N-(18-hydroxylinolenoyl)-L-glutamine: a newly discovered analog of volicitin in Manduca sexta and its elicitor activity in plants.
Running title:


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Abstract

Plants attacked by insect herbivores release a blend of volatile organic compounds (VOCs) that serve as chemical cues for host location by parasitic wasps, natural enemies of the herbivores. Volicitin [N-(17-hydroxylinolenoyl)-L-glutamine] is one of the most active VOC elicitors found in herbivore regurgitants. Our previous study revealed that hydroxylation on the 17th position of the linolenic acid moiety of N-linolenoyl-L-glutamine increases by more than 3 times the elicitor activity in corn plants. Here, we identified N-(18-hydroxylinolenoyl)-L-glutamine (18OH-volicitin) from tobacco hornworm (THW) Manduca sexta larval gut contents. Eggplant and tobacco, two solanaceous host plants of THW larvae and corn, a non-host plant responded differently to this new elicitor. Eggplant and tobacco seedlings emitted twice the amount of VOCs when 18OH-volicitin was applied to damaged leaf surfaces compared to N-linolenoyl-L-glutamine, while both these fatty acid amino acid conjugates (FACs) elicited a similar response in corn seedlings. In both solanaceous plants, there was no significant difference in the elicitor activity of 17OH- and 18OH-volicitin. Interestingly, other lepidopteran species that have 17OH-type volicitin also attack solanaceous plants. These data suggest that plants have developed herbivory-detection systems customized to their herbivorous enemies.

Key words Volicitin · Elicitor · Tobacco hornworm · Maize · Eggplant · FACs.

Abbreviations

FACs fatty acid amino acid conjugates
LC/MS liquid chromatography/mass spectrometry
GC/MS Gas chromatography/mass spectrometry
ESI electron spray ionization
LC/MS-IT-TOF liquid chromatography/mass spectrometry-ion trap-time-of-flight
Introduction

Plants have developed a variety of defense mechanisms against herbivorous insects. A typical example of indirect plant defenses is an intervention by natural enemies (Turlings et al. 1990; Kessler and Baldwin 2001). For example, parasitoids use VOCs released by herbivore-attacked plants as chemical cues to locate their hosts. In many cases, release of VOCs is triggered by a perception of wounding. If in addition to the wounding signal the plant perceives insect elicitor(s), which are contained in oral secretions, the volatile profile is augmented. Four types of such elicitors are hitherto known: β-glucosidase (Mattiacci et al. 1995), fatty acid amino acid conjugates (FACs) (Alborn et al. 1997), inceptin (Schmelz et al. 2006), and caeliferins (Alborn et al. 2007).

Among FAC type elicitors, volicitin is the most active and the precursor, \(N\)-linolenoyl-\(L\)-glutamine, takes second place against maize cultivars such as Delprim (Alborn et al. 1997). Subsequent to the identification of volicitin, \(N\)-linolenoyl-\(L\)-glutamic acid was identified in oral secretions of tobacco hornworm \textit{Manduca sexta} larvae, and found to be active against tobacco plants (Halitschke et al. 2001; Alborn et al. 2003). These three compounds have been widely considered as typical FAC elicitors; other analogs of different fatty acids like linoleate showed negligible activity (Alborn et al. 2003). Previously we showed that the amino acid moiety is also crucial for bioactivity; of the artificially designed FACs \(N\)-linolenoyl-\(L\)-leucine, -phenylalanine, -proline, -threonine, and \(L\)-glutamine, only \(L\)-glutamine is active. Further, the strength of elicitor activity on corn seedlings (Delprim) is increased three-fold by the hydroxylation at the 17\text{th} position, but the absolute configuration at position 17 carbon has no effect on activity (Sawada et al. 2006).

FACs have been identified in several species of lepidopteran caterpillars. Pohnert et al. (1999) identified FACs in 6 species from Noctuidae and Geometridae.
and found the same glutamine/glutamate-conjugates that had been previously reported for other species. The composition of the fatty acid moiety is correlated with the composition of dietary fatty acids (Pare et al. 1998; Aboshi et al. 2007), while the glutamine/glutamic acid pattern of FACs is clearly specific to the herbivore species. In a previous study we screened 29 species out of 16 families and found glutamine-conjugated FACs in two-thirds of them. Only 7 species among them had glutamic acid-conjugates as well. No other amino acid was found in association with FACs. Looking at the result from a phylogenetic perspective, glutamic acid-conjugates were found in relatively primitive species while hydroxylation of fatty acids was limited to macrolepidopteran species (Yoshinaga et al. 2010).

Considering the FAC patterns in relation to tritrophic interactions becomes even more confusing because a plant can have more than one pest species and each herbivore might show different elicitor patterns. Schmelz et al. (2009) demonstrated the complexity of plant-elicitor (herbivore) interactions by assaying VOC emission as well as phytohormone levels in several plants after treatment by volicitin and \( N \)-linolenoyl-L-glutamine together with other insect elicitors. This was significant in providing a larger picture of plant-herbivore interaction. Our interest, however, rather focuses on adaptation, hypothesizing that the selection pressure on phytophagous insects, generated by plants’ defensive responses to FACs, should have affected the combination and quantitative ratio of FACs in each insect species. On the other hand, the influence of physiological constraints of the insect on FAC composition should not be ignored. In fact, we showed that \( N \)-linolenoyl-L-glutamine functions as a form of glutamine storage, promoting uptake of glutamic acid as well as recycling ammonia, to enhance total nitrogen assimilation in \textit{Spodoptera litura} larvae (Yoshinaga et al. 2008). These findings suggest a direct benefit of \( N \)-linolenoyl-L-glutamine to the insect and possibly the reason why only glutamine- or glutamate-type FACs have so far been found in insects (Yoshinaga et al. 2007). Although further studies are necessary to explore the role of other analogs including hydroxylated FACs, not only an ecological
suppression (external) but also physiological requirements (internal) seem to be involved in determining patterns of FAC activity.

Our question here is whether or not a plant could have adapted its response to elicitors indicative of its traditional pest species. With the evidence so far available it was hard to make a good case for this idea, but the identification of a novel and unique FAC from *Manduca sexta* larvae enabled us to examine this hypothesis. *M. sexta* is a known pest for various plants in the family Solanaceae but it does not feed on corn plants. We therefore tested elicitation activity of different FACs on seedlings of corn and solanaceous hosts, eggplant and tobacco, to reveal a possible linkage between elicitor activity and pest-host relationships.

**Materials and Methods**

*Insect rearing and analysis of FACs in gut contents.*

Colonies of *S. litura* were reared in the laboratory on an artificial diet (Insecta-LFS, Nihon Nosan Kogyo Ltd.) under 16L:8D, at 28 °C. Eggs of tobacco hornworm *Manduca sexta* were obtained from Dr. Alan Renwick at Boyce Thompson Institute, Ithaca, NY. The caterpillars were reared on artificial diet (Southland products, Arkansas) under 16L:8D, at 28 °C. Last instar larvae were placed on host plants (tomato leaves for *M. sexta* larva and *Fagopyrum tataricum* leaves for *S. litura* larva) and allowed to feed for 24 hrs before regurgitant was collected. Gut content was boiled immediately to avoid enzymatic decomposition and centrifuged at 11,000 g for 10 min. The supernatant was diluted to 10 times its volume with 50 % acetonitrile solution and analyzed by LC/MS. Negative ESI mass spectral measurements were carried out by using an LC/MS 2010A instrument (Shimadzu, Kyoto) combined with an HPLC system (LC-10ADvp pump, CTO-10ACvp column oven, and SCL-10AVvp system controller; Shimadzu). A reversed-phase column (Mightysil RP-18 GP, 50 × 2.0 mm i.d.; Kanto Chemical, Co, Inc., Tokyo) was eluted (0.2 ml/min) with a solvent gradient...
of 40–95 % acetonitrile containing 0.08 % acetic acid, in water containing 0.05 % acetic acid, over 15 min. The column temperature was maintained at 40 ºC (CTO-10Avp column oven; Shimadzu). FACs were identified by comparison of their retention times and quasi-molecular ions with previously identified FAC standards (Alborn et al. 2003; Mori et al. 2003; Yoshinaga et al. 2007). Sample solutions were further analyzed by using a Prominence HPLC system coupled to LC/MS-IT-TOF (Shimadzu, Kyoto). The MS was operated with probe voltage of 4.50 kV, CDL temperature of 200 ºC, block heater temperature of 200 ºC, nebulizer gas flow of 1.5 l/min, ion accumulation time of 10 msec, MS range of m/z 200 to 500, MS² range of m/z 100 to 500, CID parameters as follows: energy 80 %; collision gas 100 %.

Identification of new FACs.

For identification of novel FACs, 100 ml of gut contents were collected from 30 last instar larvae of M. sexta which fed on linolenic acid enriched diet (500 µl of linolenic acid in 200 ml diet) for 24 hrs, as previously reported (Yoshinaga et al. 2005). In the boiled gut contents, 18-hydroxylinolenic acid conjugated with glutamate (18OH-18:3-Glu, [M−H]⁻ at m/z 422) and 18OH-volicitin ([M−H]⁻ at m/z 421) were detected. After HPLC purification as described in Sawada et al. (2006), 18OH-18:3-Glu and 18OH-volicitin were evaporated to dryness and subjected to acid-catalyzed methanolysis using methanol/acetic anhydride by the method of Mori et al. 2001. For comparison 17OH-volicitin (synthesized or purified from S. litura larval gut contents) was processed in the same way. These samples were analyzed by GC/MS in CI mode to determine the position of the hydroxyl group in the linolenic acid moiety. Aliquots of samples (1.0 µl) were run on an Agilent 6890N Network GC system with a 30 m × 0.32 mm, 0.25 µm film thickness, HP-5MS capillary column, interfaced to an Agilent 5975 inert XL mass selective detector. The column temperature was held at 100 ºC for 5 min after injection and then programmed at 10 ºC/min to 290 ºC. Methane was used as the reagent gas for chemical ionization, and the ion source temperature
was set to 250 °C. The retention time and mass spectrum of methyl 18-acetoxylinolenate were compared with those of synthesized methyl 17- and 18-acetoxylinolenates. Furthermore, purified 18OH-volicitin was analyzed by 1H-NMR spectroscopy. Proton nuclear magnetic resonance spectra were measured with a Bruker Avance 400 FT-NMR (1H: 400 MHz) and JEOL ECP500 (1H: 500 MHz) using TMS as an internal standard.

Chemical synthesis of volicitin and N-(18-hydroxylinolenoyl)-L-glutamine.

17OH- and 18OH-Volicitin were synthesized by the procedures reported by Pohnert et al. (1999) with a minor modification. 3-Hydroxypropionic acid was used for the synthesis of 18OH-volicitin, instead of lactic acid for that of 17OH-volicitin.

Plants and elicitor treatments.

Dent corn Zea mays L., B73 (USDA-ARS Inbred line) was germinated and grown in autoclaved commercial soil at 25 °C under a 16L:8D long-day. Plants were used at v3 stage (5 leaves, 3 fully developed leaf collars, 15 days from seeding), cut off near the stem base and the end surface was immediately immersed in 200 μl of elicitor solution (25 mM Na₂HPO₄ buffer, pH 8) containing 1 nmol of each elicitor. After the solution was taken up, the plants were transferred into a small bottle of water set in VOC chambers (30 cm long, 4 cm i.d.) and kept under darkness overnight. Commercially available seeds of eggplant Solanum melongena, var. esculentum and tobacco plant, Nicotiana tabacum var. K326 (GoldLeaf Seed Co., Hartsville, SC) were also grown in pots with autoclaved soil at 25 °C under a 12L:12D long-day in a greenhouse. Plants, 6–7 weeks after germination, at approximately 25–30 cm in height were used for the experiments. The two newest fully developed leaves were scratched in the middle, crosswise, with a razor and treated with 20 μl of elicitor solution (1 nmol of each elicitor in phosphate buffer), and placed into a VOC collection chamber. The chambers consisted of a modified Pyrex bottle (7 l) with a Teflon stopper with an inlet for
charcoal filtered air (1.5 l/min) and an aluminum guillotine at the bottom of the chamber (Halloran et al., 2013). The plant remained in its growing container and the stem was wrapped in cotton with the guillotine surrounding the stem.

**VOC collection and GC analysis.**

Fabricated filter traps containing an adsorbent (30 mg, Hayesep Q, Hayes Separation Inc., Texas) were attached to a vacuum pump (1.0 l/min) via ports at the bottom of the chamber. Volatiles were collected for 6 hrs from the beginning of the light cycle until the emission decreased to almost baseline levels. To analyze the emitted volatiles, filter traps were eluted with 100 μl, 1:1 v/v hexanes: dichloromethane, and internal standard was added (10 μl, of 40 ng nonyl acetate/μl dichloromethane). Samples (1 μl injection volume, split-less mode) were analyzed with a gas chromatograph equipped with a flame ionization detector (Agilent 6890N) and an HP-1 column (15 m × 0.25 mm × 0.25 μm film thickness; Agilent) using helium as the carrier gas at an average linear flow velocity of 24 cm/second. The oven program was 40 ºC, 1 min; 8 ºC/min to 180 ºC; followed by a program of 30 ºC /min to 300 ºC, and held for 5 min. In order to identify major components, the samples were run on an Agilent 6890 N GC equipped with an Agilent 5973 N mass selective detector configured for electron impact mode and a HP-1MS column (30 m × 0.25 mm × 0.25 μm, Agilent). The column was held at 40 ºC for one min and then increased by 8 ºC/min to reach a maximum temperature of 300 ºC. The column flow rate was 0.7 ml/min. Mass spectra were compared to spectra for standards available in the National Institute of Standards and Technology (NIST) library as well as known standards from the lab. All bioassays also included a negative control (buffer only) and were replicated four to twelve times within each bioassay.

**Statistical analysis.**

Total amounts of VOC emitted per plant among different treatments were compared using a non-parametric Kruskal-Wallis rank sums test followed by nonparametric
comparisons for all pairs using Steel-Dwass method. Analyses were conducted using JMP 11.1.1 (SAS, Cary, NC, USA).

Results

Identification of 18OH-FACs.

Large amounts of acyl glutamates with small amounts of acyl glutamines are typical FAC patterns in M. sexta larval gut contents, and hydroxyacyl glutamines/glutamic acids (Fig. 1A, peaks 1, 3, 4) have been missed in previous studies (Alborn et al. 2003; Halitschke et al. 2001). However, feeding caterpillars with linolenic acid-enriched diet for 48 hrs enabled us to find and identify these novel hydroxylated FACs. Considering the [M-H]⁻ ions and retention times, compound 1 (m/z 421) and 3 (m/z 422) were supposed to be hydroxylinolenoyl-L-glutamine (OH-18:3-Gln) and hydroxylinolenoyl-L-glutamic acid (OH-18:3-Glu), respectively. The amount of OH-18:3-Glu was larger than that of OH-18:3-Gln, as is expected from the typical FAC pattern in this species. The MS² spectrum of compound 1 analyzed by LC/MS-IT-TOF was characteristic with a daughter ion at m/z 329, while 17OH-volicitin from S. litura shows a daughter ion at m/z 385 and 315 (supplemental figure 1). To determine the hydroxyl position, OH-18:3-Gln purified from larval gut contents was subjected to methanolysis and analyzed by GC/MS in CI mode. The major peak at m/z 291, derivatized hydroxylinolenate, showed up at tᵣ 23.1 min. The same methanolysis of both synthesized and natural 17OH-volicitin (purified from S. litura larval gut contents) gave methyl 17-acetoxylinolenate ion at m/z 291 (M+1–CH₃COOH) but the peak retention time was 21.5 min (Supplemental figure 2). There was no difference in the mass spectra of these three methyl acetoxylinolenate peaks but the mismatch of the tᵣ suggests the hydroxyl group is not located on carbon 17.

Further analysis to determine the position of the hydroxyl group was conducted with ¹H-NMR analysis. The ¹H-NMR spectrum (CD₃OD) of OH-18:3-Gln
was as follows: δ 1.34 (8H, s-like), 1.62 (2H, m), 1.95–1.98 (1H, m), 2.07–2.08 (2H, m), 2.11–2.18 (1H, m), 2.22–2.32 (4H, m), 2.80–2.86 (4H, m), 3.55 (2H, t, J=6.9 Hz), 4.30–4.31 (1H, s), 5.33–5.98 (6H, m). The doublet signals of the methyl protons at 1.05 ppm and a methine signal at 4.6 ppm shown in synthetic (17S)-volicitin (Sawada et al. 2006) were not found in OH-18:3-Gln. Instead, there was a clear triplet signal at 3.55 ppm proving the hydroxylation was located at 18\textsuperscript{th} position. This \textsuperscript{1}H-NMR data agreed well with that of synthetic N-(18-hydroxylinolenoyl)-L-glutamine. The GC/MS retention time and mass fragment pattern of methyl 18-acetoxylinolenate derived from the natural compound also matched those of synthetic N-(18-hydroxylinolenoyl)-L-glutamine. In a similar way, methanolysis of compound 3 (Fig. 1) gave a product with the major mass spectrum peak at m/z 291 and t\text{R} 23.1 min, suggesting the compound to be N-(18-hydroxylinolenoyl)-L-glutamate. Compound 4 was strongly suggested to be N-(18-hydroxylinoleoyl)-L-glutamate, but the presence of N-(18-hydroxylinoleoyl)-L-glutamine was not clear enough in the LC/MS analysis.

Plant assays.

The profile of major VOCs released from B73 corn seedlings was not different from previous studies using Delprim seedlings (Sawada et al. 2006). Among the released VOCs, sesquiterpenes such as (E)-β-farnesene, (E)-α-bergamotene, and (E)-β-caryophyllene were clearly induced by elicitor treatments (Fig. 2). Although the ratio of components was rather constant, the total amount of released VOCs distinctly differed by treatments. In particular 17OH-volicitin reproducibly induced more VOCs than N-linolenoyl-L-glutamine. However, 18OH-volicitin was not more active than N-linolenoyl-L-glutamine, suggesting the hydroxylation at 18\textsuperscript{th} position did not enhance the elicitor activity in corn seedlings.

The only compound common to both eggplant and corn plant VOCs was (E)-α-bergamotene. Other major components of eggplant VOCs were (Z)-3-hexenyl acetate, 4,8-dimethyl-1,3,7-nonatriene and (E)-β-ocimene (Fig. 3). Again, in eggplant,
there was a clear difference between the activity of 17OH-volicitin and
N-linolenoyl-L-glutamine, but no significant difference was observed between 17OH-
and 18OH-volicitin treatments.

In a similar way, tobacco plants released 3-fold greater amounts of its major
VOCs, \((E)\)-\(\beta\)-caryophyllene and \((E)\)-\(\beta\)-ocimene, when treated with 17OH- or
18OH-volicitin than when treated with N-linolenoyl-L-glutamine but the hydroxyl
position was of no importance (Fig. 4).

The elicitor activity of \(N\)-(18-hydroxylinolenoyl)-L-glutamate was only
examined with corn and eggplant seedlings (Fig. 5). As previously reported (Alborn et
al. 2003), corn plants did not react as strongly to \(N\)-linolenoyl-L-glutamate as to the
corresponding glutamine analog. We did not assay
\(N\)-(17-hydroxylinolenoyl)-L-glutamate but in corn seedlings the elicitor activity of
\(N\)-(18-hydroxylinolenoyl)-L-glutamate was not greater than that of
\(N\)-linolenoyl-L-glutamate, again suggesting that the hydroxylation at the 18\(^{\text{th}}\) position
had no meaning to corn plants. On the other hand, eggplant seedlings did not
differentiate between the conjugated amino acids glutamate and glutamine. More
surprisingly hydroxylation did not enhance the elicitor activity of
\(N\)-linolenoyl-L-glutamate.

**Discussion**

Our previous study (Yoshinaga et al. 2010) showed more than 10 lepidopteran species
have hydroxylated FAC elicitors and, until recently, in all cases the hydroxyl group of
the C18 fatty acid moiety was thought to be in the \(\omega\)-1 (at position 17). However, the
data presented here show that the novel FAC elicitors identified from *M. sexta* larval
midgut contents are hydroxylated on the 18\(^{\text{th}}\) carbon. Although we fed larvae with
linolenate-enriched diet in order to obtain large enough quantities for identification of
the chemical structure of FACs, a possibility that the FACs are artifactual products can
be eliminated by the fact that these new FACs were determined to be present in small
amounts in *M. sexta* larvae fed on natural diet (Fig. 1A). GC analysis clearly revealed that in *M. sexta* FACs only show hydroxylation at carbon 18 and no 17-hydroxylation, which suggests they have a ω-hydroxylase in place of (ω-1)-hydroxylase. Since no hydroxylase of FACs has yet been identified from lepidopteran species, we don’t know if these hydroxylases are related in any way. Among species that have hydroxylated FACs, only a few have been confirmed to have hydroxylation in position 17. For example, *Spodoptera exigua* was the species volicitin was first identified from, and the chemical structure was carefully determined (Alborn et al. 1997). Several species have been reported to have 17OH-volicitin, identified by GC analysis after methanolysis (Mori et al., 2001; Spiteller et al., 2001; Sawada et al. 2006). Other cases relying on single LC/MS to identify FACs leave the possibility that the hydroxylation could be other than position 17. In our preliminary experiments using LC/MS-IT-TOF, 17OH-volicitin and 18OH-volicitin gave a different daughter ion. Although we have not yet proven the reproducibility, among 6 species so far examined *Acherontia styx* (Sphingidae) was the only species whose volicitin showed the same mass spectral pattern with *M. sexta* (Supplemental figure 1).

The (ω-1)-hydroxylation in FACs is known to be important for the elicitor activity against corn plants (Alborn et al. 1997), regardless of absolute configuration of the hydroxylation (Sawada et al. 2006). Our results showed that not only corn but also eggplant and tobacco seedlings responded strongly to hydroxylated glutamine-based FACs. However, hydroxylation at the position 18 did not enhance its elicitor activity against corn seedlings. The 18OH-type FACs are, as far as we know now, limited to two species in Sphingidae, *M. sexta* and *A. styx*, which do not feed on corn. If such 18OH-type FACs are completely new to corn plants in natural conditions, it might explain why the assayed corn seedlings could not discriminate the 18OH-volicitin from *N*-linolenoyl-L-glutamine. The two Sphingid species are pests of solanaceous plants including eggplant and tobacco, which actually responded to and discriminated 18OH-volicitin from *N*-linolenoyl-L-glutamine. In both of these solanaceous species,
17OH- and 18OH-volicitin were the two top elicitors. This might be explained by the fact that 17OH-volicitin is widely found in pest species such as *Heliothis virescens*, *Helicoverpa armigera*, *S. litura*, and *S. littoralis*, which have host plants in common with two Sphingid species (Mori et al., 2001; Spiteller et al., 2001; Sawada et al., 2003). These solanaceous plants might have adapted to respond to both types of volicitin.

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**References**


Active role of fatty acid amino acid conjugates in nitrogen metabolism in Spodoptera litura larvae. Proc Natl Acad Sci USA 105: 18058–18063. doi: 10.1073/pnas.0809623105


Legends for figures

Fig. 1 LC/MS chromatogram of larval gut content extracts of M. sexta fed on tomato leaves (A) and S. litura fed on Fagopyrum tataricum leaves (B).

1, N-(18-hydroxylinolenoyl)-L-glutamine (m/z 421); 1', volicitin (m/z 421); 2, N-(17-hydroxylinoleoyl)-L-glutamine (m/z 423); 3, N-(18-hydroxylinolenoyl)-L-glutamate (m/z 422); 4, N-(18-hydroxylinoleoyl)-L-glutamate (m/z 424, tentative identification); 5, N-linolenoyl-L-glutamine (m/z 405); 6, N-linoleoyl-L-glutamine (m/z 407); 7, N-linolenoyl-L-glutamate (m/z 406); 8, N-linoleoyl-L-glutamate (m/z 408); 9, linolenic acid (m/z 277); 10, linoleic acid (m/z 279).

Fig. 2 B73 corn seedling volatile compounds induced by elicitor treatments (N=10–12, Mean ± SEM). Different letters indicate significant difference by Steel-Dwass test (P<0.002).

Fig. 3 Eggplant volatile compounds induced by elicitor treatments (N=9, Mean ± SEM). Different letters indicate significant difference by Steel-Dwass test (P<0.02).

Fig. 4 Tobacco volatile compounds induced by elicitor treatments (N=4, Mean ± SEM). There was no statistical difference among the treatments, probably owing to the
small sample size.

Fig. 5  B73 corn seedling (A) and eggplant (B) volatile compounds induced by elicitor treatments (N=9–12, Mean ± SEM). Different letters indicate significant difference by Steel-Dwass test (P<0.008 for A and P<0.004 for B).

Supplemental fig. 1  MS² spectra of hydroxylinolenoyl-L-glutamine (OH-18:3-Gln) from M. sexta (A), Acherontia styx (B) and 17OH-volicitin from S. litura larval gut contents (C). Larval gut content of wild caught A. styx was prepared in the same way.

Supplemental fig. 2  GC/MS chromatogram of methyl acetoxylinolenate (M+1-CH₃COOH, m/z 291) derived from (A) 17OH-volicitin purified from S. litura larval gut contents, (B) 18OH-volicitin from M. sexta, and (C) synthesized 18OH-volicitin.
Fig. 1

A

B

1' 2

min
Fig. 3

- **17OH-volicitin**
- **18OH-volicitin**
- **Linolenoyl glutamine**
- **Buffer**

- **(E)-α-Bergamotene**
- **4,8-Dimethyl-1,3,7-nonatriene**
- **(E)-β-Ocimene**
- **(Z)-3-Hexenyl acetate**
Fig. 4

The graph shows the levels of different compounds in ng/plant:

- **17OH-volicitin**
- **18OH-volicitin**
- **Linolenoyl glutamine**
- **Buffer**

The compounds are classified by their chemical names:

- **(E)-β-Caryophyllene**
- **(E)-β-Ocimene**
**Fig. 5**

<table>
<thead>
<tr>
<th>A</th>
<th>B</th>
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<tr>
<td><strong>ng/plants</strong></td>
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</tr>
<tr>
<td>3000</td>
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<td>1000</td>
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<tr>
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- **A**
  - a: Linolenoyl glutamine
  - ab: Linolenoyl glutamic acid
  - b: 18OH-linolenoyl glutamic acid
  - c: Buffer

- **B**
  - x: (E)-β-Farnesene
  - x: (E)-α-Bergamotene
  - x: 4,8-Dimethyl-1,3,7-nonatriene
  - y: (E)-β-Ocimene
  - y: (E)-β-Caryophyllene
  - y: (Z)-3-Hexenyl acetate
A Precursor ion: $m/z$ 421.26

B Precursor ion: $m/z$ 421.30

C Precursor ion: $m/z$ 421.26

Supplemental fig. 1