Confirmation of sex pheromone biosynthesis from (16-D_3)palmitic acid in the turnip moth using capillary gas chromatography

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Summary. (16-D_3)palmitic acid was applied topically to pheromone glands of the turnip moth. After incubation omega labeled analogues of fatty acid methyl esters and acetates were identified by gas chromatography with flame ionization detection and selected ion monitoring. The pheromone components *(Z)*-5-decenyl-, *(Z)*-7-dodecenyl- and *(Z)*-9-tetradecenyl acetate were all biosynthesized from palmitic acid along a common pathway.

Key words. Pheromone; Lepidoptera; *Agrotis segetum;* biosynthesis; palmitic acid; capillary gas chromatography; deuterium; polyethylene glycol.

Biosynthesis of moth pheromones has earlier been studied with radiolabeled precursors[1-3] or with precursors labeled with stable isotopes, followed by NMR[4] or GC-MS detection[5]. In several moths palmitic acid has been shown to be the precursor of the pheromone components, which are biosynthesized by the combined action of a delta 11-desaturase and chain-shortening enzymes[6]. The sex pheromone of the turnip moth *Agrotis segetum* (Schiff.) (Noctuidae; Lepidoptera) is a mixture of *(Z)*-5-decenyl acetate (Z5–10:OAc), *(Z)*-7-dodecenyl acetate (Z7–12:OAc) and *(Z)*-9-tetradecenyl acetate (Z9–14:OAc)[7,8]. We now report an experiment which shows that all the sex pheromone components are synthesized from palmitic acid along a common pathway. This is easily demonstrated by capillary GC on a polyethylene glycol stationary phase that separates omega deuterated methyl esters and acetates from non-labeled specimens. The results were confirmed by GC-MS analysis in the selected ion monitoring mode. The possibility of demonstrating a deuterium label with standard capillary gas chromatography and flame ionization detection should greatly facilitate biochemical experiments in many areas of fatty acid chemistry.

Materials and methods. Labeled fatty acids in DMSO (4 µg in 0.2 µl) were applied topically to the pheromone glands of one to four day-old *Agrotis segetum* females as earlier described by Bjostad and Roelofs for other Lepidoptera[3]. After the application the glands were kept everted for 1–4 h, until the glands were dissected from the ovipositors and extracted with the preferred solvent. For analysis of fatty acids 10 µl of a 2:1 (v/v) mixture of chloroform and methanol was the choice. Fatty acyl moieties were converted to methyl esters by base methanolysis as described in detail in Löfstedt et al.[9]. For analysis of acetates and alcohols gland extracts were prepared in 10 µl of hexane with 5 ng *(Z)*-8-tridecenyl acetate added as internal standard. Capillary gas chromatography with flame ionization detection (GC-FID) was performed on a Hewlett Packard (HP) model 5880 GC equipped with a 30 m × 0.25 mm i.d. Supelcowax 10 column (Supelco Inc., Bellefonte, PA), or on a model 5830 HP GC with a 60 m × 0.25 mm i.d. DB-wax column (J&W Scientific Inc., Rancho Cordova, CA). Both stationary phases are crosslinked polyethylene glycols. Conditions of chromatography were: Hydrogen carrier gas flow 40 cm/s at 80 °C, splitvalve opened 1 min after injection; temperature maintained at 80 °C for two min following injection and then programmed at 5 or 10 °C per minute to 230 °C.

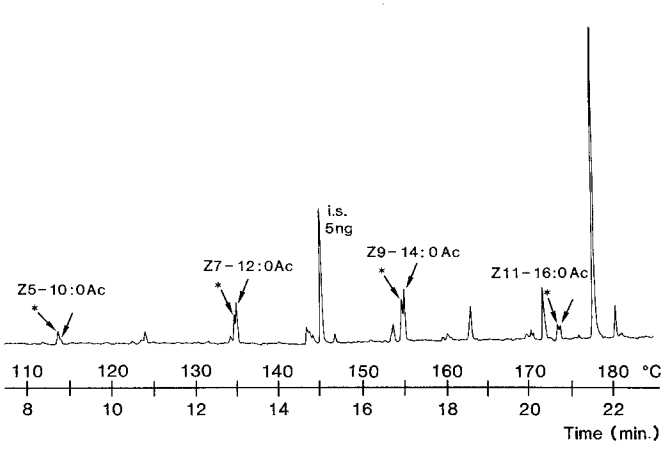

Figure 1. Incorporation of deuterium label from topically applied (16-D_3)hexadecanoic acid into pheromone components in glands of 10 female *Agrotis segetum*, monitored by capillary gas chromatography with flame ionization detection. The first peak (*) in each pair has the predicted retention time of omega labeled (D_3) analogues of the pheromone components.

Chemical ionization mass spectrometry with ammonia as the reagent gas was performed on a Ribermag R 10-10C quadropole GC/MS system equipped with a Carlo Erba model 4160 GC. Chromatographic conditions were as described above, using the Supelcowax 10 column. Recording of mass spectra showed that with ammonia as the reagent gas, the adduct ions *m/z* (M+18) are the most abundant species formed for both acetates and methyl esters. Acquisition programs were designed for the selected ion monitoring of methyl esters and acetates, respectively. For each set of compounds the deuterated analogues were monitored together with the non-labeled specimen. The pair of ions monitored was changed during the separation according to the retention-times of different compounds, to optimize detection of compounds of different chain-length and different degree of saturation. Equivalent chain-lengths (ECL) of methyl esters were calculated relative to a series of saturated straight-chain methyl esters. ECL of acetates were calculated relative to a series of straight-chain acetates.

Deuterium-labeled fatty acids were purchased from Larodan Fine Chemicals, Malmö, Sweden. The deuterium enrichment of the omega labeled acids was 99% and for the perdeuterated (fully labeled) palmitic acid 98%.

Results and discussion. GC-FID analysis of omega deuterated methyl esters on the Supelcowax column showed that these consistently eluted somewhat earlier than the non-labeled specimen. ECL of methyl(12-D_3)dodecanoate, methyl(14-D_3)tetradecanoate, methyl(16-D_3)hexadecanoate and methyl (18-D_3)-octadecanoate were found to be 1197, 1397, 1597 and 1796, respectively. Analysis of aliphatic acetates with deuterium label in the acetate group has shown that these also elute prior to the non-labeled specimen on a similar column[10]. These results indicated that although baseline separation was not achieved, incorporation of deuterium-labeled precursors in the moth fatty acid metabolism could probably be sufficiently monitored by GC-FID. The methylated perdeuterated palmitic acid with a 1569 ECL eluted well before the methyl ester of palmitic acid, on the DB wax column.

GC-FID analysis of methanolyzed fatty acid extracts of turnip moth pheromone glands incubated with (16-D_3)hexadecanoic acid revealed significant peaks eluting prior to methyl hexadecanoate, methyl tetradecanoate, methyl *(Z)*-11-hexadecenoate and methyl *(Z)*-9-tetradecenoate. The peaks eluting prior to the saturated methyl esters had the same ECL as the omega deuterated analogues. Selected ion monitoring supported that the molecular weight of the first peak in a pair was three units higher than that of the second, i.e. corresponding to that of

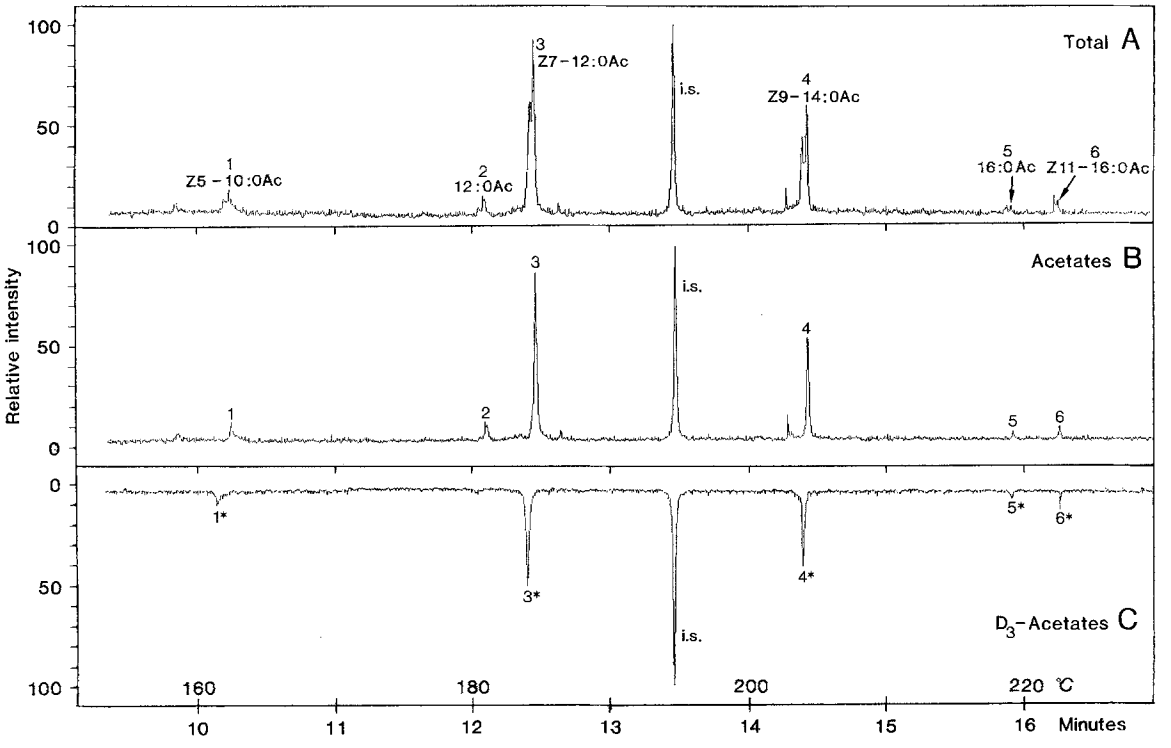

Figure 2. Incorporation of deuterium label from topically applied (16-D_3)hexadecanoic acid into pheromone components in glands of 10 female *Agrotis segetum*, detectd by selected ion monitoring; total ion chromatogram (A), sum of mass chromatograms showing *m/z* (M+18) of the pheromone acetates (B), and sum of chromatograms showing *m/z* (M+18) for the omega deuterated analogues (*) of the pheromone acetates.

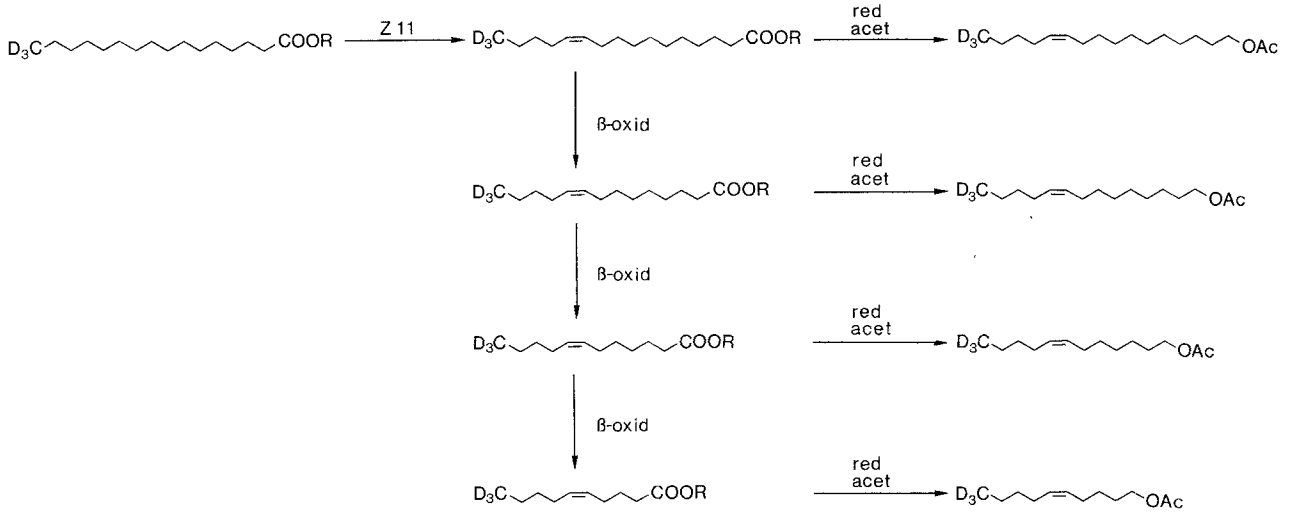

Figure 3. Biosynthesis of sex pheromone components from palmitic acid in the turnip moth, *Agrotis segetum*. The proposed reactions involved are Z11 desaturation, chain-shortening (*β*-oxidation), reduction and acetylation.

an omega deuterated analogue. We concluded that omega deuterated analogues in general should elute just prior to the non-labeled specimen on the type of stationary phase used. The labeled specimens amounted to 10–50% of the non-labeled ones after 1–4 h of incubation. The degree of incorporation was not correlated to the time of incubation. In conclusion the retention times of the double peaks (and their MS-data) indicated that palmitic acid is delta-11 desaturated to produce (16-D_3)*(Z)*-11-hexadecenoic acid and subsequently chain-shortened in the sex pheromone gland. An alternative route to *(Z)*-9-tetradecenoate would be chain-shortening of palmitic acid followed by delta 9-desaturation. However, incubation with (14-D_3)tetradecanoic acid did not result in any deuterium label in the methyl *(Z)*-9-tetradecenoate.

GC-FID analysis of acetates from pheromone glands incubated with (16-D_3) hexadecanoic acid revealed significant peaks eluting prior to all of the pheromone components Z5–10:OAc, Z7–12:OAc and Z9–14:OAc, as well as hexadecyl acetate and *(Z)*-11-hexadecenyl acetate (Z11–16:OAc) (fig. 1). The first peak in each pair was missing in control incubations with DMSO, except in case of Z5–10:OAc. However in this case the compound eluting prior to Z5–10:OAc increased in size, following incubation with (16-D_3)palmitic acid. Double peaks were observed also for *(Z)*-7-dodecenol and *(Z)*-9-tetradecenol. Se-

lected ion monitoring of the pheromone acetates and their corresponding deuterated analogues confirmed the presence of compounds tentatively assigned as (10-D_3)Z5–10:OAc, (12-D_3) Z7–12:OAc, (14-D_3)Z9–14:OAc, (16-D_3)16:OAc and (16-D_3) Z11–16:OAc, in the glands incubated with (16-D_3)palmitic acid (fig. 2). Incubation with other saturated fatty acids ((12-D_3) 12:COOH, (14-D_3)14:COOH and (18-D_3)18:COOH) did not result in any incorporation of label into the pheromone components. These results were confirmed by application of perdeuterated palmitic acid to pheromone glands. In this case a new set of compounds was generated in the female moth pheromone glands, which according to the retention time of perdeutero methyl palmitate relative to methyl palmitate were perdeuterated pheromone analogues.

Our interpretation of the experimental results is that delta-11 desaturation of hexadecanoic acid produces Z11–16:COOH, which is subsequently chain-shortened through beta-oxidation to produce Z9–14:COOH, Z7–12:COOH and Z5–10:COOH. The chain-shortening products are finally reduced and acetylated to produce the pheromone acetates. Thus all the turnip moth pheromone components are produced along a *common* biosynthetic pathway (fig. 3). This corroborates the generality of the results of Bjostad and Roelofs obtained with *Trichoplusia ni*[3]. They showed that in this species Z7–12:OAc was a chain-shortening product of Z11–16:COOH. Our results demonstrate a straight forward method of detection and quantification of labeled fatty acids and fatty acid derivatives. Deuterium-labeled saturated fatty acids are commercially available and unsaturated ones can be prepared[11]. It has recently been shown that the separation characteristics of crosslinked polyethylene glycol columns differ slightly, according to fabrication[12]. The separation of unlabeled and labeled specimen can be improved to allow precise quantifications if the analytical conditions, including the choice of stationary phase, are optimized.

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Biodegradation of nitrilotriacetic acid (NTA) in the absence of oxygen

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Summary. A microbial culture able to grow in the absence of oxygen with nitrilotriacetate (NTA) as the sole source of carbon and energy was enriched from river sediment. Growth was strictly dependent on the presence of nitrate and the results indicate that nitrate was exclusively used as the electron acceptor and was reduced to dinitrogen (N_2). The stoichiometry of anaerobic NTA degradation by the enrichment culture was determined.
Key words. Nitrilotriacetate (NTA); biodegradation; enrichment culture; denitrification; sewage treatment.

One of the main causes of the widespread eutrophication of lakes and rivers is the extensive use of phosphates in washing powders and agricultural fertilizers[1]. In order to reduce such effects, the Swiss Federal Council has passed an amendment, effective from July 1986, prohibiting the use of phosphates in washing powders. One of the potential substitutes for sodium tripolyphosphates (STPP) in domestic detergents is nitrilotriacetic acid (NTA). Being a highly effective complexing agent, NTA will bind primarily to calcium, magnesium and heavy metal ions under conditions occurring in natural waters and wastewater treatment plants. It is expected that the increased use of NTA will result in an enhanced concentration of this compound in natural waters which are used as sources for drinking water production. For this reason, in spite of the fact that NTA has been employed in Canada since 1970 without apparent negative results, there is considerable concern, e.g., in West Germany, particularly in the Ruhr region, that NTA usage could result in significant remobilization of heavy metals at present bound in considerable concentrations in both soils and sediments. As a consequence NTA/heavy metal complexes could be transported through the process steps involved in drinking water production. The microbial degradation of NTA in the presence of oxygen is well documented for freshwater environments, soil and biotreatment plants as well as for pure cultures of NTA-utilizing bacteria. The relevant literature is cited in refs 2 and 3. The catabolic pathway for NTA was studied in two *Pseudomonas* spp.[4–6] and it was shown that in both bacteria the first step in NTA oxidation is catalyzed by an O_2/NADH-dependent mono-oxygenase.

The disappearance of NTA in complex systems such as anaerobic digesters, and sediments in the absence of oxygen, is also documented[7–9]. It proceeds at a markedly slower rate than aerobic degradation and is often only partially complete. Until now, anaerobic degradation of NTA by a pure culture has only been demonstrated by Enfors and Molin[10]. Because the research was apparently discontinued the data reported by these authors is rather incomplete and neither the taxonomy of the bacterium isolated nor the enzymology of anaerobic NTA degradation have been elucidated. Because aerobic NTA utilization, in all strains investigated so far, involves an O_2-dependent mono-oxygenase it must be assumed that degradation under anoxic conditions must proceed via an alternative metabolic pathway.

Materials and methods. The enrichment medium contained per liter: $MgSO_4 \cdot 7H_2O$, 1.0 g; $CaCl_2 \cdot 2H_2O$, 0.20 g; $Na_2HPO_4 \cdot 2H_2O$, 0.41 g; KH_2PO_4, 0.26 g; $NaNO_3$, 1.40 g; trace element solution according to Widdel[11], 1.0 ml; pyridoxin·HCl, 100 µg; 50 µg of each thiamine·HCl, riboflavin, nicotinic acid, D-Ca-pantothenic acid, p-amino benzoic acid, lipoic acid, nicotinamide, vitamin B_{12}; biotin, 20 µg; folic acid, 20 µg. The enrichment culture was started with an NTA concentration of 20 mg l^{-1} in the inflowing medium and, after complete utilization of NTA was observed in the culture vessel, its concentration in the reservoir was increased stepwise to 1.0 g l^{-1}.