the pretreated antenna of each insect was treated with 150,000 dpm tritium-labeled compound (1.5×10^{-3} nmol either T-Z-11-14:OAc or T-2F-Z11-14:OAc) in 0.25 µL of heptane. The males were then incubated for 0.3, 1, 3, 5, 10, or 15 min. Treatments were replicated six times. At the end of each incubation period, treated antennae were excised and placed individually in 50 µL 1:1 methanol:chloroform to stop catabolism and to extract metabolites. Previous tests (Klun et al. 1996) showed that 98.8% of radiolabel applied to the antennae was recovered by extraction with chloroform:methanol. Extracts were stored in a –4°C freezer to await analysis. Extracts were analyzed by reverse phase high pressure liquid chromatography using a Beckman System Gold chromatograph fitted with a Beckman 5 micron ODS Ultrasphere column [25 cm × 4.6mm(ID)]. The eluting solvent was 90:10 methanol:water pumped at 1 mL/min. Tritiated compounds in the extracts were detected with a Beckman 171 radioisotope detector using a 1 mL liquid cell and Beckman ReadyFlow III scintillation fluid pumped at 1 mL/min. The system was calibrated with standard solutions of T-Z11-14:OAc, T-Z11-14:OH, T-2F-Z11-14:OAc, T-2F-Z11-14:OH, and tritiated water. The water was purchased from NEN, DuPont Company, Boston, MA. Detection efficiency was 23% for tritiated water and 39% for tritiated organic compounds. Because the absolute amount of recoverable radioactivity varied from one antennal application to another, we recorded proportional amounts of radioactivity associated with compounds detected in extracts of treated antennae as a function of incubation time. Proportions of radiolabeled compounds detected in each treatment were analyzed as two factor general linear mixed models using PROC MIXED (SAS Institute 1994) for the two experiments. Treatment and time were the fixed effects. Treatment-time combinations were grouped into similar variance sets for the analyses to account for variance heterogeneity in the data.

Results and Discussion

T-Z-11-14:OAc, T-2F-Z-11-14:OAc, corresponding alcohols, and tritiated water were the only radiolabeled compounds detected in the antennal extracts. Trace catabolic intermediates, [11,12-$^3H_2$]-Z-11-tetradecenoic acid and 2-fluoro-[11,12-$^3H_2$]-Z-11-tetradecenoic acid, were not detected in analyses of the individual antennae; however, analyses of combined extracts of about 30 antennae verified the presence of the respective carboxylic acid intermediates (Klun et al. 1992, Klun et al., unpubl. data). Mean proportions (±SEM) of radiolabeled acetate, alcohol, and water formed in

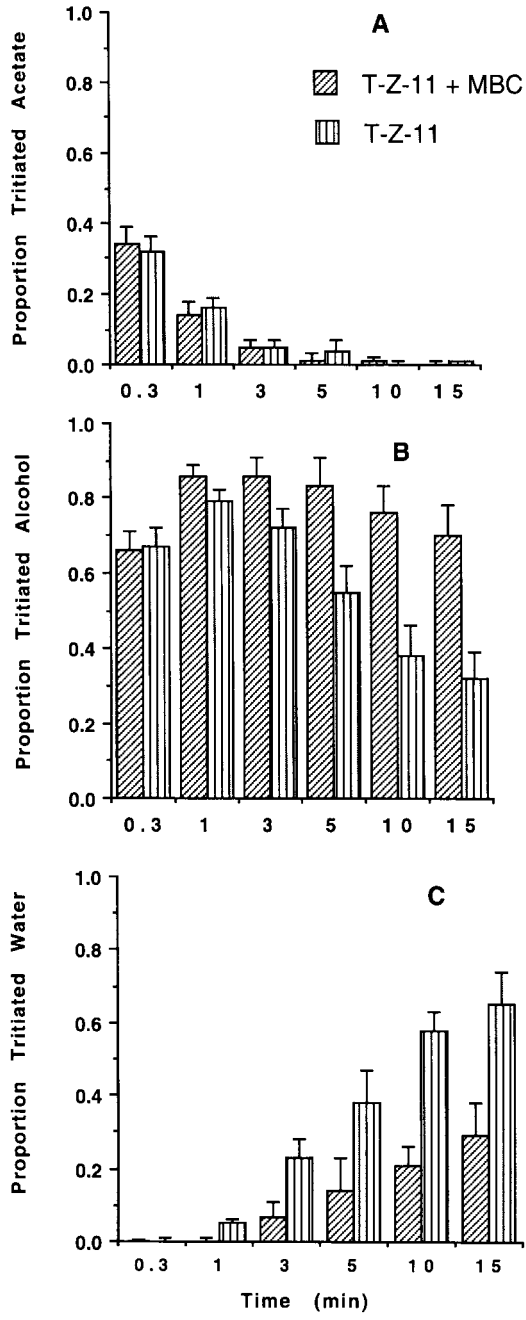

Fig. 2. Mean proportion (SEM indicated by bar) of radiolabeled compounds in extracts of antennae incubated following treatment with [11,12-$^{3}H_{2}$]-Z-11-tetradecen-1-ol acetate (T-Z-11) alone or T-Z-11 plus methyl-4-bromocrotonate (MBC).

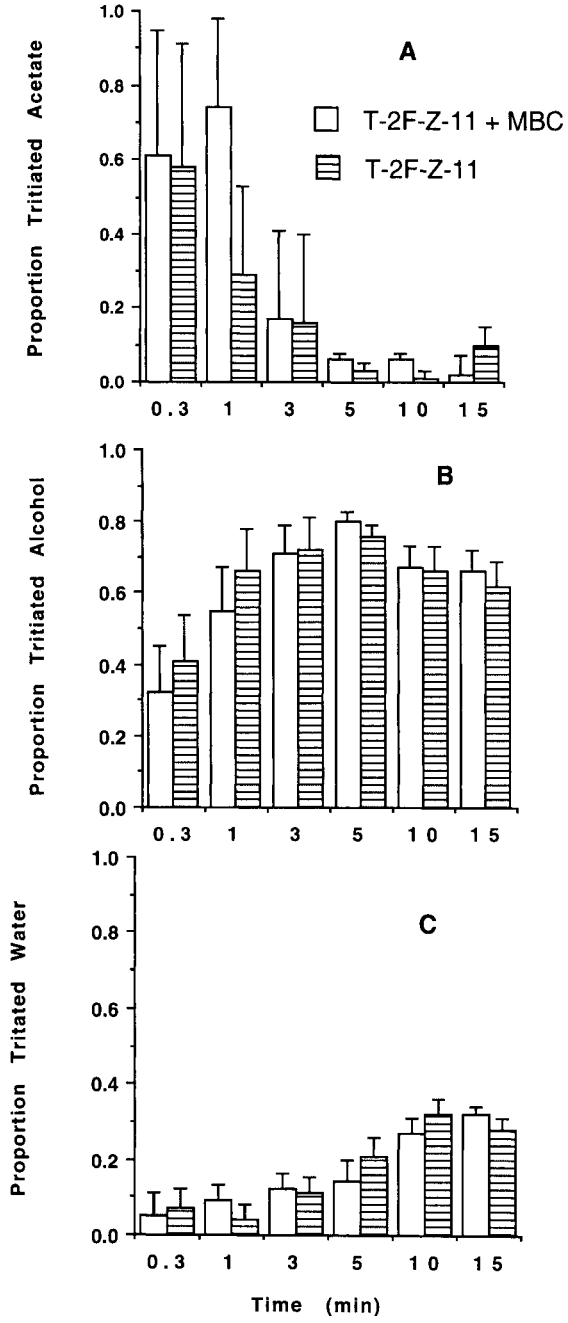

Fig. 3. Mean proportion (SEM indicated by bar) of radiolabeled compounds in extracts of antennae incubated following treatment with 2-fluoro-[11,12-$^{3}H_{2}$]-Z-11-tetradecen-1-ol acetate (T-2F-Z-11) alone or T-2F-Z-11 plus MBC.

incubations of antennae with T-Z-11-14:OAc and T-2F-Z-11-14:OAc are shown in Fig. 2 and 3, respectively. The proportional amount of labeled acetate present in MBC-treated antennae was not significantly ($P = 0.05$) different from the corresponding controls across all incubation times (Fig. 2A, 3A). This proves that MBC had no influence upon the hydrolysis of T-Z-11-14:OAc or T-2F-Z-11-14:OAc. With the 2-fluoro analog, the hydrolysis rate was highly variable in the first 3 min of incubation (Fig. 3A) and that significantly less T-2F-Z-11-14:OH was produced at 0.3 min (Fig. 3B) compared the amount of T-Z-11-14:OH produced from T-Z-11-14:OAc in 0.3 min. (Fig. 2B). Therefore, there is indication that 2-fluoro substitution may have interfered with esterase activity.

In the presence of MBC the proportional amount of radiolabeled alcohol generated from T-Z-11-14:OAc increased in the first minute of incubation and then remained almost unchanged thereafter (Fig. 2B). This shows that processing of T-Z-11-14:OH was significantly inhibited by MBC. In contrast, without MBC, the proportional amount of radiolabeled alcohol in the antennae steadily declined after 1 min. Concomitantly, there was a proportional increase in the amount of water in the same antennae (Fig. 2C) which was produced by alcohol oxidation. In the presence of MBC, the proportional amount of labeled water increased only slightly. The small increase in amount of labeled water with MBC-inhibited antennae (Fig. 2C) suggests that oxidation was not completely inhibited or that other, but less aggressive, oxidative processes such as ω-oxidation may be operative on the antennae.

Similarly, the proportional amount of T-2F-Z-11-14:OH increased slightly in the first 3 min of incubation and remained unchanged thereafter (Fig. 3B). Proportionately little tritiated water was formed with time (Fig. 3C) whether or not MBC was present. These results show the MBC had no effect on the catabolism of the 2-fluoro analog and the lack of oxidative activity against the alcohol was due to the intrinsic effect of the 2-fluoro substitution which made it resistant to oxidation. This effect is consistent with the report of Plettner et al. (1996) that honey bees were incapable of chain-shortening 2-fluorostearic acid because the compound was not susceptible to β-oxidation. As before, the insignificant increase in the proportional amount of labeled water in the analog-treated antennae likely occurs with other oxidative processes such as ω-oxidation.

In the first case, the results show that normal catabolic processing of radiolabeled natural pheromone was inhibited by MBC. This inhibition was most likely caused by direct poisoning of the β-oxidation pathway by MBC. In the second case, substitution of a hydrogen atom with fluorine on carbon 2 in the pheromone analog made it impossible for the compound to enter into the β-oxidation pathway and, thus, interfered with degradation of the compound. The combined observations provide evidence that β-oxidation is a major catabolic pathway in the post-chemoreceptive processing of sex pheromone in the antenna of the male European corn borer.

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