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RAPID COMMUNICATION

Fatty Acid Amides, Previously Identified in Caterpillars, Found in the Cricket *Teleogryllus taiwanemma* and Fruit Fly *Drosophila melanogaster* Larvae

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Abstract Fatty acid amides (FAAs) are known elicitors that induce plants to release volatile compounds that, in turn, attract foraging parasitoids. Since the discovery of volicitin [*N*-(17-hydroxylinolenoyl)-L-glutamine] in the regurgitant of larval *Spodoptera exigua*, a series of related FAAs have been identified in several other species of lepidopteran caterpillars. We screened 13 non-lepidopteran insects for the presence of FAAs and found that these compounds were present in adults of two closely related cricket species, *Teleogryllus taiwanemma* and *T. emma* (Orthoptera: Gryllidae), and larvae of the fruit fly, *Drosophila melanogaster* (Diptera: Drosophilidae). When analyzed by liquid chromatography/mass spectrometry-ion trap-time-of-flight (LCMS-IT-TOF), the gut contents of both crickets had nearly identical FAA composition, the major FAAs comprising *N*-linolenoyl-L-glutamic acid and *N*-linoleoyl-L-glutamic acid. There were also two previously uncharacterized FAAs that were thought to be hydroxylated derivatives of these glutamic acid conjugates, based on their observed fragmentation patterns. In addition to these four FAAs containing glutamic acid, *N*-linolenoyl-L-glutamine and a small amount of volicitin were detected. In *D. melanogaster*, *N*-linolenoyl-L-glutamic acid and *N*-linoleoyl-L-glutamic acid were the major FAAs found in larval extracts, while hydroxylated glutamic acid conjugates, volicitin and *N*-linolenoyl-L-glutamine, were detected as trace components. Although

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these FAAs were not found in ten of the insects studied here, their identification in two additional orders of insects suggests that FAAs are more common than previously reported and may have physiological roles in a wide range of insects besides caterpillars.

Keywords Volicitin · Lepidoptera · *Drosophila* · Teleogryllus · Larvae · Elicitors · LCMS-IT-TOF · Fatty acid amides · Volicitin

Introduction

Volicitin and its related analogs, generically called fatty acid amides (FAAs), were first identified as elicitors that induce plant volatile emission in the context of plant–herbivore interactions (Alborn et al. 1997). The mechanism by which volicitin triggers the systemic release of volatiles by corn plants has been studied in detail (Truitt et al. 2004), but how and why plants have evolved such a complex chemical signaling response to compounds of herbivore origin continues to engage researchers. Equally intriguing from an evolutionary perspective, volicitin and other FAAs exist in the oral secretions of some caterpillar species despite the propensity of some FAAs to elicit volatile emission in plants, thus, ultimately attracting the caterpillar's natural enemies. Lait et al. (2003) reported a key enzyme involved with the biosynthesis of *N*-linolenoyl-L-glutamine, a structural analog of volicitin, in alimentary tissues of *Manduca sexta* larvae. Our recent study of volicitin synthesis by *Spodoptera litura* revealed that glutamine is selectively incorporated into FAAs even though other amino acids are also found in the hemolymph and lumen of the gut (Yoshinaga et al. 2005). This suggests that FAAs could be involved with glutamine metabolism, and we hypothesize that they likely play a fundamental role in the physiology of lepidopteran larvae by virtue of their occurrence in multiple species (Alborn et al. 1997, 2003; Pare et al. 1998; Pohnert et al. 1999; Halitschke et al. 2001; Mori et al. 2001, 2003). Thus far, the search for FAAs has been limited to a small number of caterpillar species. The objective of this study was to search for FAAs in orders of insects other than Lepidoptera. We performed liquid chromatography/mass spectrometry-ion trap-time-of-flight (LCMS-IT-TOF) analysis on extracts from five species of Orthoptera, three species each of Hemiptera and Hymenoptera, and one species each of Coleoptera and Diptera (Table 1).

Methods and Materials

Insect Material and Gut or Larval Extracts Laboratory strains of *Drosophila melanogaster* (Canton S, Oregon R) were reared on artificial diet Formula 4-24 (Carolina Biological Supply Co.) that was supplemented with α-linolenic acid (10 μl/6 g wet diet) to compensate for dietary deficiency. Final instars were removed from the diet, rinsed with distilled water, and blotted dry with a paper towel. Larvae (20 per sample) were boiled in a microcentrifuge tube for 20 min with 10 μl of distilled water to prevent FAA hydrolysis by endogenous enzyme(s). Whole larvae were homogenized in 50 μl of acetonitrile, containing 0.3 μg of *N*-palmitoleoyl-L-glutamine as an internal standard, and then centrifuged for 10 min at 13,000×*g* to obtain a supernatant for LCMS and LCMS-IT-TOF analysis. Adult *Teleogryllus taiwanemma* (laboratory strain) were fed a natural diet of gramineous weeds for 48 hr and then frozen. Other adult specimens of Orthoptera, Hemiptera, Hymenoptera, and Coleoptera were collected from the field and frozen immediately after taxonomic identification. Each of these insects was sliced longitudinally with a razor, and the frozen

Table 1 Insect species used for LCMS-IT-TOF screening and FAA composition of extracts from gut contents or whole larval body

Order	Family	Species	Food Plants	FAAs							
				(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
Diptera	Drosophilidae	*Drosophila melanogaster*	Artificial diet	o	o	+	+	o	o	++	++
Orthoptera	Pyrgomophidae	*Atractomorpha lata*	*Eleusine indica*	−	−	−	−	−	−	−	−
	Acrididae	*Acrida cinera*	*Setaria viridis*	−	−	−	−	−	−	−	−
	Catantopidae	*Patanga japonica*	Unknown plants	−	−	−	−	−	−	−	−
	Gryllidae	*Teleogryllus taiwanemma*	*Eleusine indica*	+	o	+	+	+	o	++	++
		Teleogryllus emma	Unknown plants	+	o	+	+	+	o	++	++
Hemiptera	Pemtatomidae	*Eurydema rugosa*	*Brassica rapa* L.	−	−	−	−	−	−	−	−
	Coreidae	*Acanthocoris sordidus*	*Ipomoe batatas*	−	−	−	−	−	−	−	−
	Cicadellidae	*Bothrogonia ferruginea*	Unknown Plants	−	−	−	−	−	−	−	−
Coleoptera	Scarabaeidae	*Oxycetonia jucunda*	Unknown Plants	−	−	−	−	−	−	−	−
Hymenoptera	Argidae	*Arge* sp	*Rosa* sp.	−	−	−	−	−	−	−	−
	Tenthreddnidae	*Athalia rosae ruficornis*	*Brassica oleracea* L.	−	−	−	−	−	−	−	−
	Cimbicidae	*Zeraea akebii*	*Akebia* sp.	−	−	−	−	−	−	−	−

(1) volicitin, *(2)* *N*-hydroxylinoleoyl-L-glutamine, (*3*) *N*-hydroxylinolenoyl-L-glutamic acid, (*4*) *N*-hydroxylinoleoyl-L-glutamic acid, *(5)* *N*-linolenoyl-L-glutamine, *(6)* *N*-linoleoyl-L-glutamine, *(7)* *N*-linolenoyl-L-glutamic acid, *(8)* *N*-linoleoyl-L-glutamic acid, ++ major component (>30% of total component), + minor component (>3%), *o* trace component (< 3%), − not detected

midgut contents were transferred, by forceps, to microcentrifuge tubes. Midgut contents (approximately 30 μl) were boiled with 10 μl of distilled water, homogenized with 50 μl of acetonitrile, containing 0.3 μg of *N*-palmitoleoyl-L-glutamine, and centrifuged as described above to obtain a supernatant for LCMS and LCMS-IT-TOF analysis.

LCMS and LCMS-IT-TOF Analyses MS detection focused on ions at *m/z* 421, 422, 423, 424, 405, 406, 407, 408, 277, and 279, by using selected ion monitoring mode. Negative ESI mass spectral measurements were conducted with an LCMS-2010A instrument (Shimadzu, Kyoto) combined with an HPLC system (LC-10ADvp pump, CTO-10ACvp column oven, and SCL-10AVvp system controller; Shimadzu, Kyoto). A reverse-phase column (Mightysil RP-18 GP, 50×2.0 mm i.d.; Kanto Chemical Co., Inc., Tokyo) was eluted (0.25 ml min^{-1}) with a gradient of 20–95% acetonitrile, containing 0.08% acetic acid, in water containing 0.05% acetic acid, over 15 min with column temperature maintained at 40°C. Sample solutions containing FAAs were further analyzed by using a Prominence HPLC system coupled to LCMS-IT-TOF (Shimadzu, Kyoto). A Cosmosil 5C_{18}-AR-II column (50×2.0 mm i.d.; Nakalai Tesque, Kyoto) was eluted (0.2 ml min^{-1}) with a gradient of 20–90% acetonitrile, containing 0.08% acetic acid, in water containing 0.05% acetic acid, over 15 min with column temperature maintained at 40°C. The MS was

operated with probe voltage of 4.50 kV, CDL temperature of 200°C, block heater temperature of 200°C, nebulizer gas flow of 1.5 L min^{-1}, ion accumulation time of 10 msec, MS range of *m/z* 200 to 500, MS^2 range of *m/z* 100 to 500, CID parameters as follows: energy, 80%; collision gas, 100%. The precursor ions of volicitin, *N*-linolenoyl-L-glutamine, *N*-hydroxylinolenoyl-L-glutamic acid, *N*-linolenoyl-L-glutamic acid were *m/z* 421.27 (calculated for $C_{23}H_{37}N_2O_5^-$, 421.2702), 405.27 (calculated for $C_{23}H_{37}N_2O_4^-$, 405.2753), 422.27 (calculated for $C_{23}H_{36}NO_6^-$, 422.2543), 406.27 (calculated for $C_{23}H_{36}NO_5^-$, 406.2593), respectively. MS data were processed with LCMS solution ver. 3.4 software (Shimadzu).

Results and Discussion

From the thirteen non-lepidopteran insect species analyzed by LCMS, we determined that crickets (two species) and the fruit fly possess several kinds of FAAs (Table 1). These compounds were identified by comparison of their retention times and quasi-molecular ions with previously identified FAAs (standards) obtained from regurgitants of larval *S. litura* (Mori et al. 2003) and *M. sexta* (Alborn et al. 2003). *N*-Linolenoyl-L-glutamic acid (compound 7) was the main FAA component in extracts of *T. taiwanemma* (16.6±3.4 μg/insect, *N*=3) and *D. melanogaster* (8.9±1.7 μg/20 larvae, *N*=4). The quantity of *N*-linolenoyl-L-glutamic acid per mass of individual larva from each species was comparable with reported values for *M. sexta* (Alborn et al. 2003) or *N*-linolenoyl-L-glutamine in *S. litura* (unpublished).

For identification of FAAs found in crickets and fruit fly larvae, LCMS-IT-TOF analysis was conducted to obtain additional structural information. The following fragmentation ions were tentatively assigned: Volicitin, obtained from the regurgitant of larval *S. litura*, produces daughter ions at *m/z* 403.26 (dehydrated ion, calculated 403.2597), 385.25 (doubly dehydrated ion, calculated 385.2491), 315.24 (unknown), 231.21 (dehydrated and decarboxylated hydroxylinolenate ion, calculated 231.2113), and 145.06 (glutamine-derived ion, calculated 145.0614). *N*-Linolenoyl-L-glutamine produces daughter ions at *m/z* 387.27 (dehydrated ion, calculated 387.2648), 343.27 (further decarboxylated ion, calculated 343.2749), 233.23 (decarboxylated linolenate ion, calculate 233.2269), and 145.06 (glutamine-derived ion). *N*-Linolenoyl-L-glutamic acid, obtained from *M. sexta* regurgitant, produces daughter ions at *m/z* 388.25 (dehydrated ion, calculated 388.2488), 362.27 (decarboxylated ion, calculated 362.2695), and 277.22 (linolenic acid-derived ion, calculated 277.2168). The fragmentation pattern of FAAs containing glutamine, such as volicitin and *N*-linolenoyl-L-glutamine, typically includes a common glutamine-derived ion. Further MS analysis included manual scanning for daughter ions at *m/z* 277.22, 279.23, and 145.06, corresponding to other linolenoyl-, linoleoyl-, or glutamine conjugates. However, we could not detect any ions suggesting the presence of the related conjugates.

Figure 1 shows MS total ion chromatograms (TICs) with extracted ion chromatograms (XICs) of FAAs obtained from *T. taiwanemma* and *D. melanogaster* and MS^2 mass spectra with corresponding structures of FAAs obtained from *D. melanogaster* larvae. The spectra of *N*-linolenoyl-L-glutamic acid (d) and *N*-linolenoyl-L-glutamine (c) were nearly identical to those of authentic standards. Furthermore, *N*-linoleoyl-L-glutamic acid (e) was identified by its fragmentation pattern, which agreed reasonably with that of *N*-linolenoyl-L-glutamic acid (d). As the volicitin content was too low to yield fragment ions with enough intensity to measure accurately, only the dehydrated ion (*m/z* 403) and glutamine-derived ion (*m/z* 145) were detected along with ions from impurities (a). Spectrum (b) represents an

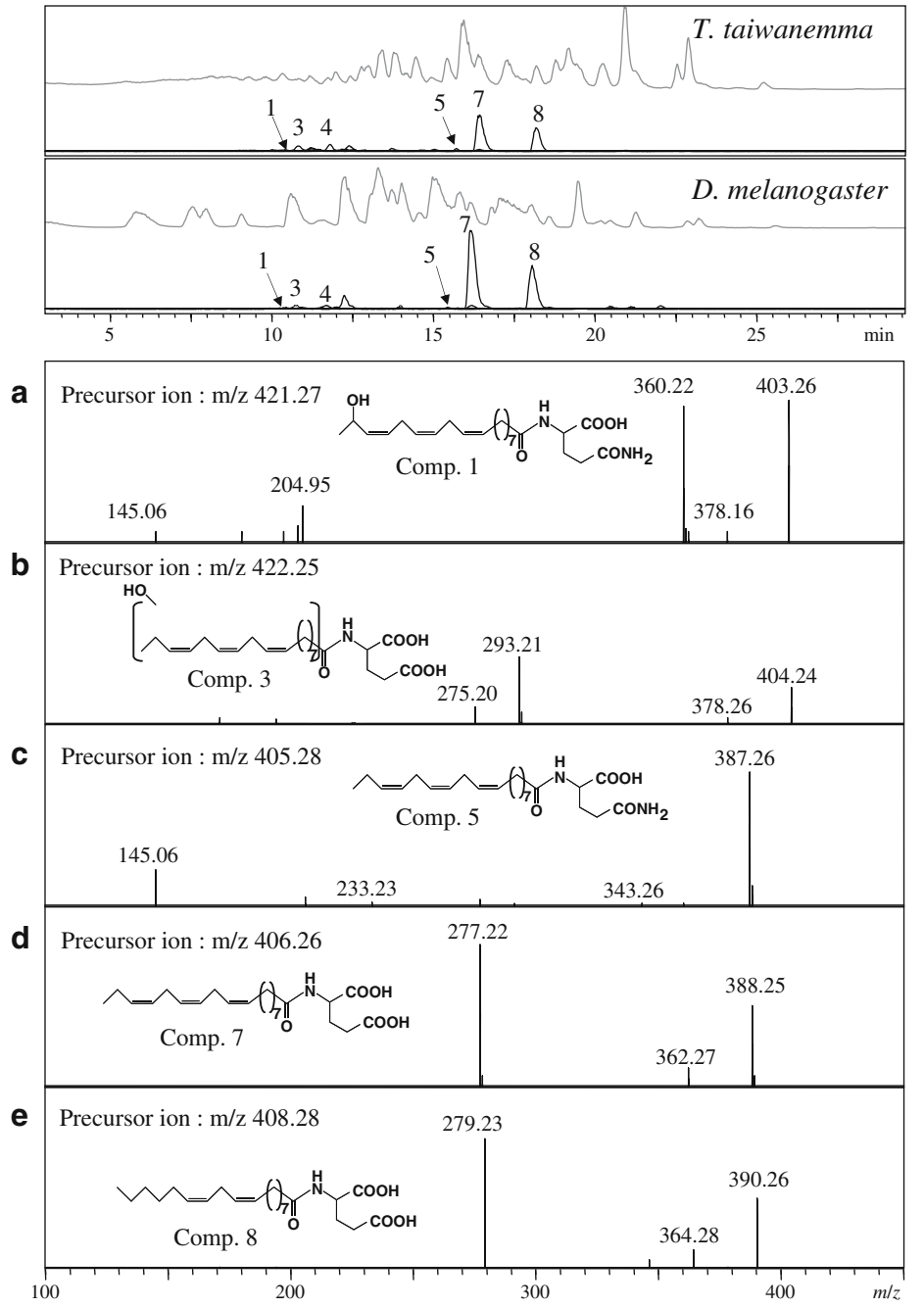

Fig. 1 Mass spectral total ion chromatograms and extracted ion chromatograms of FAAs obtained from *T. taiwanemma* and *D. melanogaster* and MS^2 spectra and structures of **a** volicitin, **b** *N*-hydroxylinolenoyl-L-glutamic acid, **c** *N*-linolenoyl-L-glutamine, **d** *N*-linolenoyl-L-glutamic acid, and **e** *N*-linoleoyl-L-glutamic acid found in the *D. melanogaster* larval extracts (structural analysis incomplete). The compound numbers represent the same compounds as those in Table 1

incompletely characterized FAA, which is thought to be *N*-hydroxylinolenoyl-L-glutamic acid (theoretical *m*/*z* 422.2543), as a presumed quasi-molecular ion appears at *m*/*z* 422.25. The fragmentation pattern agreed with the expected structure; dehydrated ion at *m*/*z* 404.24, doubly dehydrated ion at *m*/*z* 378.26, hydroxylinolenic acid-derived ion at *m*/*z* 293.21, and its dehydrated ion at *m*/*z* 275.20. When *D. melanogaster* larvae were reared on linolenic acid-supplemented artificial diet, peaks corresponding to FAAs containing a linolenic acid moiety had greater intensities and were more easily identified. However, it is not clear whether these FAAs came only from the larval gut. A compound with the same mass fragment patterns (not shown) was also found in the midgut contents of both cricket species. The absolute configuration and position of hydroxylation of the fatty acid moiety in the hydroxylated FAAs have yet to be confirmed. However, these findings are significant, as they indicate that FAAs might have physiological roles in additional orders of insects besides Lepidoptera. Halitschke et al. (2001) postulated that FAAs might simply act as biosurfactants or oil emulsifiers in the caterpillar gut, and indeed, this hypothesis may be applicable to crickets and fruit flies. However, the facts that FAAs identified thus far contain glutamic acid and/or glutamine, but not other amino acids, and hydroxylated FAAs are present in species from at least three different orders, suggest that a specific rather than general function in insect metabolism could also exist. As yet, we have no definitive explanation for their presence.

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References

ALBORN, H. T., TURLINGS, T. C. J., JONES, T. H., STENHAGEN, G., LOUGHRIN, J. H., and TUMLINSON, J. H. 1997. An elicitor of plant volatiles from beet armyworm oral secretion. *Science* 276:945–949.

ALBORN, H. T., BRENNAN, M. M., and TUMLINSON, J. H. 2003. Differential activity and degradation of plant volatile elicitors in regurgitant of tobacco hornworm (*Manduca sexta*) larvae. *J. Chem. Ecol.* 29:1357–1372.

HALITSCHKE, R., SCHITTKO, U., POHNERT, G., BOLAND, W., and BALDWIN, I. T. 2001. Molecular interactions between the specialist herbivore *Manduca sexta* (Lepidoptera, Sphingidae) and its natural host *Nicotiana attenuata*. III. Fatty acid-amino acid conjugates in herbivore oral secretions are necessary and sufficient for herbivore-specific plant responses. *Plant Physiol.* 125:711–717.

LAIT, C. G., ALBORN, H. T., TEAL, P. E. A., and TUMLINSON, J. H. 2003. Rapid biosynthesis of *N*-linolenoyl-L-glutamine, an elicitor of plant volatiles, by membrane-associated enzyme(s) in *Manduca sexta*. *Proc. Natl. Acad. Sci. USA* 100:7027–7032.

MORI, N., ALBORN, H. T., TEAL, T. E. A., and TUMLINSON, J. H. 2001. Enzymatic decomposition of elicitors of plant volatiles in *Heliothis virescens* and *Helicoverpa zea*. *J. Insect Physiol.* 47:749–757.

MORI, N., YOSHINAGA, N., SAWADA, Y., FUKUI, M., SHIMODA, M., FUJISAKI, K., NISHIDA, R., and KUWAHARA, Y. 2003. Identification of volicitin-related compounds from the regurgitant of lepidopteran caterpillars. *Biosci. Biotec. Biochem.* 67:1168–1171.

PARE, P. W., ALBORN, H. T., and TUMLINSON, J. H. 1998. Concerted biosynthesis of an insect elicitor of plant volatiles. *Proc. Natl. Acad. Sci. USA* 95:13971–13975.

POHNERT, G., JUNG, V., HAUKIJA, E., LEMPA, K., and BOLAND, W. 1999. New fatty acid amides from regurgitant of lepidopteran (Noctuidae, Geometridae) caterpillars. *Tetrahedron* 55:11275–11280.

TRUITT, C. L., WEI, H. X., and PARE, P. W. 2004. A plasma membrane protein from *Zea mays* binds with the herbivore elicitor volicitin. *Plant Cell* 16:523–532.

YOSHINAGA, N., MORIGAKI, N., MATSUDA, F., NISHIDA, R., and MORI, N. 2005. *In vitro* biosynthesis of volicitin in *Spodoptera litura*. *Insect Biochem. Mol. Biol.* 35:175–184.