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Biosynthesis of 8-hydroxyquinoline-2-carboxylic acid, an iron chelator from the gut of the lepidopteran *Spodoptera littoralis*

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In the regurgitate (foregut content) of *Spodoptera* larvae we found high concentrations (0.5 - 5 mM) of 8-hydroxyquinoline-2-carboxylic acid (8-HQA). In a survey of different lepidopteran species, this compound was only detected in species belonging to the family of Noctuidae. 8-HQA was shown to derive from tryptophan metabolism. The amount of 8-HQA in the regurgitate was strongly dependent on the tryptophan content of the diet. In the insect 8-HQA is generated from tryptophan via kynurenine and 3-hydroxykynurenine. 8-HQA is produced by the larvae and not by their commensal gut bacteria. Analysis of different life stages of *Spodoptera* larvae revealed that 8-HQA is formed during the larval stage, probably acting as an iron chelator to control the gut microbiome.

Introduction

Quinolinic carboxylic acid derivates are widely distributed in nature. They were found in insects, bacteria and plants. Examples are shown in Figure 1.

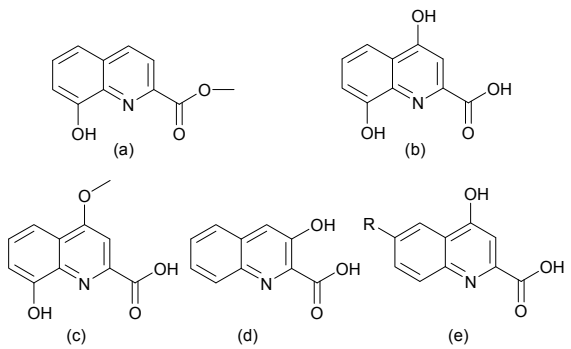
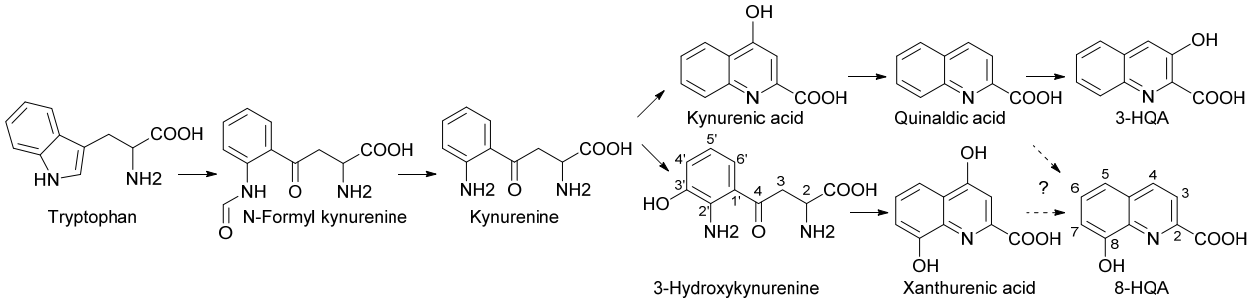


Figure 1 Quinolinic carboxylic acid derivatives: (a) methyl-8-hydroxy-quinoline-2-carboxylate (b) xanthurenic acid (c) quinolobactin (d) 3-hydroxyquinoline-2-carboxylic acid (e) kynurenic acid (R=H), 6-hydroxykynurenic acid (R=OH), 6-methoxykynurenic acid (R=OCH_3)

Methyl-8-hydroxyquinoline-2-carboxylate was isolated by H. Schildknecht from the defensive secretion of the water beetle *Ilybius fenestratus*[1]. Xanthurenic acid (4,8-dihydroxyquinoline-2-carboxylic acid) was found in Drosophila melanogaster [2, 3] and several mosquito species[4, 5]. Elucidation of its biosynthetic pathway showed that it derives from tryptophan and is formed via 3-hydroxykynurenine[5-7] (Scheme 1). As this compound has toxic effects, a conversion into xanthurenic acid has been proposed. Moreover, xanthurenic acid is known as a gametogenesis inducing factor of the malaria causing agent Plasmodium falciparum in mosquitoes[4]. Quinolinic carboxylic acids also occur abundantly in bacteria. An important quorum sensing factor is the siderophore quinolobactin (8-hydroxy-4-methoxyquinoline-2-carboxylic acid)[8] from Pseudomonas fluorescens. 3-Hydroxyquinoline-2-carboxylic acid (3-HQA) is a precursor of the antibiotic cinropeptin from Streptomyces griseoflavus[9, 10] and of thiocoralin, an antitumor compound from the marine actinomycete Micromonospora sp.[11]. Biosynthetic studies demonstrated that thiocoralin is derived from tryptophan via kynurenic acid and quinaldic acid intermediates (Scheme 1)[11]. Derivatives of 4-hydroxyquinoline-2-carboxylic acid[12-15] are also found in plants, for example 6-hydroxykynurenic acid in Gingko biloba[14]. The biological function in Gingko is unknown.

Spodoptera littoralis Boisduval (Lepidoptera: Noctuidae), widespread in Africa and Asia but also found in Southern Europe, is one of the most devastating pests of crops worldwide. Its host range covers over 40 families, containing at least 87 species of economic importance like cotton, corn, peanuts, vegetables, and soybean.[16] Extensive use of insecticides has caused the development of resistant strains.[17]

During LCMS analysis of the regurgitate (foregut content) of *S. littoralis* larvae we observed substantial amounts of free 8-hydroxyquinoline-2-carboxylic acid (8-HQA, 0.5 - 5 mM)[18, 19] To learn more about the function of 8-HQA in the insect gut, the knowledge of the producing organism, either the insect or members of the gut microbial community, is relevant since 8-HQA is produced over the whole life span of the Spodopteran larva in high concentrations.



Scheme 1 Biosynthesis of quinolinic acids according to Han[7] et al. and Lombo et al.[11]

Here we report on the evaluation of the biosynthetic pathway of 8-HQA from tryptophan in the insect gut using antibiotics and labeled precursors such as L-tryptophan-(indole-d_5) ([$^2H_5$]-Trp), 4-hydroxyquinoline-2-carboxylic-3-d acid ([^2H]-kynurenic acid), 2-amino-4-(2-amino-3-hydroxyphenyl-5-d)-4-oxobutanoic-3,3-d_2 acid ([$^2H_3$]-3-hydroxykynurenine), and 4,8-dihydroxyquinoline-2-carboxylic-3,5,6,7-d_4 ([$^2H_3$]-xanthurenic acid). Our data suggest a conversion of Trp via 3-hydroxykynurenine and ring closure to give 8-HQA. Since 8-HQA is a potent siderophore, a production by the insect may substantiate its role as a control element for the gut microbiome used by the insect host.

Results and Discussion

Detection of 8-HQA in the regurgitate of *S. littoralis* and other noctuid larvae

LCMS analysis of the regurgitate of *S. littoralis* larvae revealed an intense signal at m/z 190 $(M+H)^+$, which could be attributed to 8-HQA. Treatment of an isolated sample with CH_2N_2 afforded the methylester m/z = 203 ($M^{+\cdot}$) (Fig. S1)[16]. As this compound was present in high concentrations (up to 5 mMol)[17], we also analyzed closely related species for the presence of 8-HQA (Table S1). The regurgitate of three additional *Spodoptera* species, *S. exigua, S. frugiperda* (JS3C, a "corn" and OnaR, a "rice strain")[20], and *S. eridania*, three *Heliothis* species, *H. armigera, H. subflexa,* and *H. virescens* (JEN2 a wild type strain[21] and YHD3, a strain highly resistant against Cry1-toxins from *B. thuringiensis*[22]), and *Orthosia cerasi* were investigated.

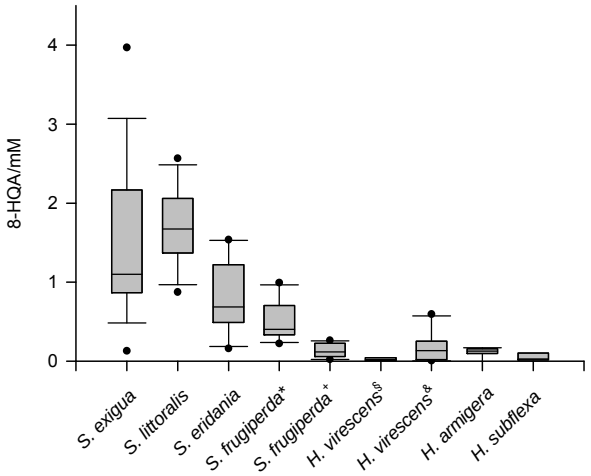


Fig. 2 Concentration of 8-HQA in the regurgitate of different lepidopteran larvae Two different populations were studied for *S. frugiperda* (*: JS3C, +: OnaR) and *H. virescens* (§: JEN2, &: YHD3) respectively. Boxplots (n = 6-15) indicate median (horizontal line in the box), 25th and 75th (box boundaries), 10th and 90th percentile (whiskers), respectively. Outliers are marked as black dots.

As non-noctuid members were included larvae of *Malacosoma* spp., *Plutella xylostella* and *Yponomeuta evonymellus* as well as *Manduca sexta* and *Aglais urticae.* 8-HQA was detected in the regurgitate of all *Spodoptera* and *Heliothis* species (Fig. 2) and in *O. cerasi*, but not in *M. sexta*, *A. urticae, Malacosoma* sp., *P. xylostella* and *Y. evonymellus*. Interestingly, *Spodoptera*, *Heliothis* and *O. cerasi* belong to the Noctuidae family, whereas the other five species belong to other families (Sphingidae, Nymphalidae, Lasiocampidae, Plutellidae and Yponomeutidae, respectively)[23].

Different compartments of *S. littoralis* were tested for the presence of 8-HQA. The compound could not be localized in the Malpighian tubules and the fat body, but it was present in low amounts in the hemolymph, ca. 3-44 ng μl^{-1} (16 - 233 µM). The concentration in the regurgitate was substantially higher, ca. 0.1-1 µg μl^{-1} (0.5 - 5 mM). Testing different gut segments of larvae of *S. littoralis* revealed that the amounts of 8-HQA was highest at the end of the foregut with a steady decline via midgut to the hindgut[17], Pupae and adults of *S. littoralis* do not contain 8-HQA, but in pupae an accumulation 3-hydroxykynurenin(ca. 0.18 µg mg^{-1}) was found instead of 8-HQA[18].

8-HQA is continuously synthesized from tryptophan and excreted

Since the majority of quinolinic compounds derives from Trp metabolism[24, 25], first [$^2H_5$]-Trp was fed to larvae of *S. littoralis*. In addition, [$^2H_5$]-Trp was injected into the hemolymph. Both application modes resulted after 24 h in a signal for 8-hydroxyquinoline-2-carboxylic-5,6,7-d_3 acid ([$^2H_3$]-8-HQA) in the regurgitate and in the frass, documented by m/z 193 with the same retention time as natural 8-HQA (m/z 190) (Fig. 3). Since the food also provided natural Trp, the proportion of labeled 8-HQA did not exceed 40%.

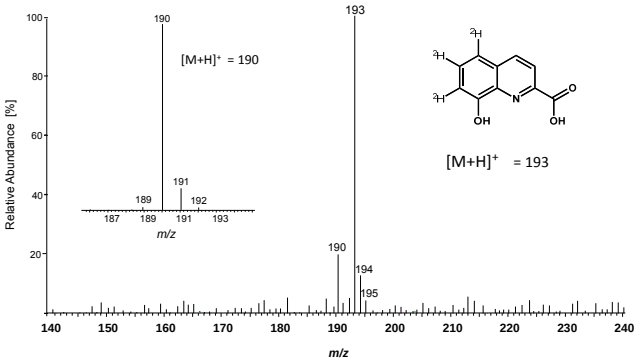


Fig. 3 MS spectra of 8-HQA and [$^2H_3$]-8-HQA

Feeding larvae with defined diets, containing Trp at 0, 1.95 or 19.5 mmol g^{-1} demonstrated that the formation of 8-HQA in the insect is strongly dependent on the amount of ingested Trp. Larvae that fed on a normal Trp diet (1.95 mmol g^{-1}) contained 1.69 ± 0.94 mM 8-HQA in the regurgitate and at 2.4 ± 1.03 nmol mg^{-1} in the excrements. Feeding on a Trp-depleted diet caused a strong decrease (0.1 ± 0.07 mM) of 8-HQA after three days, whereas ingestion of a Trp-rich diet caused an increase of 8-HQA (10.4 ± 2.2 mM) in the regurgitate. The results demonstrate that 8-HQA is produced continuously in the gut tissue from the incoming diet and is excreted with the feces. The regurgitate itself showed no biosynthetic activity. Quantitative feeding experiments with [$^2H_5$]-Trp allowed to estimate that ca. 10% of the ingested Trp are converted into 8-HQA.

8-HQA is synthesized via 3-hydroxykynurenine

The ultimate precursor of 8-HQA is tryptophan, but details of the pathway remained to be elucidated. Two known biosynthetic pathways for quinoline carboxylic acids[7, 11], could principally apply for 8-HQA (Scheme 2), but none of them is compatible with the substitution pattern of 8-HQA. To obtain first information on potential intermediates, larvae were fed on Trp-depleted diet supplemented with Trp, kynurenine, kynurenic acid, 3-hydroxykynurenine, quinaldic acid and xanthurenic acid. After two days of feeding, the concentration of 8-HQA was quantified in the regurgitate. In the presence of Trp, kynurenine and 3-hydroxykynurenine a significant increase of 8-HQA was observed compared to the Trp-depleted control. Feeding of [^{2}H]-labeled and/or unlabeled xanthurenic acid, kynurenic acid and quinaldic acid showed no labeled 8-HQA in the regurgitate or any effect on the 8-HQA concentration, respectively . However, feeding of [$^2H_3$]-3-hydroxykynurenine (70 µg, 313 nmol) led to the formation of 8-hydroxyquinoline-2-carboxylic-3,6-d_2 acid ([$^2H_2$]-8-HQA) after 3 hr (Fig. 4). ESI-HR-MS: 8-HQA, $[M+H]^+$, $C_{10}H_8O_3N$, *m/z* = 190.0498, calc. 190.0498 and [$^2H_2$]-8-HQA, $[M+H]^+$, $C_{10}H_6{}^2H_2O_3N$, *m/z* 192.0623, calc. 192.0624.

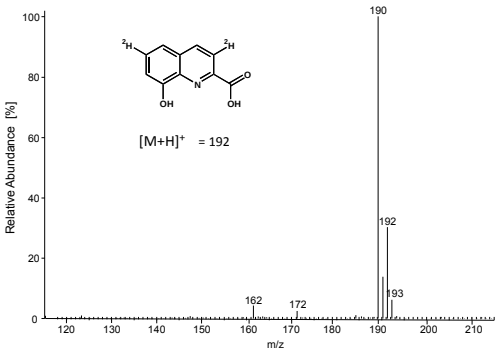
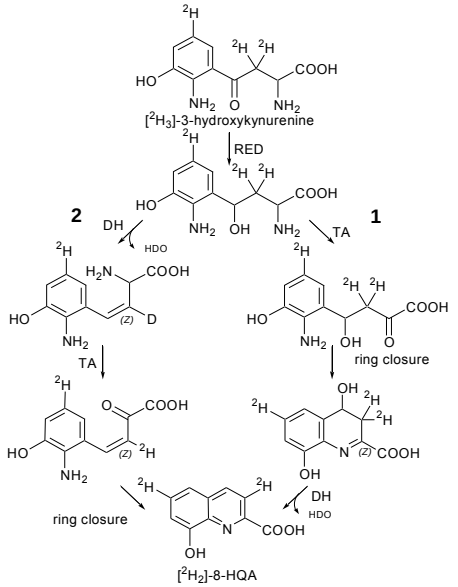


Fig. 4 MS spectrum of 8-HQA and [$^2H_2$]-8-HQA.



Scheme 2 Alternative routes to 8-HQA downstream 3-hydroxykynurenine. Spontaneous or enzymatic ring closure. (RED: reductase, DH: dehydratase, TA: transaminase)

Thus, 3-hydroxykynurenine is a precursor, whereas xanthurenic acid is not. The presence of two deuterium atoms in the product (m/z = 192) allows to reconstruct the pathway to 8-HQA as proposed in Scheme 2.

3-Hydroxykynurenine is most likely first reduced to an alcohol followed by regioselective transamination of the α-amino group. Ring closure between the resulting keto group and the aromatic amine yields a 4,8-dihydroxy-3,4-dihydroquinoline-2-carboxylic acid as a potential intermediate. Final elimination of HDO generates [$^2H_2$]-8-HQA. Although the sequence of reactions is not proven and an alternative sequence with elimination of water prior to transamination is possible, at least the occurrence of a β-diketone intermediate (transamination prior to reduction of the keto group) is unlikely as this would cause the loss of both deuterium atoms from the C_4-side chain due to extensive enolization.

Reduction of 8-HQA concentration in the larval gut after inhibition of the kynurenine 3-monooxygenase

Feeding of the larvae of *S. littoralis* with *m*-nitrobenzoylalanine (*m*NBA), a powerful inhibitor of the kynurenine 3-monooxygenase[26] (KMO converts kynurenine to 3-hydroxykynurenine), reduces the production of 8-HQA by up to 83 % after 5 days (cf. Fig. 5). Toxic side effects were not observed. Moreover, inhibition of the oxidation of kynurenine directs the metabolism of Trp to the formation of quinaldic acid (HRMS: $C_{10}H_8O_2N$ *m/z* = 174.0543, calc. 174.0555), which was not detected in the regurgitate of the control group (cf. Scheme 1). Accordingly, also in insects the biosynthesis of quinaldic acid may proceed directly from kynurenine via similar intermediates as outlined in Scheme 2. Injection of *m*NBA into the hemolymph remained without effect.

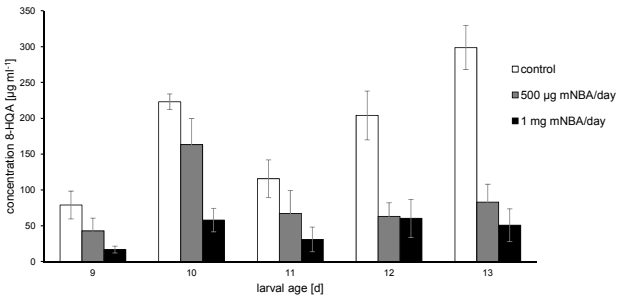


Fig. 5 Concentration of 8-HQA (mean ±SD) in the regurgitate of *S. littoralis* larvae after feeding the KMO-inhibitor *m*NBA (control □, 500 µg day^{-1} $larvae^{-1}$ ■, 1 mg day^{-1} $larvae^{-1}$ ■) for several days. Starting of feeding: at a larval age of 8 days.

Although *m*NBA was developed as an inhibitor for the human KMO[18], the high efficiency against the insect enzyme is remarkable and points to a highly conserved biocatalyst in the tryptophan-kynurenine pathway. Inhibition of the kynurenine aminotransferase (KAT) by 3-(2-aminobenzoyl)-propionate, as described by Rossi[27] was not successful, but resulted in an enhanced mortality of the larvae.

8-HQA is formed by the insect and not by intestinal microorganisms

To determine the source of 8-HQA, the diet of *S. littoralis* was supplemented with antibiotics (erythromycin, polymyxin,

tetracycline and vancomycin) and [$^2H_5$]-Trp. The broad spectrum of antibiotics guaranteed a bacteriostatic and bactericidal impact on gram-positive and gram-negative bacteria present in larval gut. After two days, the regurgitate was collected and plated onto LB agar to count the colony-forming units (CFUs). As expected, a strong decrease (factor 10^4) of the CFUs was observed in presence of antibiotics (Fig. 6a). On the other hand, the formation of 8-HQA was not affected, suggesting that the intestinal bacteria are not involved in the biosynthesis of 8-HQA (Fig. 6b).

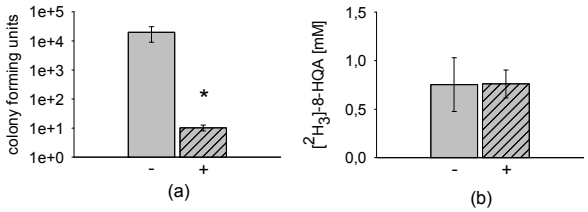


Fig. 6 Impact of antibiotics onto CFUs **(a)** and [$^2H_3$]-8-HQA concentration **(b)** in the regurgitate after 2 days of treatment; (mean ± SD). The asterisk indicates a statistically significant difference ($p < 0.05$; Student`s *t*-test) (- without antibiotics; + with antibiotics)

Conclusions

For the first time large quantities of 8-HQA have been found in the gut fluid of several Noctuid larvae (Fig. 2). The concentrations can be substantial (up to 5 mmolar), but a biological function has as yet not been described. The biosynthesis proceeds via kynurenine and 3-hydoxykynurenin, as the production is effectively blocked by the KMO-inhibitor *m*NBA. Xanthurenic acid and quinaldic acid are not precursors, demonstrating that the last steps from 3-hydroxykynurenine towards 8-HQA are not following known biosynthetic routes (Scheme 1) but utilize an alternative pathway (Scheme 2).. As 8-HQA is a strong chelator of Fe(II)[19] a function as a siderophore is feasible. Our data demonstrate that the insect itself produces continuously large amounts of 8-HQA from Trp of the ingested diet. Accordingly, the concentration of free Fe(II) in the gut should be low (stability of the complexes along the pH-gradient along the gut has to be considered)[17,19]. Thus, the growth of strongly Fe-dependent bacteria should be reduced or inhibited, as already shown by *in vitro* experiments[23] using 8-HQA at typical gut concentrations (ca. 1 mMol). To which extent such an Fe-mediated control of the gut microbiome is utilized by the insects remains to be established.

Experimental

Insects

Rearing and maintenance of lepidopteran larvae

Larvae of *S. littoralis* (Syngenta Crop Protection Münchwilen AG, Stein, Switzerland), *S. exigua* (Bayer Cropscience AG, Monheim, Germany) and *S. eridania* (BASF Corporation, Florham Park NJ USA) were hatched from eggs and reared on a standard bean-based diet[28] at 23-25 °C with a 16:8 (L:D) photoperiod. *S. frugiperda*, *H. armigera*, *H. subflexa*, *H. virescens* and *P. xylostella* were kindly provided by the department of Entomology and larvae of *M. sexta* were kindly provided by the department of Biochemistry, Max-Planck-Institute for Chemical Ecology, Jena, Germany. *Y. evonymellus* larvae were collected in Eschweiler, Germany. *Malacosoma* sp. and *O. cerasi* were collected in Jena, Germany. *A. urticae* was collected in Heubach-Lautern, Germany.

For incubation experiments 10-14 days old larvae of *S. littoralis* were reared on a chemically defined diet containing only casein as protein source[29]. The Trp-depleted diet contained hydrolyzed casein (Carl Roth), which is stated as Trp-free. This diet was supplemented with Trp at 1.95 (normal) or 19.5 mmol g^{-1} (rich).

Feeding experiments

In order to evaluate potential precursors and intermediates *en route* to 8-HQA, tryptophan (Sigma-Aldrich), kynurenine (Enzo Life Sciences), 3-hydroxykynurenine (Sigma-Aldrich), xanthurenic acid (Sigma-Aldrich), kynurenic acid (Sigma-Aldrich) and quinaldic acid (Enzo Life Sciences), aqueous solutions (20-100 µl at 10 mg ml^{-1}) were applied per larva onto a Trp-depleted diet.

Significant reduction of larval gut bacteria was achieved by feeding sub-lethal concentrations of antibiotics. A mixture of erythromycin (73 µg ml^{-1}, Sigma-Aldrich), polymyxin (500 ng ml^{-1}, Sigma-Aldrich), tetracycline (25 µg ml^{-1}, Carl Roth) und vancomycin (10 µgml^{-1}, Sigma-Aldrich) was used. For each larva 100 µl were applied daily onto 1.0 g chemically defined normal-Trp diet.

Inhibitor experiments were done by feeding *m*NBA (synthesized according to Pellicciari[20]) impregnated bean diet (1 mg or 500 µg *m*NBA respectively per1 g standard bean diet) to larvae starting at a larval age of 8 days. Diet was replaced daily; control groups were fed with the same amount (1g day^{-1}) of *m*NBA free diet. Regurgitate was collected daily before replacing the diet. Regurgitate of 3 individuals per group (5 groups of treatment and control respectively) was pooled and 8-HQA was quantified by HPLC-MS.

Microinjection

2 µl of a solution of inhibitor in water (34 µg ml^{-1}) or water as control, respectively was injected into the hemolymph. A FemtoJet® microinjection system (Eppendorf) was used; compensation pressure P_c = 150 hPa, injection pressure P_i = 50 hPa, injection time t_i = 3 s.

Whole insects

For analysis of 8-HQA or 3-hydroxykynurenine, whole insects were homogenized in 1-2 ml methanol (containing 10 µg ml^{-1} of internal standard, kynurenine or kynurenic acid respectively) with a Polytron PT 1300 D (Kinematica) or Ultra-Turrax T25 (IKA). The homogenate was centrifuged for 5 min at 10,000 rpm, supernatant was evaporated to dryness, re-dissolved in 100 µl methanol and used for HPLC-MS analysis.

Dissections

Insects were dissected under a stereomicroscope (Zeiss Stemi

2000 C) in ringer solution[30]. Dissected parts (fat body, Malpighian tubules, gut) where sonicated (15 min) and homogenized in 100-300 µl methanol (containing kynurenine 10 µg ml^{-1} as internal standard), centrifuged for 5 min at 10,000 rpm. Supernatant was evaporated to dryness, re-dissolved in 50 µl methanol and used for HPLC-MS analysis.

Collection of regurgitate

Oral secretions of larvae were collected with a pipet by gently squeezing the larva with a forceps behind the head which caused immediate regurgitation or by applying slight partial vacuum. Amount of collectable regurgitate per larva ranged between 1 to 15 µl, depending on species and larval age. For *S. littoralis* e.g., harvested samples of 3-10 larvae (age dependent) were pooled into pre-weighted Eppendorf vials. After weighing 20 µl methanol (containing kynurenine 10 µg ml^{-1} as internal standard) was added and the suspension was centrifuged for 5 min at 10,000 rpm. Supernatant was transferred into a GC vial with micro insert and injected without further clean-up into HPLC-MS or stored at -20 °C for further analysis.

HPLC-MS

For HPLC-MS an Agilent HP1100 HPLC system connected to a LCQ (Thermoquest, Engelsbach, Germany) was used. Solvent A was quartz-distilled water, B was acetonitrile, each with v/v 0.5% acetic acid.

8-HQA in biological samples was quantified in APCI+ mode (vaporizer temperature: 430°C) using a Grom™ Sil column, ODS-3 CP, 120 Å, 3 µm, 125 x 2 mm (Grace). Gradient elution at 0.2 ml min^{-1}: 0% B for 3 min, up to 100% in 27 min, 100% B for 15 min, 0% B for 15 min was used to separate compounds. Kynurenine (not detected in the biological samples) served as an internal standard

3-Hydroxykynurenine in biological samples was quantified in APCI+ mode using a Luna NH_2 column 100 Å, 5 µm, 150 x 2 mm (Phenomenex). Gradient elution at 0.2 ml min^{-1}: 100% B for 3 min, down to 0% in 27 min, 0% B for 2 min, 0% B for 12 min was used for analysis. Kynurenic acid served as internal standard.

Deuterium-labeled precursors

[$^2H_5$]-Trp (L-2',4',5',6',7'-[$^2H_5$]-tryptophan) was purchased from Isotec. Deuterated kynurenic acid and xanthurenic acid were prepared by a modified method of Derdau *et al.*[31]. The respective quinolinic acid (0.4 mmol), sodium borohydride (10-15 mg, 60-80 mol.%) and palladium/charcoal (17-23 mg) were suspended in deuterium oxide (3 ml), and the slurry was heated to mild reflux. After removal of the catalyst by filtration over celite and concentration *in vacuo,* a yellow-colored product was obtained. Yield: 38-42%.

3-Hydroxykynurenine was not stable under these conditions and a modified method of Trachtenberg *et al.*[32] was used. 3-Hydroxykynurenine (0.35 µmol) was dissolved in conc. deuterium chloride in deuterium oxide (340 µl) and the solution was stirred at 100 °C overnight. After removal of solvent *in vacuo,* the degree of deuteration was quantified by ^{1}H NMR.

^{1}H NMR

[^2H]-kynurenic acid (4-hydroxyquinoline-2-carboxylic-3-d acid)

^{1}H NMR (500 MHz, DMSO-d_6) δ ppm 6.53 (s, 0.54 H) 7.27 (ddd, *J*=8.05, 6.96, 1.09 Hz, 1 H) 7.59 (ddd, *J*=8.42, 6.93, 1.37 Hz, 1 H) 7.92 (d, *J*=8.48 Hz, 1 H) 8.04 (dd, *J*=8.13, 1.49 Hz, 1 H

[$^2H_4$]-xanthurenic acid (4,8-dihydroxyquinoline-2-carboxylic-3,5, 6,7-d_4 acid)

^{1}H NMR (500 MHz, D_2O) δ ppm 6.72 (s, 0.79 H) 7.04 (d, *J*=7.56 Hz, 0.39 H) 7.18 (dd, *J*=8.25, 7.79 Hz, 1 H) 7.44 (d, *J*=8.25 Hz, 0.53 H)

[$^2H_3$]-3-hydroxykynurenine (2-amino-4-(2-amino-3-hydroxyphenyl-5-d)-4-oxobutanoic-3,3-d2 acid):

^{1}H NMR (500 MHz, DCl in D_2O, 333K) δ ppm 4.54 (s, 1 H) 7.36 (s, 1 H) 7.65 (s, 1 H)

NMR indicated for Kynurenic acid (DMSO-d_6) a proton exchange at C3 (46%) whereas Xanthurenic acid (D_2O) showed a random label that was quantified relative H-C6 (set to 0% exchange) H-C3 (21%), H-C5 (61%) H-C7 (47%). 3-Hydroxykynurenine (DCl in D_2O) was completely labeled at C5' and C3. Hydrogen atoms at C4' and C6' were not affected.

Notes and references

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