Pyrrolidonyl and Pyridyl Alkaloids in *Lymantria dispar*

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The occurrence and metabolism of nicotine and related *N*-containing compounds in body fluids of the gipsy moth were addressed. Thin layer chromatographic studies clearly showed the simultaneous presence of GABA and 2-pyrrolidone but not of GABamide in the larval haemolymph and osmeterial secretion of *Lymantria dispar* as well as in the corresponding body fluids of the saturniids, *Saturnia pavonia* and *Attacus atlas*. Furthermore, feeding and injection experiments using alkylated precursors and combined gas chromatography/mass spectrometry gave evidence of the transformation of 2-pyrrolidone to nicotine and of nicotinic acid to nicotinamide in caterpillars of *L. dispar*. Based on these results, on the earlier described variation of the secondary-compound patterns of *L. dispar* during its development, and on literature data, metabolic pathways for the hitherto detected pyridyl and pyrrolidonyl alkaloids in Lymantriidae (and possibly Saturniidae) are proposed.

Key words: Chemical Defense, Lymantriidae, Saturniidae, Secondary Metabolites

Introduction

The gipsy moth, *Lymantria dispar* (L.) (Lep.: Lymantriidae), is one of the most famous pests in forestry. Deml and Dettner (1995a, b) and Aldrich *et al.* (1997) investigated secondary metabolites in *L. dispar* and found aromatics, aliphatics, and heterocyclics in haemolymph, hairs, and secretions of this species. The exocrine secretions are discharged by osmeteria on the larval abdomen and probably serve for chemical defense from enemies, due to feeding deterrent effects of several secondary compounds therein such as the unusual alkaloid nicotine. Blocking nicotine synthesis, thereby reducing protection from enemies, would be an attractive new way of controlling outbreaks of *L. dispar*, an alternative to the use of the more unspecific dimilin (*e.g.*, Schmidt, 1986).

In this study, a few key questions regarding relevant metabolic pathways in *L. dispar* had to be clarified. The one problem was that GABA (γ-aminobutyric acid), its lactam 2-pyrrolidone, and probably also its amide GABamide produce identical mass spectra due to decomposition processes (Budavari *et al.*, 1989). By means of derivatizations, Aldrich *et al.* (1997) showed that the secretion of larvae reared on an artificial diet may contain only GABA. However, Deml and Dettner (1995a, b) had fed the caterpillars with *Quercus* leaves, and it was unclear which of the substances may occur in *L. dispar*. This problem was resolved herein by a study using thin layer chromatography (TLC). The second question was whether the larvae actually produce nicotine or sequester it from their food; nicotine is wide-spread in plants (Frohne and Jensen, 1998). Therefore, potential, alkylated precursors of nicotine were administered to the larvae, and subsequently *L. dispar* samples were analyzed by gas chromatography/mass spectrometry (GC/MS). Finally, the new findings were combined with biogenetic data from literature in order to obtain a plausible metabolic map for nicotine in *L. dispar*.

Materials and Methods

Insect material

Caterpillars of *L. dispar* were collected in the field from various *Quercus* species near Hallstadt (Bamberg/Bavaria) and were further reared in order to get eggs resulting from this breeding. Furthermore, egg masses of *L. dispar* were collected in a park in Hollfeld (Bavaria) in autumn. All eggs were hibernated outdoors. In spring, the hatched larvae were reared in aerated plastic boxes at 22 °C and 60–70% relative humidity and were fed only with *Q. robur* L. (English oak; Fagaceae) for their whole lives. Eggs as well as first and last instar larvae were frozen and stored at – 20 °C until used.

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Prior to analysis, eggs were triturated in a mortar. The resulting pulp was diluted with water, then sucked up with a glass capillary. "Balloon hairs" were cut from freshly emerged L1 larvae with microscissors. Secretion was obtained by pressing living last instar caterpillars on their thorax, or by defrosting the frozen larvae and pressing the integument on both sides of the osmeteria gently with forceps. Secretion that flowed out was sucked up with a glass capillary. To sample the haemolymph of last instar larvae, a small cut was made with a scalpel in the ventral integument; then the haemolymph droplets were sucked up with a glass capillary.

Chemicals and derivatizing procedure

Authentic *N*-methylnicotinamide chloride, 6-methylnicotinamide, and 6-methylnicotinic acid were purchased from Sigma, *N*-ethyl-2-pyrrolidone from Aldrich. Authentic GABamide was prepared by ester-ammonolysis: GABA was treated with a surplus of Methyl-8® (Pierce) for 15 min at 60 °C, the resulting GABA methyl ester solution was evaporated, then mixed with aqueous 25% ammonia, boiled for 4 h, and evaporated.

The methyl ester of 6-methylnicotinic acid was prepared by methylating 6-methylnicotinic acid with a surplus of Methyl-8® for 15 min at 60 °C. In order to detect potentially occurring 6-methylnicotinic acid methyl ester in the larval samples, the samples were dried above P_2O_5 and subsequently treated with Methyl-8® as described. Subsequently, the reaction mixtures were injected directly into the GC.

TLC

In order to separate GABA, GABamide, 2-pyrrolidone, and *N*-methyl-2-pyrrolidone, several TLC systems suitable for separations of amino acids were tested (Brenner *et al.*, 1967; Randerath, 1972). At first, aqueous solutions of the substances were prepared [GABA 1:20 (w/v), GABamide 1:15, 2-pyrrolidone 1:10, *N*-methyl-2-pyrrolidone 1:15]. TLC was performed in a developing tank at pre-saturated conditions. The plates used were silica gel on aluminium with or without fluorescent indicator (Kieselgel 60F254 and Kieselgel 60; Merck). 1-μl droplets, in each case, of the dilutions of the chemicals were applied to the plates. After development, the chromatograms were colorated by spraying with 0.2% ethanolic ninhydrin reagent and were inspected in visual light and UV. Several solvents were used in various ratios: cyclohexane/ethyl acetate (1:2, 1:3, 1:5, 1:7, pure ethyl acetate), isopropanol/acetic acid/water (6:1:1), and methanol/chloroform/17% ammonia (2:2:1, 2:4:1, 3:4:1, 3:2:1, 4:2:1, 5:1:1 v/v/v). The solvents tested differed largely with regard to their capability of separating the authentic chemicals. With cyclohexane/ethyl acetate, GABA and 2-pyrrolidone did not migrate far and were not separated at all. With isopropanol/acetic acid/water, the substances migrated but GABA and GABamide were only poorly separated from each other but better from 2-pyrrolidone. All methanol/chloroform/ammonia mixtures separated 2-pyrrolidone well from GABA and GABamide whereas the latter substances started to migrate only from 3:2:1 and were separated only from 4:2:1. Because the best result was achieved with the ratio 5:1:1 (distinct R_f values for all the authentic chemicals were obtained), this mixture was used with all larval samples: 0.25 μl and 0.125 μl haemolymph of *L. dispar*, 0.75 μl osmeterial secretion of *L. dispar*, as well as, for comparison, 0.25 μl and 0.125 μl haemolymph of *Saturnia pavonia* (L.) (Lep.: Saturniidae), 0.75 μl scolus secretion of *S. pavonia*, and 0.25 μl haemolymph of *Attacus atlas* (L.) (Lep.: Saturniidae); the two saturniids were previously also known to contain a substance with the relevant mass spectrum in their haemolymph and in the exocrine secretion of their scolus glands (Deml and Dettner, 1997).

Administration of alkylated compounds

Three chemicals were applied to last instar caterpillars as potential substrates for the synthesis of the corresponding nicotines: *N*-methylnicotinamide chloride, 6-methylnicotinic acid, and *N*-ethyl-2-pyrrolidone. Each substance was administered to individual larvae in one of two different ways. Either 1 μl of a 1 M aqueous solution/suspension was injected laterally into the larval body cavity (subdermal administration) or 1 μl of the solution was applied to leaves of the foodplant, allowed to dry, and given to one caterpillar which ate the whole plant material readily (oral administration). The larvae were allowed to pupate, and the result-

ing adults laid eggs which were hibernated in order to get freshly emerged L1 caterpillars. Samples analyzed were balloon hairs from L1 larvae (subdermally treated larvae) as well as eggs, balloon hairs from L1 larvae, haemolymph and secretion from last-instar larvae (orally treated animals). Because of the small amounts of sample per caterpillar, the samples were pooled for analysis from three to five larvae from the respective assays, in each case.

GC/MS

Samples were chemically analyzed by transferring them from the capillary (liquid samples) or from the microscissors (balloon hairs) onto a Solid Injektor SI 1 (SGE) syringe and injecting into a Carlo Erba GC 6000 Vega gas chromatograph. The chromatograph contained a 15-m fused silica capillary column (either CP-SIL 19 CB or DB 1701; Chrompack). The columns were coupled to a Finnigan-MAT Ion Trap Detector. The temperature program was 50 °C to 260 °C (10 °C/min). The carrier gas was helium. Electron impact ionization (EI; 70 eV) mass spectra were obtained in total ion chromatograms. For confirmation of nicotine and precursor compounds, authentic chemicals were injected and retention times and mass spectra compared. Because no authentic chemicals were available for the possible products (methylnicotines, ethylnornicotine), their mass spectra were calculated mainly on the base of the fragmentation of nicotine and according to remarks made by Budzikiewicz *et al.* (1967).

Results

TLC

In all larval samples, several spots were visualized by ninhydrin, most of them probably corresponding to free amino acids; altogether, haemolymph yielded seven (*L. dispar*, *A. atlas*) and eight (*S. pavonia*) spots, and secretion five (*L. dispar*) and seven (*S. pavonia*) spots. Due to their R_f values, spots of GABA and of the typically blue colored 2-pyrrolidone, but not of GABamide, were identified in all samples (Table I). Based on measurements of diameters and optical estimations of spot areas, the spots of GABA were larger than the spots of 2-pyrrolidone, in all cases (ratios about 2:1–3:1 with the haemolymphs, 4:1 with the *L. dispar* secretion, and 5:1–6:1 with the *S. pavonia* secretion). A spot of the light-violet colored *N*-methyl-2-pyrrolidone was additionally found in secretion of *L. dispar* in about the same size as compared with 2-pyrrolidone, but not in the two body fluids of *S. pavonia* while larger amounts of the latter larval samples unfortunately resulted in overloading. Obviously the relatively small amounts of this component in the saturniid detected in corresponding GC/MS analyses (Deml and Dettner, 1997) fell below the detection limit of the TLC method whereas the larger amounts in *L. dispar* were still detectable.

GC/MS

Three alkylated chemicals which were detectable by GC/MS (in case of 6-methylnicotinic acid

Table I. Occurrence and R_f values of GABA and structurally related compounds in lepidopteran larval samples, obtained after separations by means of TLC[1].

Sample[2]	Compound			
	GABA	GABamide	2-Pyrrolidone	*N*-Methyl-2-pyrrolidone
Authentic chemicals	0.52 – 0.62	0.20 – 0.26	0.86 – 0.89	0.91 – 0.93
HL *Lymantria dispar*	0.50	–	0.89 – 0.92	–
GS *L. dispar*	0.52	–	0.89	0.93
HL *Saturnia pavonia*	0.59	–	0.88	–
GS *S. pavonia*	0.63	–	0.88	–
HL *Attacus atlas*	0.59	–	0.87	–

[1] TLC was performed using silica gel on aluminium (Kieselgel 60 plates), a mixture of methanol/chloroform/17% ammonia (5:1:1) as solvent, and ninhydrin for coloration.

[2] HL = haemolymph; GS = glandular secretion; – = not detected. Four to seven runs with authentic chemicals, two runs with HL *L. dispar*, one run each with the other samples.

as the methyl ester) had been administered to caterpillars of *L. dispar*. Either of them was assumed to be a suitable precursor to yield *N*- or 6-methylnicotine and *N′*-ethylnornicotine, respectively, while 6-methylnicotinamide was considered to be a possible intermediate of conversion of one of the alkylated chemicals. Their mass spectral characteristics are as follows (interpretation of EI mass spectra mainly according to the general remarks of Budzikiewicz *et al.*, 1967). The methyl ester of 6-methylnicotinic acid (M^+ 151) is characterized by fragments at *m/z* 137 ($M-CH_2$), 122 ($M-CH_2-CH_3$), 121 ($M-OCH_2$), 107 ($M-OCH_2-CH_2$), 93 ($M-COOCH_2$), 92 ($M-COOCH_3$), 79, 65, 51, and 45 (base peak). The fragmentation of 6-methylnicotinamide is principally identical with that of nicotinamide but with all important fragments + 14 amu (additional methyl group): *m/z* 136 (M^+), 120 (M-16, loss of the NH_2 group), 108, 92 (base peak; M-44, *i.e.*, further release of -CO), and 65. Distinctly distinguished from this, *N*-methylnicotinamide chloride yields typical fragments at *m/z* 155, 131, 119, 105, 97, 85, 77, 69, 57, and 44 (base peak). *N*-Ethyl-2-pyrrolidone (M^+ 113) shows peaks at *m/z* 114 (M+1), 113 (M-1) (uptake and loss of a hydrogen atom, respectively), 98 ($M-CH_3$), 84 ($M-C_2H_5$), 70 (further elimination of carbon monoxide; base peak), 57, and, alike 2-pyrrolidone and *N*-methyl-2-pyrrolidone, at *m/z* 56 and 44 through 40. In order to find the possible products (methylnicotines, ethylnornicotine), attention was directed to peaks with mass spectra showing the expected M^+ and M-1 peaks, and fragments at *m/z* 92 or 93 (methylated pyridyl residue) accompanied with *m/z* 84 (*N*-methylpyrrolidyl residue), or alternatively at *m/z* 98 (*N*-ethylpyrrolidyl residue) together with *m/z* 78 (pyridyl residue).

From the three possible metabolism-products of the alkylated substances (Fig. 1), only one could be definitely detected: *N′*-ethylnornicotine in eggs of *L. dispar*, obtained after oral administration of *N*-ethyl-2-pyrrolidone. Its mass spectrum showed important peaks at *m/z* 176, 133, 131, 113, 101, 98, 78, 67, 53, and 45. Furthermore, an *N*-methylnicotine salt was possibly contained in the secretion of last instar larvae after oral application of the corresponding nicotinamide. However, the mass spectrum obtained did not fit those of methylnicotinamide as well as authentic nicotine very well although the typical fragments searched for (*m/z* 177, 93, 92) were clearly present besides other important fragments at *m/z* 135, 98, 81, 72, 60, and 45. Additionally, nicotine itself which was already known from untreated *L. dispar* could be found in most larval samples investigated (Table II). Its fragmentation pattern has already been described (Deml and Dettner, 1995a, b). The third possible product, 6-methylnicotine, could not be found in any of the samples. *N*-methylnicotinamide chloride was detected in the haemolymph of last instar larvae which means that the administered substances are actually taken up from the ingested food into the larval body. 6-Methylnicotinamide was found in the balloon hairs after subdermal application of the corresponding acid, indicating a conversion of the acid into the amide. This finding is also striking because normally the balloon hairs do not contain nicotinamide (Deml and Dettner, 1995b).

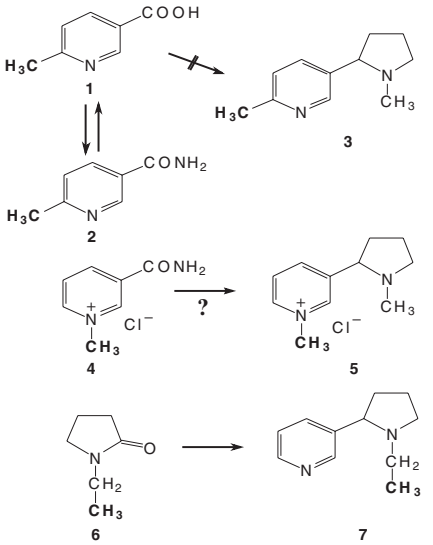

Fig. 1. Alkylated precursors administered to caterpillars of *Lymantria dispar* (**1**, **4**, **6**), and their possible products (**2**, **3**, **5**, **7**). Actual performance of the reactions is indicated. (**1**), 6-Methylnicotinic acid; (**2**), 6-methylnicotinamide; (**3**), 6-methylnicotine; (**4**), *N*-methylnicotinamide chloride; (**5**), *N*-methylnicotine chloride; (**6**), *N*-ethyl-2-pyrrolidone; (**7**) = *N′*-ethylnornicotine.

Discussion

Altogether eight compounds potentially involved in the metabolism of the alkaloid nicotine have now been definitely identified from *L. dispar*

Table II. Alkylated compounds and regular nicotine detected by GC/MS of larval body fluids, larval hairs, and eggs of *Lymantria dispar*[1].

Assay[2,3]	*Compound*[2]							
	6-Methyl-nicotinic acid (methyl ester) (**1**)	6-Methyl-nicotinamide (**2**)	6-Methyl-nicotine (**3**)	*N*-Methyl-nicotinamide chloride (**4**)	*N*-Methyl-nicotine chloride (**5**)	*N*-Ethyl-2-pyrrolidone (**6**)	*N′*-Ethyl-nornicotine (**7**)	Nicotine
1, O, eggs	–	–	–					–
1, O, balloon hairs	–	–	–					+
1, S, balloon hairs	–	+	–					+
1, O, haemolymph	–	–	–					+
1, O, secretion	–	?	–					+
4, O, eggs				–	?			–
4, O, balloon hairs				–	–			–
4, S, balloon hairs				–	–			+
4, O, haemolymph				+	–			+
4, O, secretion				–	+?			+
6, O, eggs						–	+	–
6, O, balloon hairs						–	?	+
6, S, balloon hairs						–	–	+
6, O, haemolymph						?	–	+
6, O, secretion						–	–	+
U, eggs			–		–		–	+
U, balloon hairs			–		–		–	+
U, haemolymph			–		–		–	+
U, secretion			–		–		–	+

[1] + = detected; ? = trace compound not definitely identified due to only incomplete mass spectrum (*e.g.*, no M^+ peak; quantity-dependent) as compared with authentic chemical at corresponding retention time; – = not detected.
[2] Numbers refer to Fig. 1.
[3] Sample taken after oral (O) or subdermal (S) administration of alkylated compound to last instar larvae. Balloon hairs were taken from L1 larvae, haemolymph and secretion from L5/6 larvae. U = untreated.

(Fig. 2). Probably their chronologic occurrence and increasing number along ontogeny (Deml and Dettner, 1995b; Aldrich *et al.*, 1997; Deml, in press) are adapted to the specific needs of each developmental stage, such as chemical defense. Because all the compounds but GABA and possibly 2-pyrrolidone were not detected in *Q. robur* (Kasai *et al.*, 1978; Deml and Dettner, 1995b), they must be biosynthesized by the caterpillars. As was shown herein, the pathway from 2-pyrrolidone/*N*-methyl-2-pyrrolidone towards nicotine is realized in the gipsy moth. A conversion of nicotinic acid into nicotinamide also takes place. However, a direct origin of the pyridyl moiety of nicotine from nicotinic acid/nicotinamide could not be confirmed. Perhaps the mimicing precursors used have been unsuitable for the responsible enzymes and/or there is an alternative pathway via an intermediary activation (yielding a nicotinamide nucleotide, *e.g.*, NADH).

Considering all these results as well as data on metabolism of nicotine and related compounds in plants and animals (*e.g.*, Fischer and Brander, 1960; McKennis *et al.*, 1962; Leete, 1967; Blum, 1981; Teuscher and Lindequist, 1988; Budavari *et al.*, 1989; Urich, 1990; Stryer, 1991; Habermehl and Hammann, 1992; Snyder *et al.*, 1994), plausible biosynthetic pathways can be proposed without constraint (Fig. 2). Three points shall be stressed:

(i) Free amino acids in *Q. robur* (Kasai *et al.*, 1978; Hegnauer, 1962–1996) might be suitable precursors for anabolism of nicotine in *L. dispar*: glutamic acid and its product GABA, proline, and asparagine.

(ii) Due to the temporary, simultaneous presence of glycerol, nicotinic acid, and nicotinamide in last instar larvae, it appears convincing that *L. dispar* synthesizes nicotinic acid *de novo*, alike higher plants do, from aspartate (from asparagine from *Q. robur*) and glyceraldehyde-3-phosphate [from glycerol (Leete, 1967)], and uses nicotinamide as an additional storage form of the pyridyl ring. Consequently, L1 hairs contain none of these substances but large amounts of nicotine (Deml and Dettner, 1995b). Such biosynthesis of nicotinic

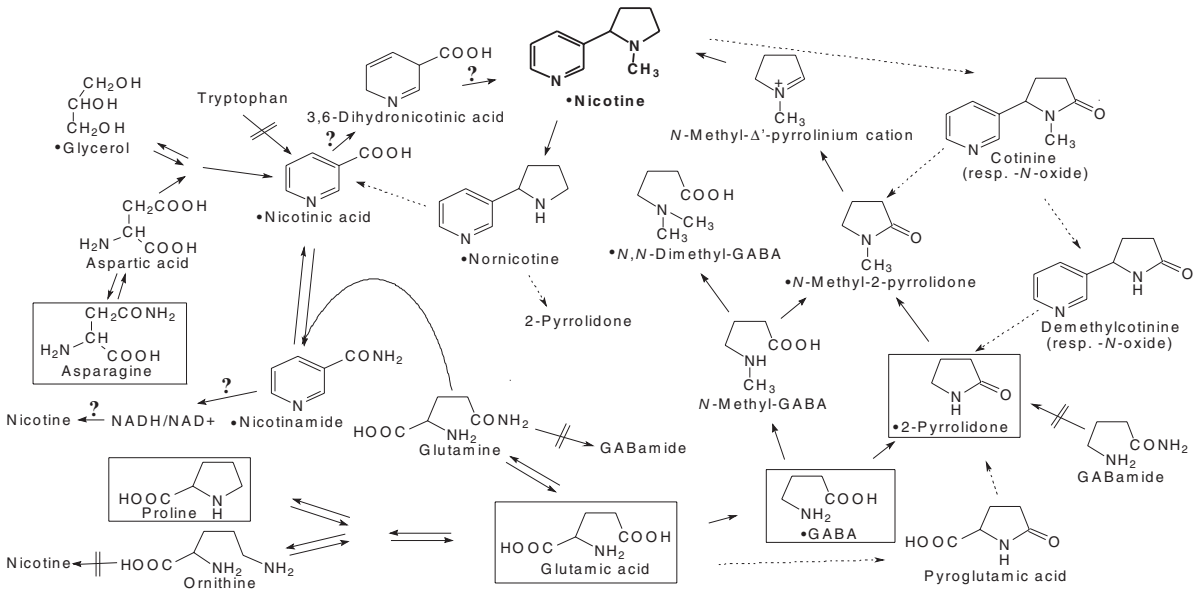

Fig. 2. Hypothetical, biogenetic correlations of detected *Lymantria dispar* components structurally related to nicotine. The detected compounds are marked by a dot (nornicotine has been added but has been identified only from *L. monacha*). Possible precursors (from the foodplant of *L. dispar*: *Quercus robur*) are shown in boxes. Dotted arrows = hypothetical pathways still without concrete evidence; crossed out arrows = hypothetical pathways defeated by the presented results.

acid is unusual because this acid normally represents an essential element of food for insects (Linzen, 1974).

(iii) The nicotine-catabolic reactions specified (via cotinine in *L. dispar*; via nornicotine in *L. monacha*) could be useful for detoxifying the alkaloid when it penetrates the larval body from hairs or defensive glands. A minor compound revealing the mass spectrum of the non-toxic cotinine was actually detected in L1 haemolymph (Deml and Dettner, 1995b).

Clearly, further parts of the presented metabolic map have to be confirmed, so that the biogenesis and degradation of pyrrolidonyl and pyridyl alkaloids by the gipsy moth can be understood in such detail that meaningful new starting points of control are in sight.

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