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N-(15,16-Dihydroxylinoleoyl)-glutamine and *N*-(15,16-epoxylinoleoyl)-glutamine isolated from oral secretions of lepidopteran larvae

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Abstract—*N*-(15,16-Dihydroxylinoleoyl)-glutamine (**1**) and *N*-(15,16-epoxylinoleoyl)-glutamine (**2**) and were identified in the regurgitant of lepidopteran larvae (*Spodoptera exigua* and *Spodoptera frugiperda*) by LC–MS. After methanolysis and derivatisation with MSTFA, the positions of the hydroxy groups of **1** were identified by GC–MS. The structures of both conjugates were confirmed by synthesis.

1. Introduction

N-Acylamino acid conjugates, such as *N*-acylornithines,[1,2] *N*-acylserines[3] and *N*-acylisoleucines[4] are well known bacterial metabolites. Some of them have pharmacological and industrial importance as antibiotics and surfactants.[5–7] The compounds are produced by many different microorganisms, among them *Streptomycetes*, *Flavobacteria* and *Pseudomonas* species.[1,5,6] In 1997, Alborn et al. isolated the *N*-acylglutamine 'volicitin', (17*S*)-(17-hydroxylinolenoyl)-L-glutamine, from oral secretions of the larvae of the beet armyworm (*Spodoptera exigua*).[8] After introduction of the compound into the damaged leaf by the feeding larvae, volicitin may act as an elicitor of plant volatile biosynthesis. The compound is not generally active, but triggers volatile biosynthesis in corn (*Zea mays*)[8,9] or sweet potato (*Ipomoea batatas*).[10] Besides volicitin, several other *N*-acyl-L-glutamines were found in the regurgitates of caterpillars (*Noctuidae*, *Geometridae*),[11,12] containing fatty acids varying in chain length (C_{14}–C_{18}) and in the number of double bonds. Previous work on the biosynthesis of these conjugates has provided the first evidence that the amide bond between the plant derived[11] fatty acid and L-glutamine is made by microorganisms living in the fore- and midgut of the insect larvae.[13] Moreover, the introduction of the (17*S*)-hydroxy group[14] into the linolenic acid moiety of volicitin represents another transformation which is typical for bacterial metabolism. Well known are the ω, ω-1, or ω-2 hydroxylations[15] which are often achieved by enzymes from the family of cytochrome P450 enzymes.[16] Since these biocatalysts not only introduce hydroxy groups, but also convert double bonds into epoxides,[17,18] we screened the regurgitant from lepidoteran larvae (*S. exigua* and *Spodoptera frugiperda*) for additional oxygen-containing fatty acid metabolites, in particular epoxy- and dihydroxy fatty acids.

2. Results and discussion

Compounds from freshly collected regurgitant from larvae of *S. exigua* or *S. frugiperda* were separated by HPLC on reverse phase under programmed conditions and analysed by mass spectrometry using atmospheric pressure chemical ionization (APCI). Two peaks in the midpolar region of the chromatographic run displayed quasimolecular ions as expected for an epoxide or a dihydroxy derivative of *N*-(linolenoyl)-glutamine. First eluted the polar *N*-(dihydroxylinoleoyl)-glutamine **1** ($[M+H]^+$=441; t_{ret}=21.1 min; ca. 5% with respect to the major constituent volicitin followed by the less polar *N*-(epoxylinoleoyl)-glutamine **2** $[M+H]^+$=423, t_{ret}=24.7 min; ca. 3% with respect to volicitin).

The APCI mass spectrum of the dihydroxy conjugate **1** (Fig. 1(a)) showed a strong quasimolecular ion at *m*/*z*=441 followed by the loss of two molecules of water (*m*/*z*=441→423→405). The fragment at *m*/*z*=147 is characteristic for the amino acid fragment (Gln) and results from fragmentation across the protonated amide bond. The epoxide **2** displayed a similar spectrum with a quasimolecular ion at *m*/*z*=423 and fragments at *m*/*z*=405 and 388. For further analysis, the fraction of the dihydroxy

Keywords: *N*-(15,16-epoxylinoleoyl)-glutamine; *N*-(15,16-dihydroxylinoleoyl)-glutamine; 15,16-epoxylinoleate; 15,16-dihydroxylinoleate; *Spodoptera exigua*.

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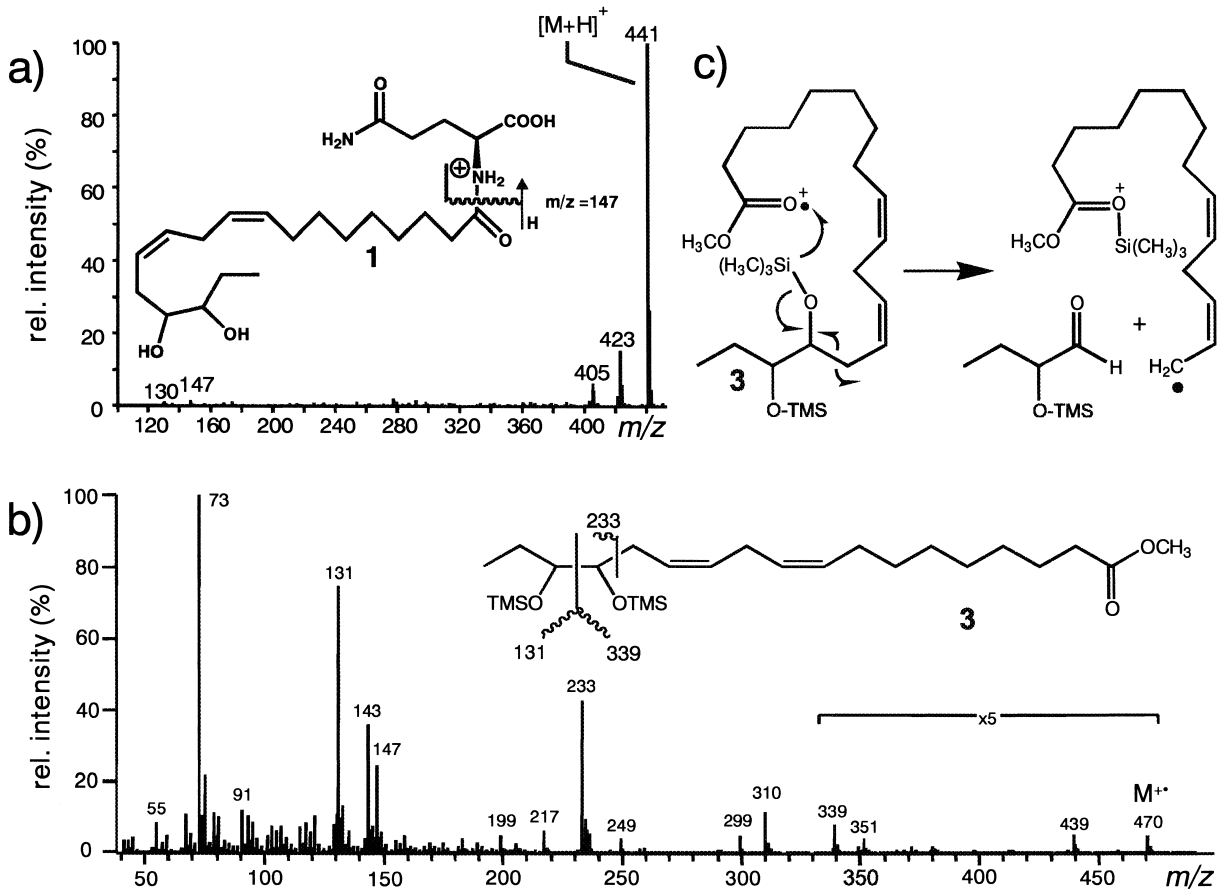
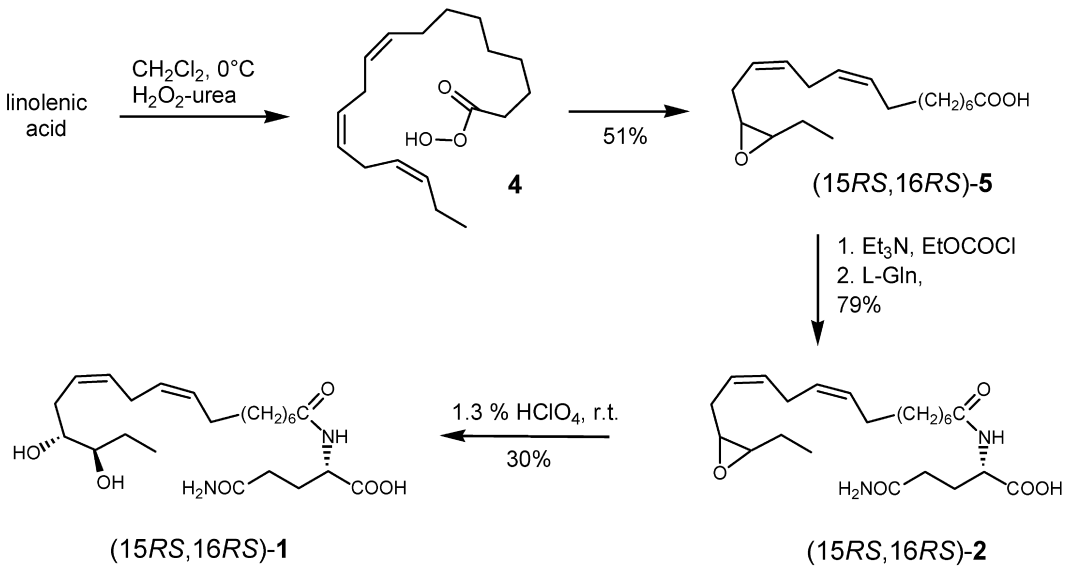


Figure 1. (a) APCI-MS of *N*-(15,16-dihydroxylinoleoyl)-L-glutamine (**1**), (b) EI-MS of 15,16-bis-trimethylsilanyloxylinoleic acid methyl ester (**3**) and (c) *O*-TMS mediated fragmentation of **3**. Reference compounds display identical mass spectra.

conjugate **1** was collected, methanolysed with Ac_2O/MeOH at 80°C for 0.5 h,[14] derivatised with MSTFA at 40°C for 1 h and investigated by GC–MS. The EI-MS of the bistrimethylsilylated ester **3** showed a molecular ion at *m*/*z*=470, and a fragment at *m*/*z*=439, indicating a loss of OMe. The position of the dihydroxy moiety was proven by the cleavage between the two vicinal *O*-TMS groups leading to the fragments at *m*/*z*=131 and *m*/*z*=339 along with the dominant allylic cleavage yielding *m*/*z*=233 characteristic for the EtCH(OTMS)CH(OTMS) fragment (Fig. 1(b)). The ion at *m*/*z*=310 (Fig. 1(c)) results from transfer of the trimethylsiloxy group to the carboxyl group followed by cleavage of the C(15)–C(16) bond resulting in loss of 2-trimethylsilyloxybutanal in analogy to well known



Scheme 1. Synthesis of (15*RS*,16*RS*)-oxygenated conjugates of octa-9,12-dienoic acid and glutamine.

degradation reactions of other trimethylsilyloxy fatty acids.[19]

The identity of both conjugates **1** and **2** was additionally proven by synthesis (Scheme 1). Linolenic acid was converted into peroxylinolenic acid **4** following a protocol of Corey et al.[20] using linolenoylimidazole in combination with urea–hydrogen peroxide[21] instead of anhydrous H_2O_2 (Scheme 1).

At low temperature (0°C), intramolecular epoxidation occurred predominantly at the terminal double bond (ca. 75%) yielding 15,16-epoxylinoleic acid **5** along with minor amounts of the 9,10- and 12,13-isomers (ca. 25%).[22]

Formation of the glutamine conjugate **2** was achieved via the mixed anhydride protocol using chloroformate and L-glutamine. Hydrolysis of **2** was accomplished with aq. $HClO_4$ (1.3%) and provided the dihydroxy conjugate **1** in moderate yield (30%). Both synthetic compounds proved to be identical with the natural products. However, the absolute configurations of the epoxide- or the diol moiety of the conjugates **1** and **2** were left open owing to the very low concentrations of the natural products.

Oxygenated octadecanoids generally display a wide range of biological activities. Free 15,16-epoxyoleic acid and all three positional isomers of epoxylinoleic acid were first isolated by Kato et al. from rice plants.[23,24] The epoxy acids induce resistance against the rice blast fungus (*Magnaporthe grisea*) by preventing spore germination (ED50 20–30 ppm). The structurally related vernolic acid (12,13-epoxyoleic acid, =leukotoxin B) exhibits bactericidal and antifungal properties.[25] Other epoxy acids modulate cellular signalling by affecting hormone levels,[26] the action of G proteins[27] and ion channels.[28] Dihydroxy fatty acids, derived from vernolic and isovernolic acid by action of an epoxide hydrolase, are associated with the acute respiratory distress syndrome.[29] The biological potential of the novel glutamine conjugates **1** and **2** is, as yet, unexplored and remains to be established with appropriate plant and animal assay systems.

First results on the mode of action of *N*-acyl glutamines on plants and the up-regulation of well-defined defense responses will be reported in due course.

3. Experimental

3.1. General

Reactions were performed under Ar; solvents were dried according to standard methods. LC–MS (APCI): Hewlett–Packard HP 1100, equipped with a Grom-Sil ODS-3 CP, 120 mm×2 mm (Fa. Grom, D-71083 Herrenberg, Germany) for LC separation, and attached to a Finnigan LCQ with Finnigan LC/MS APCI interface (CA 95134 San Jose, USA). Vaporizer temperature: 450°C. GC–MS: Trace MS, 2000 Series (Thermoquest, D-63329 Egelsbach, Germany) equipped with an Alltech EC5 (15 m×0.25 mm, 0.25 μm). Mass spectra were measured in electron impact (EI) mode at 70 eV. High resolution EI mass spectra were recorded with a Micromass MasSpec 2, for high resolution ESI mass spectra a Micromass Quattro II was used (Micromass, Manchester, UK). IR: Bruker Equinox 55 FTIR spectrophotometer. ^1H and ^{13}C NMR: Bruker Avance 400 spectrometer (Bruker, D-76287 Rheinstetten/Karlsruhe, Germany). Chemical shifts of ^1H and ^{13}C NMR are given in ppm (δ) based on solvent peaks: $DCCl_3$ 7.26 ppm (^1H NMR) and 77.70 ppm (^{13}C NMR); CD_3OD 3.31 ppm (^1H NMR) and 49.00 ppm (^{13}C NMR).

3.1.1. Isolation and degradation of *N*-(15,16-dihydroxylinoleoyl)-glutamine (1) from lepidopteran larvae. Compounds from freshly collected regurgitant[12] of *S. exigua* larvae or *S. frugiperda* (ca. 0.5 ml) were separated by HPLC on reverse phase (Grom-Sil ODS-3 CP, 120 mm×2 mm) under programmed conditions from 100% A (3 min) to 100% B (27 min). Solvent A: H_2O, 0.1% AcOH. Solvent B: CH_3CN, 0.1% AcOH. Flow: 0.2 ml min^{-1}. Fractions containing *N*-(dihydroxylinoleoyl)-glutamine (**1**) were combined and evaporated to dryness. The crude residue was dissolved in methanol (2.5 ml), acetic acid anhydride (0.25 ml) was added and then heated to 80°C for 30 min. After removal of solvent, the diol was silylated for 1 h with MSTFA at 40°C. The bis-*O*TMS ether **3** (Fig. 1(b) or (c)) was directly analysed by GC–MS. MS (EI 70 eV) *m/z* (%): 59 (8), 73 (100), 75 (32), 131 (54), 143 (40), 147 (22), 217 (5), 233 (35), 299 (2), 310 (10), 339 (4), 380 (0.5), 439 (0.5), 470 (0.5).

3.1.2. *rac*-14-(3-Ethyl-oxiranyl)-tetradeca-9,12-dienoic acid (5); [=(15*RS*,16*RS*)-15,16-epoxylinoleic acid].[20,21] Linolenic acid (0.278 g) and 1,1′-carbonyldiimidazole in dry CH_2Cl_2 (10 ml) were stirred for 20 min at rt. In a second flask, the stable urea–H_2O_2 adduct (10 g, 30%) and Na_2HPO_4 (5.0 g) were suspended in CH_2Cl_2 (50 ml). After cooling (0°C) and addition of lithium imidazolide (0.2 equiv.), the activated linolenic acid was added within 2 min. Stirring was continued for 5 min, $KHSO_4$ (5.0 g), CH_2Cl_2 (50 ml), and Na_2SO_4 (5.0 g) were added upon which the solution clarified within 5 min of stirring. The reaction was allowed to stand at 0°C. The progress of the reaction was monitored by GC–MS of aliquots after esterification with diazomethane. The reaction was terminated after ca. 60% conversion (ca. 1 week) and solids were removed by filtration and washed with 50 ml CH_2Cl_2. After removal of solvents, the residue was purified by chromatography on RP 18 with MeOH/H_2O (75:25, v/v) for elution. Yield: 0.149 g (51%, ca. 75% 15,16-epoxide). ^1H NMR (400 MHz, $CDCl_3$) δ 1.06 (t, J=7.51 Hz, 3H), 1.25–1.41 (m, 8H), 1.48–1.68 (m, 4H), 2.01–2.10 (m, 2H), 2.17–2.27 (m, 1H), 2.34 (pt, J=7.47 Hz, 2H), 2.37–2.45 (m, 1H), 2.80 (pt, J=6.89 Hz, 2H), 2.90 (dt, J=4.24, 6.31 Hz, 1H), 2.96 (dt, J=4.24, 6.41 Hz, 1H), 5.26–5.55 (m, 4H).^{13}C NMR (100 MHz, $CDCl_3$) δ 11.30, 21.72, 25.39, 26.49, 26.81, 27.89, 29.69, 29.73, 29.77, 30.18, 34.62, 57.33, 59.14, 124.82, 127.97, 131.24, 131.50, 179.36. IR (NaCl, neat): 3011 m, 2971, 2931, 2856, 1711, 1455, 1260, 1240, 1085, 910, 815, 795, 730 cm^{-1}. MS (EI 70 eV) *m/z* (%) 294 ($M^{+\cdot}$, 0.5), 276 (6), 236 (10), 222 (32), 135 (12), 121 (21), 107 (40), 95 (45), 93 (73), 91 (34), 81 (67), 80 (84), 79 (100), 67 (77), 59 (18), 55 (68); exact mass 276.2090; calcd for $C_{18}H_{28}O_2$ [$M^{+\cdot}-H_2O$] 276.2089.

3.1.3. (15*RS*,16*RS*)-4-Carbamoyl-2-[14-(3-ethyloxiranyl)-tetradeca-9,12-dienoylamino]-butyric acid (2) [=*N*-(15,16-epoxylinoleoyl)-L-glutamine]. A stirred and chilled solution of 15,16-epoxylinoleic acid (100 mg) in dry tetrahydrofuran (10 ml) was treated with triethylamine (55 μl) and ethyl chloroformate (34 μl). After 2 min, L-glutamine (125 mg), dissolved in 0.3N NaOH (7 ml), was added. After 5 min, the solution was allowed to come to rt and the reaction was stopped by careful acidification with 2N HCl. The conjugate was extracted with CH_2Cl_2 (3×10 ml). After drying (Na_2SO_4) and removal of solvent, the residue was purified by medium-pressure chromatography on reversed phase using MeOH/H_2O, 75:25, v/v) for elution. Yield: 0.114 g (79%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.05 (t, J=7.55 Hz, 3H), 1.25–1.40 (m, 8H), 1.48–1.68 (m, 4H), 1.94–2.5 (m, 11H), 2.80 (pt, J=6.71 Hz, 2H), 2.90 (dt, J=4.17, 6.27 Hz, 1H), 2.96 (dt, J=6.29, 4.17 Hz, 1H), 4.39 (br, 1H), 5.26–5.56 (m, 4H), 6.21 (br, 1H), 6.65 (br, 1H), 7.24 (br, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 11.26, 21.74, 26.28, 26.54, 26.87, 27.94, 29.85, 29.93, 30.26, 32.44, 37.02, 57.34, 59.14, 124.87, 128.03, 131.23, 131.52, 174.96, 175.22, 177.43. IR (KBr, neat): 3438, 3405, 3315, 3216, 3070, 3013, 2966, 2933, 2858, 1712, 1660, 1641, 1542, 1452, 1419, 1249, 1136, 985, 815, 797 cm^{-1}. MS (EI, 70 eV) *m*/*z* (%): 422 ($M^{+\cdot}$, 12), 404 (54), 386 (7), 335 (6,) 319 (8), 294 (7), 276 (6), 170 (9), 147 (29), 129 (29), 130 (30), 101 (33), 93 (21), 84 (100), 79 (32), 67 (31), 55 (32). APCI-MS *m*/*z* (%): 423 (100), 405 (69), 388 (7), 259 (3), 147 (3); exact mass 422.2785; calcd. for $C_{23}H_{38}N_2O_5$ [$M^{+\cdot}$] 422.2781.

3.1.4. (15*RS*,16*RS*)-4-Carbamoyl-2-(15,16-dihydroxyoctadeca-9,12-dienoylamino)-butyric acid (1); [=*N*-(15,16-dihydoxylinoleoyl)-L-glutamine]. A solution of *N*-(15,16-epoxylinoleoyl)-L-glutamine (**2**) (10 mg) in tetrahydrofuran (1.5 ml) water (0.5 ml) and $HClO_4$ (25 μl) was stirred at rt for 15 min. The acid was neutralised with 2N NaOH and, after drying (Na_2SO_4), the organic layer was removed and the residue purified by HPLC on reversed phase (Grom-Sil 120 ODS-4HE, 250 mm×8 mm) under programmed conditions from 100% A (3 min) to 100% B (30 min). Solvent A: H_2O, 0.5% AcOH. Solvent B: CH_3CN, 0.5% AcOH. Flow: 2 ml min^{-1}. Yield: 3.1 mg (30%). ^{1}H NMR (400 MHz, CD_3OD) δ 0.97 (t, J=7.40 Hz, 3H), 1.29–1.41 (m, 8H), 1.42–1.60 (m, 4H), 1.63 (pt, J=6.91 Hz, 2H), 1.90–2.01 (m, 2H), 2.08 (pq, J=6.62 Hz, 2H), 2.11–2.21 (m, 2H), 2.25 (pt, J=7.40 Hz, 2H), 2.26–2.40 (m, 2H), 2.82 (pt, J=6.50 Hz, 2H), 3.31–3.36 (m, 1H), 3.42–3.47 (m, 1H), 4.36–4.41 (m, 1H), 5.29–5.54 (m, 4H). ^{13}C NMR (100 MHz, CD_3OD) δ 10.76, 26.73, 26.86, 26.98, 28.17, 28.72, 30.21, 30.27, 30.33, 30.71, 32.19, 32.82, 36.90, 53.48, 74.89, 76.18, 127.26, 128.88, 130.93, 131.11, 175.30, 176.35, 177.80. IR (KBr, neat): 3391, 2932, 2858, 1716, 1657, 1539, 1410, 1287, 1242, 1129, 1059, 980 cm^{-1}. MS (EI, 70 eV) *m*/*z*(%): 422 ([$M^{+\cdot}-H_2O$], 3.5), 404 (1), 367 (1.5), 363 (4), 334 (5), 311 (20), 294 (6), 276 (9), 252 (34), 235 (25), 223 (58), 206 (45), 199 (5), 189 (9), 164 (20), 145 (13), 135 (26), 121 (35), 107 (38), 95 (52), 91 (36), 84 (72), 79 (73), 72 (72), 67 (66), 59 (100). APCI-MS *m*/*z* (%): 441 (100), 423 (16), 405 (7), 259 (1), 147 (1); exact mass 441.2956; calcd for $C_{23}H_{41}N_2O_6$ $[M+H]^+$ 441.2965.

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