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TETRAHEDRON

New Fatty Acid Amides from Regurgitant of Lepidopteran (Noctuidae, Geometridae) Caterpillars

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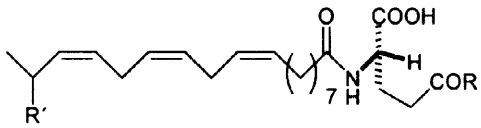
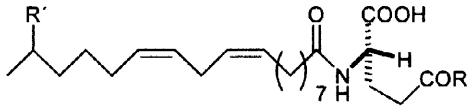
Abstract: Oral secretions of seven different species of caterpillars, feeding on natural and artificial diets have been analysed by liquid chromatography mass spectroscopy. The compounds present in the caterpillar regurgitates were identified as a structurally diverse group of conjugates of glutamine and glutamic acid linked via an amide bond to saturated and unsaturated C_{14}-, C_{16}- and C_{18} fatty acids. Proportions of different compounds in regurgitants were species specific.

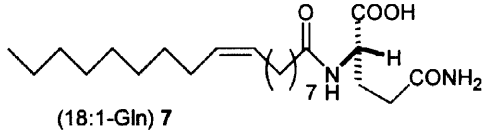
Plants under attack by a herbivore release a blend of *de novo* synthesised volatiles that may attract natural enemies of the attacking insect.[1,2] The mechanical damage of the feeding process only effects the release of preformed volatiles and that from rapid degradation processes, but components (elicitors) from introduced salivary secretions modify the plant's gene expression activating the *de novo* biosynthesis of high- and/or low molecular weight defense compounds.[3] To date two insect-derived elicitors have been established, *i)* a β-glucosidase from the regurgitant of *Pieris brassicae* caterpillars,[4] triggering the emission of volatiles from cabbage plants, and *ii)* the recently identified *N*-(17-hydroxylinolenoyl)-L-glutamine (volicitin) (**1**) from beet armyworm oral secretion.[5]



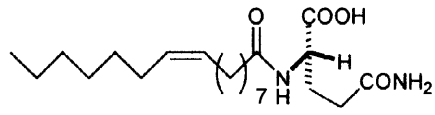
R = NH_2, R′ = OH (volicitin) **1**
R = NH_2, R′ = H (18:3-Gln) **2**
R = OH, R′ = H (18:3-Glu) **3**



R = NH_2, R′ = OH **4**
R = NH_2, R′ = H (18:2-Gln) **5**
R = OH, R′ = H (18:2-Glu) **6**



(18:1-Gln) **7**



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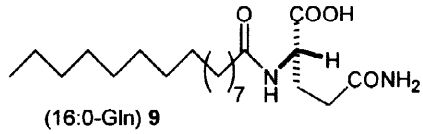
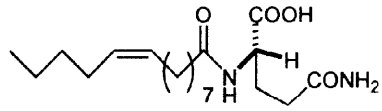
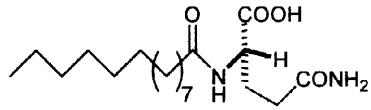


(16:0-Gln) **9**



(14:1-Gln) **10**



(14:0-Gln) **11**

Volicitin (**1**) has been reported to induce in corn seedlings (*Zea mays* L., var. Ioana) the biosynthesis of a blend of volatile terpenoids and indole, attracting females of the parasitic wasp *Cotesia marginiventris*, a na-

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tural enemy of beet armyworm larvae.[2] More recent investigations of the oral secretion of beet armyworms revealed the additional presence of the structurally related conjugates **2**, **4**, and **5**.[6] All hitherto identified metabolites comprise an nonpolar lipid moiety joined to an amino acid by an amide linkage, a structural principle that is also characteristic for the volatile inducing microbial metabolite coronatine.[7] Despite of the original report on volicitin (**1**) as an elicitor of volatile biosynthesis there exists no further information on the occurrence of this and other compounds in the oral secretions of closely and distantly related *Spodoptera* sp. (Noctuidae) or even beyond this genus of herbivorous insects. Here we report a detailed analysis of medium polar metabolites within the regurgitant from seven different caterpillar species. We selected four species from the family of Noctuidae and three from the family of Geometridae, raised on both, artificial and natural diet. Three of the four Noctuids were of the genus *Spodoptera* and are known as widespread agricultural pests on corn, alfalfa, sugar beet or cotton. *Heliothis virescens*, the tobacco budworm occurs throughout the western hemisphere on its main host plants cotton, maize, tomato and beans. In a standard assay,[7] allowing the caterpillars to feed on lima bean (*Phaseolus lunatus*), all of the above species induced a *de novo* biosynthesis of volatiles. Two of the Geometrids studied here (*Epirrita autumnata* and *Operophtera* sp.) represent serious forest- and orchard pests in northern Europe, periodically causing large scale damage to Scandinavian birch forests.[8]

Using an APCI LC/MS method, targeting medium polar to non-polar metabolites, we analysed freshly harvested caterpillar regurgitant without preceding sample preparation. Here we report that the occurrence of amino acid conjugates of fatty acids is not restricted to *Spodoptera* sp. or the family Noctuidae but is a common feature of all of the investigated caterpillar species.

The fatty acid amides **1**→**11** were identified by a characteristic $[M+H]^+$ species or by their dominant $[M-H_2O+H]^+$ peak, resulting from the loss of water in the hydroxy fatty acid moiety of **1** and **4**. The fragmentation pattern (APCI, vaporiser temperature = 560 °C) of all conjugates exhibited characteristic b fragments resulting from cleavage of the amide bond, namely *M*-145 in case of the glutamine conjugates **2**, **5**, and **7**→**11** and *M*-146 in case of the glutamic acid conjugates **3** and **6**. The b-18 fragments (*M*-163) were dominant in the spectra of **1** and **4**. In all spectra the y'' fragment ions 147 (glutamine) or 148 (glutamic acid) could be used as diagnostic fragments for the detection of conjugates. The identity of conjugates **1**-**3** and **5**-**11** was verified by comparison of retention time and fragmentation patterns with that of synthetic references, generated from the corresponding free fatty acids and unprotected amino acids following a previously published protocol.[9,10]

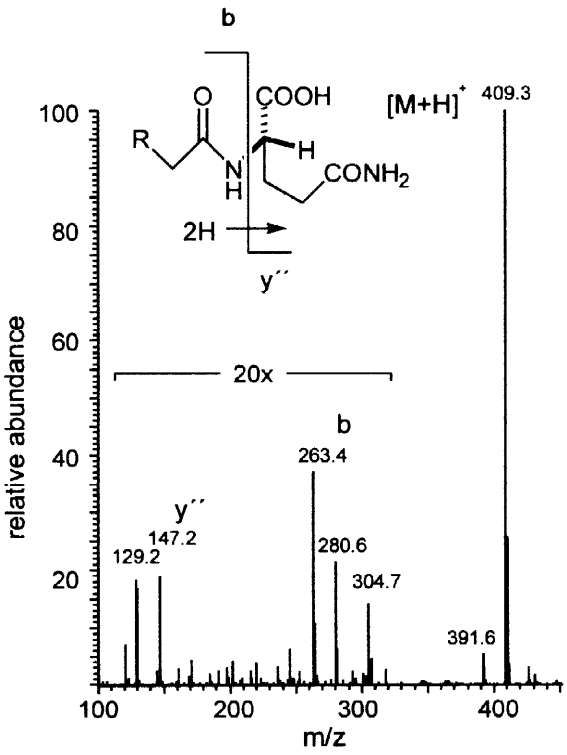

Figure 1. APCI mass spectrum and fragmentation of *N*-linolyl glutamine (**5**)

In contrast to literature[5,6] *S. exigua* reared according to our protocol (see Experimental) did produce only trace quantities of volicitin (**1**) besides larger amounts of **2** and **5** (Figure 2a). The glutamine conjugates of non functionalised C_{14}- C_{16}- and C_{18} fatty acids **2**, **5**, and **7**→**11** also dominate the regurgitant of most of the other investigated species. Only *S. frugiperda* and *S. littoralis* were found to produce significant amounts of hydroxylinolenic and hydroxylinoleic acid derived conjugates (Figure 2b).

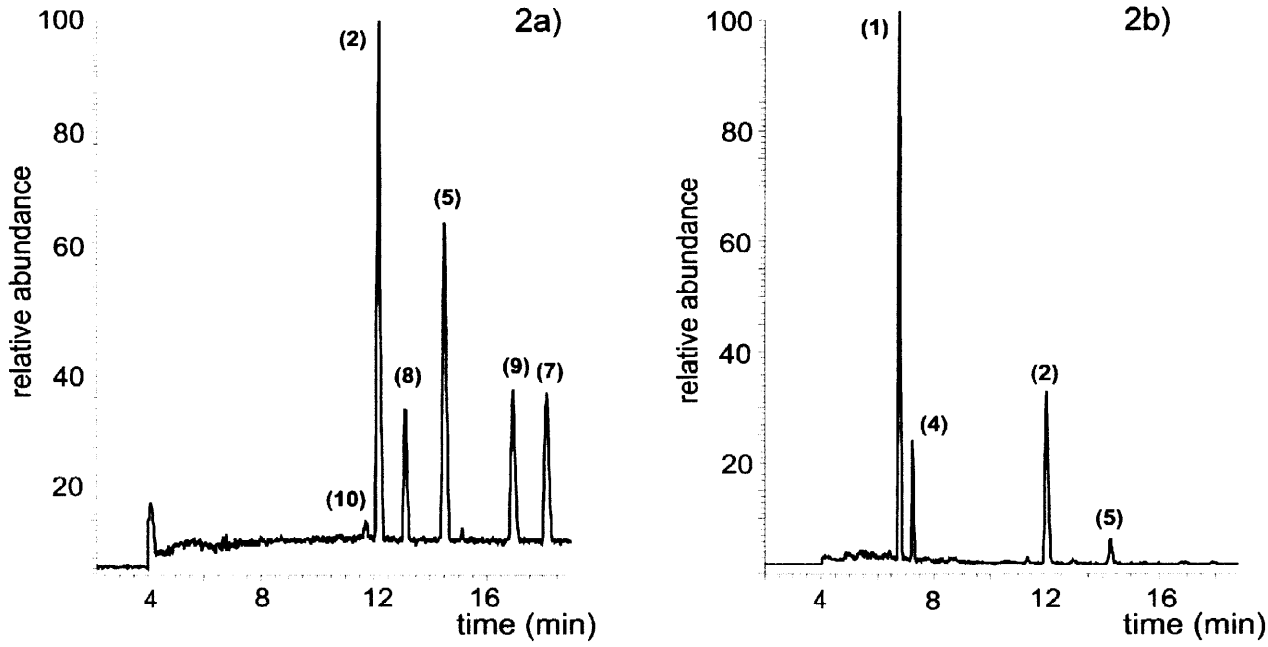

Figure 2. HPLC/MS-TIC profile (APCI) of 10 µl freshly collected oral secretion from *S. exigua* (**2a**) and *S. littoralis* (**2b**). HPLC gradient (C18): CH_3CN/H_2O; 0.5% HOAc; 1 ml/min: 40%, 7 min 68%, 18 min 80%, 28 min 100% CH_3CN, Vaporiser 560 °C.

Dependent on the different larval stages and on the environmental conditions in which the larvae were raised, substantial variation in the absolute amount of the different conjugates **1**→**11** was observed ranging from 25 to 160 ng/µl regurgitant. But, remarkably, the relative amounts of the different conjugates were species specific and did not vary between instars or diets (Table).

	volici-tin (**1**)	18:3-Gln (**2**)	18:3-Glu (**3**)	(**4**)[6]	18:2-Gln (**5**)	18:2-Glu (**6**)	18:1-Gln (**7**)	16:1-Gln (**8**)	16:0-Gln (**9**)	14:1-Gln (**10**)	14:0-Gln (**11**)
Spodoptera exigua	o	++	-	o	++	-	+	+	+	o	o
S. frugiperda	++	+	-	+	+	-	-	+	-	-	-
S. littoralis	++	++	-	+	+	o	+	o	o	-	-
Heliothis virescens	+	++	-	+	++	-	+	+	+	o	o
Epirrita autumnata	-	++	+	-	++	-	+	o	+	o	-
***Operophtera* sp.**	o	++	-	-	++	-	+	-	+	-	+
Chloroclysta truncata	-	++	-	-	+	-	+	+	+	-	-

Table: Fatty acid amides in caterpillar regurgitants: ++ major component (>30% of total conjugates); + minor component (>5%); o trace component (<5%); - not detected

These data clearly demonstrate that the new class of fatty acid glutamine and glutamic acid conjugates is ubiquitous in oral secretions of all of the investigated caterpillar larvae. As shown previously the conjugation of the amino acid and fatty acid of **1** occurs in the larva, but the fatty acid moiety of **1** is sequestered from the

diet. Here we present data demonstrating that both glutamine and glutamic acid conjugates of virtually all free fatty acids, generally detected in total lipid extracts of insects,[11] can be found in the regurgitant of larvae. Apparently conjugate formation in caterpillars does not require for specific fatty acids from the diet but reflects the pattern of fatty acids commonly found in insects. No information is available to indicate, whether this structurally diverse group of conjugates is assembled by specific enzymes, selectively forming the amide bond for each individual conjugate, or from few enzymes with broad substrate tolerance for all fatty acids present. Further work has to be carried out on the physiological function, the biosynthesis and the ecological relevance of this new class of natural products.

Experimental

General: Reactions were performed under Ar; solvents were dried according to standard methods. MS-grade solvents were purchased from Riedel de Haen (Seelze, Germany). LC-MS (APCI): Hewlett-Packard HP1100, equipped with a Merck LiChrospher 100 RP-18 column (5μm, 250x4mm) for LC separation, Finnigan LCQ with Finnigan LC/MS APCI interface. MPLC: Büchi B668 pump, Merck Lichroprep RP18 (40-63μm) 36x230mm. IR: Bruker Equinox 55 FTIR Spectrophotometer. ^{1}H- and ^{13}C NMR: Avance DRX 500 spectrometer; CD_3OD as solvent. Chemical shifts of ^{1}H- and ^{13}C NMR are given in ppm (δ) downfield relative to TMS. HR-MS: Micromass MasSpec (Micromass, Manchester, UK).

Caterpillar Rearing

Eggs of *Heliothis virescens*, *Spodoptera exigua* and *S. frugiperda* were obtained from Dr. A. Elber, Bayer AG (Agricultural Centre, D-51368 Leverkusen). Eggs of *S. littoralis* were provided by Prof. Dr. P. Proksch, University of Würzburg. First and second instar larvae of *Epirrita autumnata* were obtained from laboratory hatched eggs March to June 1999, and were reared on birch foliage. All larvae were reared at 23-25°C under a 16 h light/ 8h dark photoperiod. *H. virescens*, *S. exigua*, *S. frugiperda* and *S. littoralis* were reared on an artificial diet prepared from 150 g ground white beans, soaked overnight in 400 ml water, 20 g baker's yeast, 3 g methyl-paraben, 3 g ascorbic acid, 1 ml formalin and 180 mg gentamycin sulfate. These ingredients were homogenised and added at 40°C to 10 g agar boiled in 315 ml water. Larvae of *Epirrita* and *Operophtera* were fed on freshly collected birch (*Betula pendula*) leaves.

Collection of Oral Secretion and Sample Preparation

Regurgitant of third to fifth instar larvae was collected as described[1] and 5 μl crude oral secretions were dissolved in 15 μl MeOH; each sample was centrifuged at 16.000 g for 3 min and the supernatant was directly analysed by APCI LC/MS.

Synthesis of the Conjugates 2, 3, and 5-11

Amino acid conjugates were prepared from fatty acids (SIGMA, Deisenhofen) and L-amino acids as described.[9,10] After evaporation of the solvent the crude product was purified by MPLC reversed phase chromatography. Yields ranged from 35% to 86%. The spectroscopic data for volicitin (**1**) and for **2** have been published elsewhere.[9,10]

N-Linolenoyl-L-glutamic acid (18:3-Gln) (3)

^{1}H NMR (CD_3OD, 500 MHz) δ: 0.8 (t, *J*=7.6, 3H); 1.19-1.31 (m, 10H); 1.52 (t, *J*=6.2, 2H); 1.80-1.9 (m, 1H); 1.92-2.0 (m, 4H); 2.02-2.09 (m, 1H); 2.15 (t, *J*=7.57, 1H); 2.27 (t, *J*=6.94, 1H); 2.72 (m, 4H); 4.28 (m, 1H); 5.17-5.32 (m, 6H). ^{13}C NMR (CD_3OD, 125 MHz) δ: 14.74; 21.56; 26.5; 26.61; 27.02; 28.28; 30.35;

30.40; 30.46; 30.76; 30.82; 31.41; 37.13; 54.36; 128.34; 128.9; 129.32; 129.31; 131.21; 132.82; 176.08; 176.17; 177.21. IR (KBr): 3311, 3010, 2927, 2855, 1734, 1647, 1545, 1458, 1413, 1217, 724 cm^{-1}. MS (70 eV): 407($M^{+\bullet}$, 17), 389(12), 364(3), 260(17), 232(8), 189(17), 148(94), 130(100), 108(52), 102(81), 95(49), 84(63), 79(71), 67(63). HR-MS: *m/z* calcd. for $C_{23}H_{37}NO_5$: 407.2672, found: 407.2669.

N-Linolyl-L-glutamine (18:2-Gln) (5)

^1H NMR (CD_3OD, 500 MHz) δ: 0.81 (t, *J*=6.9, 3H); 1.18-1.31 (m, 16H); 1.53 (t, *J*=6.9, 2H); 1.81-1.89 (m, 1H); 1.93-1.99 (m, 4H); 2.02-2.09 (m, 1H); 2.12-2.22 (m, 2H); 2.68 (t, *J*=6.92, 2H); 4.25 (dd, *J*=5.04, *J*=8.19, 1H); 5.19-5.3 (m, 4H). ^{13}C NMR (CD_3OD, 125 MHz) δ: 14.7; 23.9; 26.82; 27.19; 28.43; 28.46; 29.68; 30.55; 30.63; 30.75; 30.96; 31.04; 32.94; 33.27; 37.37; 54.19; 129.35; 129.36; 131.19; 131.22; 176.29; 176.8; 178.42. IR (KBr): 3421, 3010, 2926, 2855, 1718, 1653, 1560, 1538, 1458, 1400, 1273, 1071 cm^{-1}. MS (70 eV): 408($M^{+\bullet}$, 24), 390(40), 262(74), 234(13), 178(11), 150(21), 129(87), 121(20), 95(44), 81(67), 67(91), 55(100). HR-MS: *m/z* calcd. for $C_{23}H_{40}N_2O_4$: 408.2988, found: 408.2980.

N-Linolyl-L-glutamate (18:2-Glu) (6)

^1H NMR (CD_3OD, 500 MHz) δ: 0.81 (t, *J*=7.4, 3H); 1.16-1.34 (m, 16H); 1.52 (t, *J*=6.4, 2H); 1.81-1.87 (m, 1H); 1.93-1.97 (m, 4H); 2.01-2.1 (m, 1H); 2.14 (t, *J*=7.57, 1H); 2.28 (t, *J*=6.94, 1H); 2.69 (t, *J*=6.9, 2H); 4.29 (m, 1H); 5.2-5.31 (m, 4H). ^{13}C NMR (CD_3OD, 125 MHz) δ: 14.73; 23.92; 26.83; 27.21; 28.45; 28.47; 30.56; 30.58; 30.65; 30.76; 31.03; 31.75; 31.76; 32.95; 37.19; 54.03; 129.35; 129.37; 131.2; 131.23; 175.73; 176.56; 176.87. IR (KBr): 3311, 3010, 2927, 2855, 1734, 1718, 1647, 1545, 1458, 1413, 1217, 724 cm^{-1}. MS (70 eV): 409($M^{+\bullet}$, 17), 391(12), 365(8), 280(6), 262(57), 234(8), 189(11), 164(9), 148(73), 130(100), 102(80), 95(28), 84(62), 67(67), 55(58). HR-MS: *m/z* calcd. for $C_{23}H_{39}NO_5$: 409.2828, found: 409.2825.

N-Oleyl-L-glutamine (18:1-Gln) (7)

^1H NMR (CD_3OD, 500 MHz) δ: 0.89 (t, *J*=7.56, 3H); 1.22-1.36 (m, 22H); 1.62 (t, *J*=6.9, 2H); 1.84-1.92 (m, 1H); 1.98-2.03 (m, 4H); 2.11-2.19 (m, 1H); 2.22-2.34 (m, 2H); 4.37 (dd, *J*=4.42, *J*=8.42, 1H); 5.3-5.35 (m, 2H). ^{13}C NMR (CD_3OD, 125 MHz) δ: 14.48; 23.77; 26.93; 28.17; 28.2; 28.67; 30.29; 30.34; 30.38; 30.41; 30.48; 30.64; 30.65; 30.89; 32.84; 33.1; 36.91; 53.41; 130.86; 130.9; 176.45; 177.78; 179.13. IR (KBr): 3433, 3377, 3319, 3008, 2922, 2851, 1728, 1695, 1670, 1647, 1629, 1535, 1516, 1466, 1419, 1358, 1311, 1211, 929, 722 cm^{-1}. MS (70 eV): 410($M^{+\bullet}$, 4), 392(64), 264(33), 221(7), 170(33), 129(100), 98(40), 84(60), 67(41), 55(75). HR-MS: *m/z* calcd. for $C_{23}H_{42}N_2O_4$: 410.3145, found: 410.3146.

N-Palmitoleyl-L-glutamine (16:1-Gln) (8)

^1H NMR (CD_3OD, 500 MHz) δ: 0.9 (t, *J*=6.94, 3H); 1.28-1.39 (m, 20H); 1.62 (t, *J*=6.8, 2H); 1.90-1.98 (m, 1H); 2.0-2.06 (m, 4H); 2.13-2.20 (m, 1H); 2.23-2.34 (m, 2H); 4.39 (dd, *J*=5.05, *J*=8.19, 1H); 5.31-5.39 (m, 2H). ^{13}C NMR (CD_3OD, 125 MHz) δ: 14.51; 23.81; 26.19; 26.98; 28.19; 28.67; 30.14; 30.26; 30.32; 30.39; 30.46; 32.88; 33.02; 33.03; 36.96; 53.39; 130.9; 130.96; 175.13; 176.52; 177.78. IR (KBr): 3414, 3370, 2925, 2851, 1728, 1706, 1646, 1591, 1577, 1540, 1458, 1419, 1215, 1185 cm^{-1}. MS (70 eV): 382($M^{+\bullet}$, 1), 364(59), 236(34), 193(6), 183(13), 170(28), 129(100), 112(17), 98(46), 84(67), 69(45), 55(82). HR-MS: *m/z* calcd. for $C_{21}H_{38}N_2O_4$: 382.2832, found: 382.2834.

N-Palmitoyl-L-glutamine (16:0-Gln) (9)

^1H NMR (CD_3OD, 500 MHz) δ: 0.9 (t, *J*=6.94, 3H); 1.27-1.34 (m, 28H); 1.6 (t, *J*=6.94, 2H); 1.88-1.96 (m, 1H); 2.12-2.23 (m, 1H); 2.23-2.36 (m, 2H); 4.37-4.41 (m, 1H). ^{13}C NMR (CD_3OD, 125 MHz) δ: 14.42; 20.73; 23.73; 26.1; 26.89; 28.66; 30.24; 30.31; 30.42; 30.46; 30.6; 30.64; 30.77; 32.70; 32.77; 33.07; 34.95; 53.24; 175.18; 176.43; 177.71. IR (KBr): 3364, 3313, 2957, 2918, 1734, 1686, 1670, 1539, 1465, 1275,

1207, 1124, 1070 cm^{-1}. MS (70 eV): 366($M^{+\bullet}$-18, 16), 279(6), 256(8), 200(8), 183(15), 170(100), 155(18), 128(50), 116(14), 100(19), 84(87), 57(57). HR-MS: *m/z* calcd. for $C_{21}H_{38}N_2O_3$ (M-18): 366.2882, found: 366.2881.

N-Myristoleyl-L-glutamine (14:1-Gln) (10)

^1H NMR (CD_3OD, 500 MHz) δ: 0.91 (t, *J*=6.93, 3H); 1.30-1.39 (m, 14H); 1.62 (t, *J*=6.92, 2H); 1.91-1.99 (m, 1H); 2.01-2.06 (m, 4H); 2.12-2.2 (m, 1H); 2.24 (t, *J*=7.48, 1H); 2.29-2.34 (m, 1H); 4.39 (dd, *J*=4.87, *J*=8.79, 1H); 5.31-5.38 (m, 2H). ^{13}C NMR (CD_3OD, 125 MHz) δ: 14.43; 23.44; 26.97; 27.96; 28.22; 28.68; 30.32; 30.38; 30.45; 30.92; 32.88; 33.23; 36.95; 53.42; 130.89; 130.91; 175.18; 176.5; 177.84. IR (KBr): 3367, 3325, 3009, 2924, 2851, 1724, 1689, 1641, 1539, 1465, 1456, 1417, 1327, 1212, 1099, 943 cm^{-1}. MS (70 eV): 354($M^{+\bullet}$, 1), 336(51), 208(29), 170(30), 129(100), 98(46), 84(58), 67(40), 55(85) HR-MS: *m/z* calcd. for $C_{19}H_{34}N_2O_4$: 354.2519, found: 354.2517.

N-Myristoyl-L-glutamine (14:0-Gln) (11)

^1H NMR (CD_3OD, 500 MHz) δ: 0.9 (t, *J*=6.98, 3H); 1.27-1.36 (m, 22H); 1.62 (t, *J*=7.0, 2H); 1.90-1.99 (m, 1H); 2.1-2.19 (m, 1H); 2.22-2.33 (m, 2H); 4.33 (dd, *J*=5.03, *J*=8.28, 1H). ^{13}C NMR (CD_3OD, 125 MHz) δ: 14.51; 23.81; 26.29; 27.01; 29.61; 30.36; 30.45; 30.56; 30.57; 30.73; 30.84; 30.86; 33.1; 33.16; 37.21; 54.59; 176.1; 176.73; 178.31. IR (KBr): 3421, 2956, 2918, 2850, 1719, 1686, 1670, 1655 1571, 1419, 1260, 1100, 953 cm^{-1}. MS (70 eV): 354($M^{+\bullet}$, 1), 336(51), 208(29), 170(30), 129(100), 98(46), 84(58), 67(40), 55(85) HR-MS: *m/z* calcd. for $C_{19}H_{34}N_2O_3$ ($M^{+\bullet}$-H_2O): 338.2569, found: 338.2573.

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References

1. Turlings, T.C.J., McCall, H., Alborn, T., Tumlinson, J.H. *J. Chem. Ecol.* **1993**, *19*, 411.
2. Stowe, M.K., Turlings, T.C.J., Loughrin, J.H., Lewis, W.J., Tumlinson, J.H. *Proc. Natl. Acad. Sci. USA*, **1995**, *92*, 23.
3. Parè, P.W., Tumlinson, J.H. *Nature* **1997**, *385*, 30.
4. Mattiacci, M.L., Dicke, M., Posthumus, M.A. *Proc. Natl. Acad. Sci. USA*, **1995**, *92*, 2036.
5. Alborn, H.T., Turlings, T.C.J., Jones, T.H., Stenhagen, G., Loughrin, J.H., Tumlinson J.H. *Science*, **1997**, *276*, 945.
6. Paré, P.W., Alborn, H.R. Tumlinson, J.H. *Proc. Natl. Acad. Sci. USA*, **1998**, *95*, 13971.
7. Boland, W., Hopke, J., Donath, J., Nüske, J., Bublitz, F. *Angew. Chem. Int. Ed. Engl.* **1995**, *34*, 1600.
8. Tenow, O. *Zool. Bidr. Uppsala* **1972**, *2*, 1.
9. Koch, T., Krumm, T., Jung, V., Engelberth, J., Boland, W. *Plant. Phys.* **1999**, *in press.*
10. Pohnert, G., Koch, T., Boland, W. *Chem. Commun.* **1999**, 1087.
11. Kerkut, G.A., Gilbert, L.I. (ed.) Comprehensive Insect Physiology, Biochemistry and Pharmacology, Pergamon Press (1985).