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A peculiar fatty acid, (Z,Z)-9,12,17-octadecatrienoic acid, identified in the phospholipids of the pea aphid, *Acyrthosiphon pisum* (Harris) (Homoptera: Aphididae)

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Abstract. A peculiar fatty acid previously detected in the phospholipids of the pea aphid, *Acyrthosiphon pisum*, is identified as (Z,Z)-9,12,17-octadecatrienoic acid. It is the first report of this compound in the literature. Comparison of fatty acid profiles of phospholipids between normal and aposymbiotic pea aphids shows that aphid symbionts are not responsible for the biosynthesis of this unusual fatty acid.
Key words. Homoptera; Aphididae; pea aphid; fatty acid; phospholipid; (Z,Z)-9,12,17-octadecatrienoic acid; symbiosis.

Lipids of the Aphididae differ remarkably from those of other insects. Their major triglycerides (>90%) are known to be esterified at the sn-1,3 positions of glycerol mainly by myristic acid (C14:0) and to a lesser extent by lauric and palmitic acids (C12:0 and C16:0)[1]. At the sn-2 position, these triglycerides have a short chain fatty acid: hexanoic (C6:0) or sorbic (C6:2) acids[2] as well as octanoic (C8:0) or octatrienoic (C8:3) acids[3]. Recent work on the biosynthetic origin of these conjugated polyenic fatty acids in *Acyrthosiphon pisum* revealed that neither symbionts nor plant-specific diet components were involved in its supply[4].

As in other insects, phosphatidylethanolamines (PE) and phosphatidylcholines (PC) are the major phospholipids in the pea aphid[1]. Unusually, however, the ratio of PC to PE is smaller than 1, as already mentioned for other species of aphids[5]. In contrast to triglycerides, the dominant fatty acid in these phospholipids is linoleic acid (C18:2). By tracer studies, there is good evidence that *A. pisum* may synthesize this fatty acid de novo[6,7]. For the first time, Febvay et al.[1] mentioned an unidentified fatty acid with a GC retention time close to that of linolenic acid (C18:3), and contributing 6.2% of the total fatty acids in PC and 5.4% in PE. This unusual fatty acid, which is absent from the host plant *Vicia faba*, is synthesized by the aphid or its symbionts. Moreover, pea aphids reared on a lipid-free artificial diet display a decrease in their phospholipid content, correlated with a fatty acid profile of phospholipids significantly deficient in this unidentified peculiar fatty acid[1].

The aim of this study was to identify this unknown compound and, by the use of aposymbiotic aphids, to investigate its biosynthetic origin in *A. pisum*.

Material and methods

Insects. A. pisum (clone LL01) was maintained in the laboratory on broad bean seedlings (*Vicia faba* L., var. Aquadulce) in Plexiglas cages (21 °C, 70% R.H., L16:D8). For the isolation and identification of the unknown fatty acid, phospholipids were extracted from young apterous adults within three days after emergence (1- to 3-day-old adults).

To produce aposymbiotic aphids, neonate larvae (≤16 h, m. wt = 30 µg) obtained on *Vicia* plants were transferred to Parafilm® sachets of artificial diet containing 50 µg/ml of rifampicine. The composition of the diet was that of the A5 diet already published[8] with a sucrose content adjusted to 584 mM (20% w/v). After 48 h at 21 °C, the larvae were transferred back to *Vicia* plants and grown under standard conditions. Fatty acids of phospholipids were analysed on these aposymbiotic aphids (1–to 3-day-old apterous adults). Control aphids were treated identically without antibiotic in the diet. A full description of this aposymbiosis protocol has been provided elsewhere[9]. The aphid samples were weighed immediately after removal from plants (fresh wt) and stored at −20 °C until lipids were extracted. Four samples of 50 aposymbiotic aphids (individual m.wt. = 1.60 mg) and five samples of 40 control aphids (individual m. wt = 2.48 mg) were used for fatty acid quantification in phospholipids.

Phospholipid and methyl ester fatty acid preparation. The procedure for the preparation of phospholipids and methyl esters of fatty acids was fully described in a previous paper[1]. Total lipids were extracted from whole aphids according to the method of Folch et al.[10]. Internal standards (consisting of triheptadecanoin, L-α-phosphatidylcholine, diheptadecanoyl and L-α-phosphatidylethanolamine, diheptadecanoyl, Sigma Chemical Company) were added during lipid extraction for the determination of recovery yields (heptadecanoic acid, C17:0, is not found in aphid lipids). The lipid extract was applied onto a silica Sep-Pak® cartridge (Waters Assoc.). Neutral lipids were eluted successively

with chloroform (10 ml) and diethyl ether (5 ml) and discarded. Phospholipids were then eluted with methanol (15 ml). Fatty acid methyl esters of this phospholipid fraction were prepared in a tube with a Teflon-lined screw-cap by heating at 95 °C for 1 h with 1 ml of 10% boron trifluoride in methanol (w/v) (Fluka Chemie AG). Methyl esters were extracted twice with 2 ml of hexane after adding 1 ml of distillated water.

Analyses. Methyl ester GC analyses of aposymbiotic and control aphids were performed on a Girdel 3000 chromatograph equipped with a solid injector maintained at 200 °C, a Supelco SP-2380 column (30 m × 0.32 mm I.D., 0.2 µm film thickness) and a flame ionisation detector (240 °C). The oven temperature was programmed from 140 to 205 °C at 1.2 °C/min. Linear flow rate for the helium carrier gas was 20 cm/s. Quantification was obtained by electronic integration using an internal standard (methyl pentadecanoate, Sigma Chemical Company) added in a known amount to each assay. Results were corrected for recovery as deduced from C17:0 internal standards.

GC-MS analyses were performed with a Nermag R-10-10 (or R-30-10) quadrupole instrument under computer control with the following source conditions: i) filament current, 180 µA; electron energy, 70 eV; temperature, 120 °C under the EI mode, and ii) filament current, 100 µA; electron energy, 95 eV; temperature 80–100 °C for CI with ammonia, isobutane or nitric oxide as reagent gas at approximately 10^{-4} Torr pressure (optimisation was done for isobutane and nitric oxide according to references 11–13 respectively) in the source housing. Samples were introduced via a 25 m × 0.32 mm I.D. CPWax-58 CB WCOT silica fused capillary column. ^{1}H and ^{13}C NMR spectra were obtained with a Brücker AC 400 MHz or a Varian Gemini 300 MHz equipment with C_6D_6 as solvent.

GC-FTIR was performed using an IFS-85 spectrometer coupled to a HRGC 5160 Carlo Erba (light pipe heated at 220 °C), the samples being introduced into a 12 m × 0.22 mm I.D. HP-1 column by on-column injection.

The compounds studied by IR and/or NMR were isolated by micropreparative GC with a Girdel 300 equipped with a previously described device[14] for collection. The column used was a 15 m × 0.53 I.D. WCOT Carbowax (Alltech).

Statistical analyses. Data means between aposymbiotic and control aphids were compared using Student's t-test. Before comparisons, the percentage data were transformed using angular transformation ($\text{Arcsin}\sqrt{f}$).

Results and discussion

Identification of the peculiar fatty acid. Assumption of a trienic fatty acid structure was previously made according to the GC behaviour of the methyl ester[1]. However, coinjection with standards corresponding to known homoconjugated structures was unfruitful.

GC-MS analysis of the methyl ester (phospholipid extract of young apterous adults after methylation) under the electron impact mode (EI) (fig.) led to a spectrum with the following ions at m/z: 292 ($M^{+\circ}$; very low intensity), 261, 135, 121, 107, 95, 94, 93, 81, 79, 67 (base peak), 55, 41. Although being of moderate relative abundance, other ions appeared at m/z 74 and 87 which were significant of the methyl ester group. The molecular weight was assumed to be 292 (thus possibly corresponding to a C_{18} triunsaturated methyl ester fatty acid) according to the chemical ionisation (CI)-MS performed with NH_3 which exhibited an ion (almost unique in the spectrum) at m/z 310 to be interpreted as $(M + NH_4)^+$. This was confirmed by using nitric oxide instead of NH_3 as reagent gas (e.g. NO-CIMS). This type of experiment may produce information not only about the molecular species (cf. molecular weight determination) but, more interestingly, about specific ion products (cf. localisation of CC double bonds)[13,15]. Indeed, besides ions at m/z 322, 292 and 291 corresponding to the $(M + NO)^+$, $M^{+\circ}$ and $(M - H)^+$ ions respectively, an ion of about 25% relative abundance is observed at m/z 185 (e.g. $H_3CO_2C(CH_2)_7CO^+$) indicating that the closest double bond to the ester group is at position 9.

Since further information on the other unsaturations involved could not be unambiguously obtained either from the NO-CI spectrum or from the t-$C_4H_9^+$ (originated from isobutane)-CI one (cf. conjugated dienes[11,12]), ^{1}H NMR was investigated after isolation of a sample of the pure compound (ca. 150 µg) by micropreparative GC. These data together with decoupling experiments led to the following interpretation (δ in ppm): 5.78 (m, H-17); 5.5 (m, 4H); 5.04 and 5.01 (2d, 2H, H-18); 3.38 (s, 3H, CO_2CH_3); 2.88 (dd, 2H, H-11); 2.12, 2.06 and 2.01 (m, 3 × 2H, allylic protons); $J_{18,17} = 17$ Hz; $J_{18',17} = 10$ Hz; $J_{11,10}$ and $J_{11,12} = 6.5$ Hz. The signals at 5.04 and 5.01 were unambiguously assigned to vinylic protons indicating that one double bond was at position 17. Furthermore, this double bond was neither conjugated nor homoconjugated. The two other double bonds (protons at 5.5) are homoconjugated (cf. bis-allylic CH_2 at 2.88) and are necessarily located at positions 9 (previously determined) and 12. Since no GC-FTIR absorption was observed at 960 cm^{-1} which would have accounted for CC double bond(s) of E geometry, the structure of the fatty acid was fully assigned as (Z,Z)-9,12,17-octadecatrienoic acid. The structure of the accompanying C16:1 and C18:1 fatty acids (see table) was similarly confirmed by NO-CIMS and GC-IRFT of the corresponding methyl esters. These experiments as well as ^{1}H and ^{13}C NMR spectra (and comparison with the standard) led to the confirmation of the structure of the C18:2 compound (linoleic acid).

Phospholipid fatty acids in control and aposymbiotic aphids. The total amount of phospholipid fatty acids

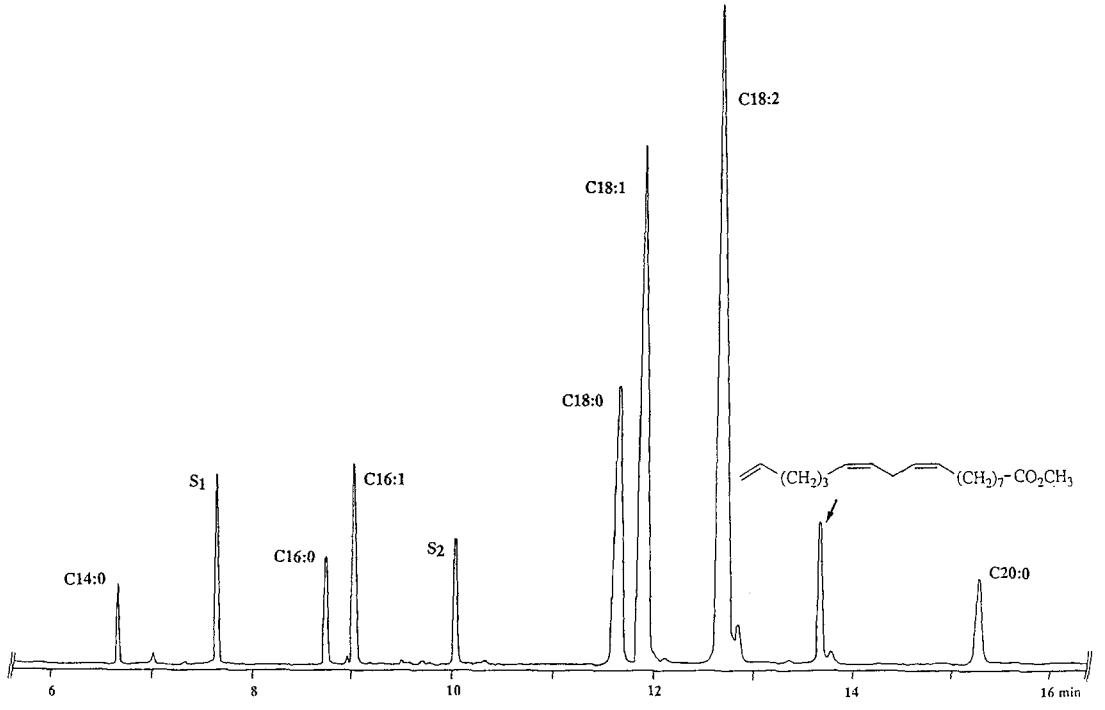

Figure. GC-MS chromatogram of phospholipid extract of whole *Acyrthosiphon pisum* adults after methylation (with S_1 and S_2, C15:0 and C17:0 respectively, as internal standards).

was significantly lower in the aposymbiotic aphids than in the control ones (table). Rifampicine treatment mainly suppressed all bacteriocytes (=mycetocytes) in the aphid haemocoel[9]. It is known, however, that reproduction is the major function affected by aposymbiosis[16,17]. With the same rifampicine treatment on *A. pisum*, Rahbé et al.[9] showed that aposymbiotic aphids could not complete reproduction, although they displayed a small number of embryos (mean of 5 red eyed embryos in aposymbiotic aphids versus 20 in control ones). Phospholipids are essential components of biological membranes and therefore it is not surprising that the phospholipid level was affected in aposymbiotic aphids which displayed a very significant reduction of embryonic tissues. Moreover, although the symbiotic bacteria do not account for a significant amount of the aphid's body mass (ca. 0.25% of fresh weight[18]) their contribution to the total membrane is probably not negligible in control aphids.

In contrast to the differences in amounts of phospholipids, the profiles of fatty acids showed only slight differences between control and aposymbiotic aphids (table). All fatty acids present in the phospholipids of control aphids were also present in the aposymbiotic aphids. In particular, the newly identified fatty acid, (Z,Z)-9,12,17-octadecatrienoic acid was also present after antibiotic treatment. The mass ratio should rule out the possibility that either this octadecatrienoic acid itself

Table. Amount of total fatty acids (µg per mg of fresh wt; mean ± SE) and proportions (%) of each fatty acid in the phospholipids of pea aphids (control and aposymbiotic aphids)

	Control aphids (n = 5)	Aposymbiotic aphids (n = 4)	Comparison
Amount of total fatty acids (µg/mg fresh wt)	9.67 ± 0.23	6.30 ± 0.03	*
Fatty acid composition (%)			
Lauric acid (C12:0)	t	t	
Myristic acid (C14:0)	1.9	1.9	NS
Palmitic acid (C16:0)	3.4	3.5	NS
Palmitoleic acid (C16:1)	5.8	4.9	*
Stearic acid (C18:0)	16.8	18.4	*
Oleic acid (C18:1)	27.6	25.7	*
Linoleic acid (C18:2)	35.5	38.5	*
(Z,Z)-9,12,17-octadecatrienoic acid (C18:3)	5.2	4.2	*
Arachidic acid (C20:0)	3.7	2.9	*

t = trace; NS = not significantly different; * = significantly different ($p \leq 0.05$).

or precursor compounds had been stored at an early stage of embryo development (before aposymbiosis). An aposymbiotic adult contains 0.42 µg of bound octadecatrienoic acid, and a single neonate larva weights about 30 µg, and it is hardly conceivable that such a compound could be stored at a level of 1.4% of fresh weight. Our results therefore clearly showed that symbionts were not involved in its biosynthesis and that aphids themselves are able to synthesize this peculiar octadecatrienoic acid. The biochemical pathways leading to the terminal double bound are now to be investigated, maybe in relation to the biosynthesis of the aphid alarm pheromone, β-farnesene, which also bears an identical terminal unsaturation.

If octadecatrienoic acid was shown to be present in aposymbiotic aphids, we must nevertheless note that its proportion was significantly lower than that in control aphids. An identical result was reported in a previous paper[1] comparing aphids grown on plants and aphids reared on an artificial diet. Aposymbiosis and diet rearing may both be regarded as affecting the reproductive functions of *A. pisum*, and it seems possible that the lowered proportion of the octadecatrienoic acid could be related to this process.

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