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Biosynthesis of the Defensive Alkaloid (*Z*)-3-(2-Methyl-1-butenyl)pyridine in *Stenus similis* Beetles

Andreas Schierling,*[,†] Matthias Schott,[‡] Konrad Dettner,[†] and Karlheinz Seifert[§]

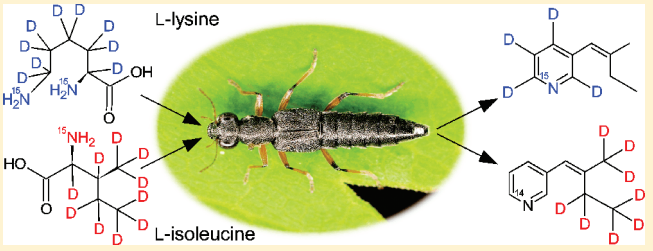
[†]Department of Animal Ecology II, University of Bayreuth, Germany

[‡]Institute of Phytopathology and Applied Zoology, Justus-Liebig-University Gießen, Germany

[§]Department of Organic Chemistry, University of Bayreuth, Germany

Supporting Information

ABSTRACT: Most rove beetles of the genus *Stenus* protect themselves against microorganisms and predators such as ants and spiders by producing the alkaloid stenusine (**1**) in their pygidial glands. The biosynthesis of **1** was previously investigated in *S. bimaculatus*, where L-lysine forms the piperidine ring, L-isoleucine the side chain, and acetate the N-ethyl group. In addition to **1**, *S. similis* keeps the pyridine alkaloid (*Z*)-3-(2-methyl-1-butenyl)pyridine (**2**) in its pygidial glands. Feeding *S. similis* beetles with [D,^{15}N]-labeled amino acids followed by GC/MS analysis showed that L-Lys yields the pyridine ring and L-Ile the 2-methyl-1-butenyl side chain. Thus the alkaloids **1** and **2** probably share two precursor molecules in their biosynthesis.



Rove beetles of the genus *Stenus* Latreille are small black beetles that occur globally and belong to the Staphylinidae family (subfamily Steninae). The typical slim habitus of this family with short elytra and a flexible but largely unprotected abdomen allows the beetles to colonize habitats with small interstices but significantly increases the danger of infestation by microorganisms and predation.[1] To avoid these hazards, the beetles synthesize antimicrobial and deterrent alkaloids such as stenusine (**1**)[2] and (*Z*)-3-(2-methyl-1-butenyl)pyridine (**2**)[3] (Figure 1) in their pygidial glands at the tip of their abdomen. While compound **1** is present in nearly every *Stenus* species,[4] especially in the ancestral species, **2** is more often found in phylogenetically advanced species.[4,5] It is also remarkable that **2** shows a higher antimicrobial activity than **1**.[3]

The biosynthesis of stenusine (**1**) in *S. bimaculatus* Gyllenhal has previously been investigated. L-Lysine forms the piperidine ring, L-isoleucine the side chain, and acetate the N-ethyl group.[6] Due to the structural similarity of **1** and **2**, the pyridine ring of **2** in *S. similis* Herbst should also be formed from L-Lys and the side chain from L-Ile. The alkaloids **1** and **2** probably share two precursor molecules in their biosynthesis.

RESULTS AND DISCUSSION

To investigate the biosynthesis of (*Z*)-3-(2-methyl-1-butenyl)pyridine (**2**), feeding experiments with [D,^{15}N]-labeled amino acids followed by GC/MS analysis of the pygidial gland secretion were performed with *S. similis* beetles. To facilitate the recognition of the labeled (*Z*)-3-(2-methyl-1-butenyl)pyridine in the MS, it is important to know in advance which fragments of **2** are going to show a shift in their *m*/*z* values due to the [D,^{15}N]-labeling. The EI mass spectrum of the native compound **2** shows the molecular ion peak at *m*/*z* 147 ($M^{+\cdot}$) and two main fragment ions *m*/*z* 132 and 117 (Figures 2 and 3A).

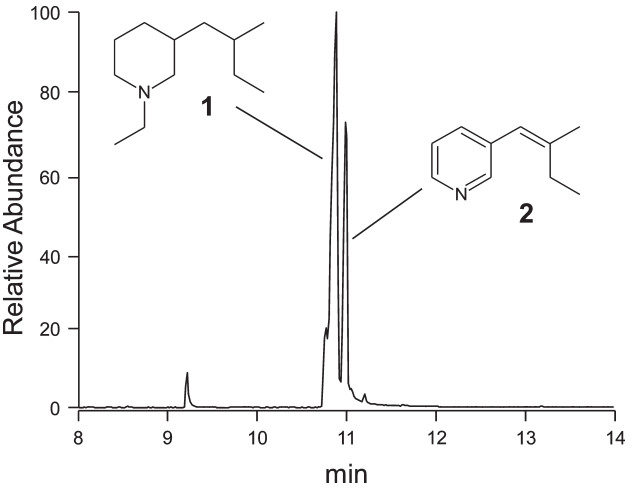


Figure 1. TIC showing the main components of the *Stenus similis* pygidial gland secretion. **1**, stenusine; **2**, (*Z*)-3-(2-methyl-1-butenyl)pyridine.

Feeding *S. similis* beetles with L-[D_9,$^{15}N_2$]-Lys should result in a mass shift of $\Delta m/z$ 5 for all three ions (Figures 2 and 3B), whereas L-[D_{10},^{15}N]-Ile feeding should give for *m*/*z* 147, 132, and 117 mass shifts of $\Delta m/z$ 8, 5, and 2, respectively (Figures 2 and 3C).

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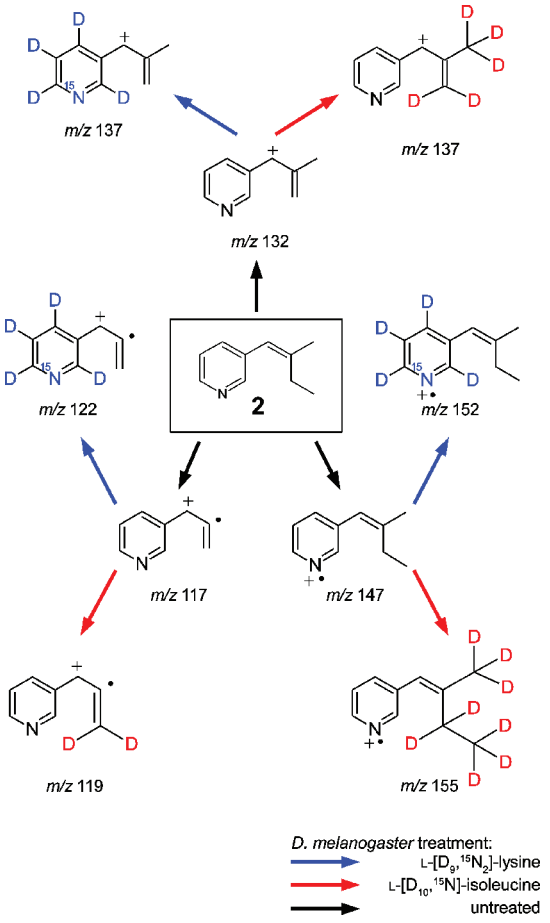

Figure 2. EIMS fragmentation of labeled and unlabeled (*Z*)-3-(2-methyl-1-butenyl)pyridine (**2**).

Four D atoms and one ^{15}N atom of L-[D_9,$^{15}N_2$]-Lys could be confirmed as incorporated into **2** (Figures 2 and 3B). From the 10 D atoms of L-[D_{10},^{15}N]-Ile eight were incorporated into **2** (Figures 2 and 3C). [D]-Labeling of a compound decreases its GC retention time.[7,8a] The 4-fold deuteration in the pyridine ring and the 8-fold deuteration in the side chain of alkaloid **2** resulted in a 1.8 s and a 6.0 s shorter retention time, respectively, in comparison with unlabeled **2**. Quantitative analysis of the secretion components revealed that 0.03% and 0.3% of **2** were labeled after feeding the beetles with *Drosophila melanogaster* fruit flies dipped in L-[D_9,$^{15}N_2$]-Lys and L-[D_{10},^{15}N]-Ile.

Since we found [D_7,^{15}N]-**1** and [D_8]-**1** as [D_4,^{15}N]-**2** and [D_8]-**2**, it could be demonstrated that **1** and **2** are derived from the same amino acids L-Lys and L-Ile (Figure 4). Both alkaloids **1** and **2** seem to share two precursor molecules **p1** and **p2** during their biosynthesis (Figure 4). We were able to identify **p1b** (free base of **p1**) by GC/MS analysis, which eluted 4.5 s after compound **2**, as a minor secretion compound in native *S. similis* secretion. Treatment of native secretion with $NaBD_4$ resulted in the [H,D]-addition product of **p1b**. The proposed structure of the precursor **p1b** is in agreement with its HRESI/MS and should be proved by synthesis and comparison of the GC/MS data of the natural with the synthesized alkaloid.

The side-chain-deuterated precursor **p1** should contain nine D atoms and is finally transformed into the 8-fold deuterated products [D_8]-**1** and [D_8]-**2** in *S. similis* (Figure 4). However,

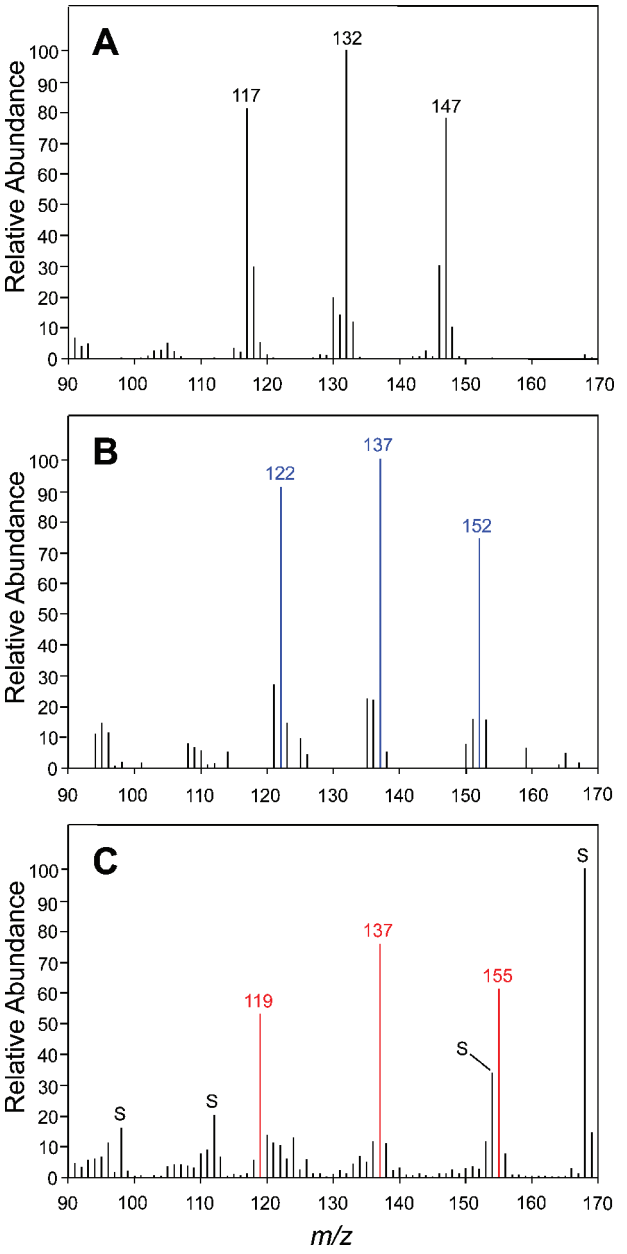

Figure 3. Full-scan GC/EIMS of (*Z*)-3-(2-methyl-1-butenyl)pyridine (**2**) from *Stenus similis* pygidial gland secretion. (A) untreated *D. melanogaster* diet, (B) L-[D_9,$^{15}N_2$]-lysine-treated *D. melanogaster* diet, (C) L-[D_{10},^{15}N]-isoleucine-treated *D. melanogaster* diet. Mass spectra B and C after subtraction of A. S = stenusine-derived fragments.

after feeding of L-[D_{10},^{15}N]-Ile to *S. bimaculatus* beetles nine D atoms[6] could be found incorporated into the side chain of (2′*S*)-stenusine (**1**). The loss of a single D atom in **1** of *S. similis* compared to **1** of *S. bimaculatus* can be explained by the inversion of the (2′*S*)-configuration of **p1** via **p2** into the (2′*R*)-configuration of [D_8]-**1**. The 8-fold deuterated **p2** should also be the precursor of [D_8]-**2** (Figure 4).

The precursor **p2** contains a Δ^2-piperideine ring, which can be reduced together with the side-chain double bond and N-ethylated to stenusine (**1**) (Figure 4). On the other side **p2** can be oxidized to (*Z*)-3-(2-methyl-1-butenyl)pyridine (**2**). The biosynthesis of most piperidine alkaloids starts with L-Lys,[8b,9] whereas the formation of a

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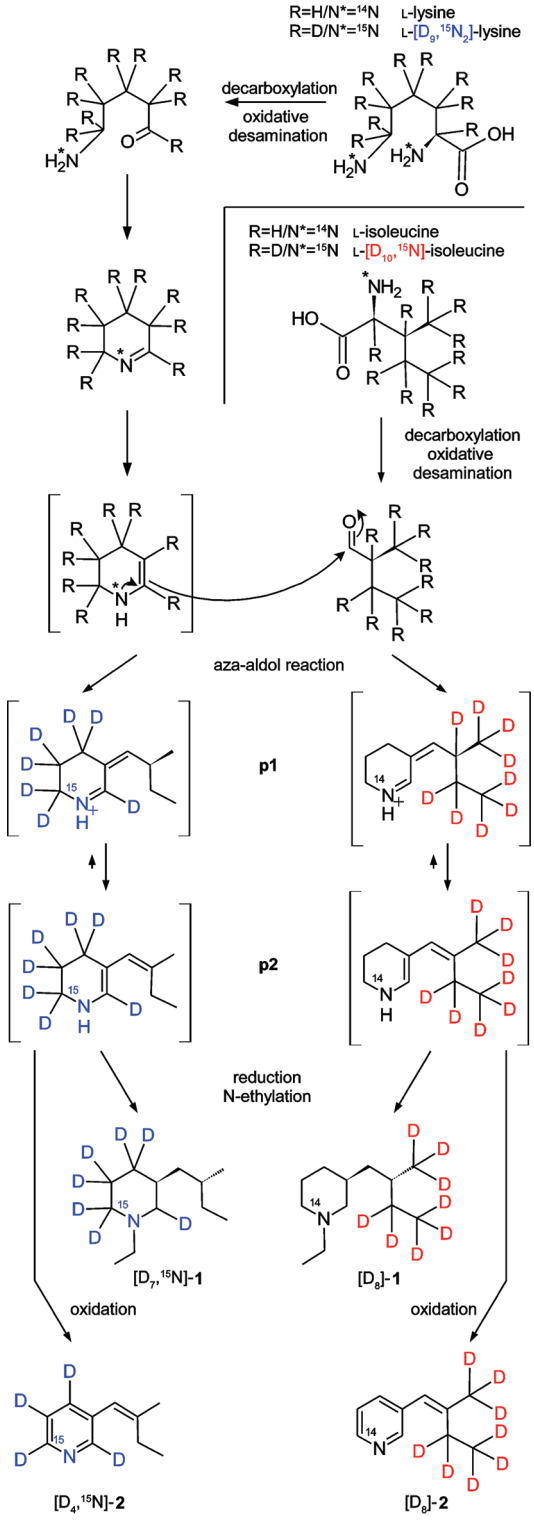

Figure 4. Proposed biosynthetic pathway to stenusine (**1**) and (*Z*)-3-(2-methyl-1-butenyl)pyridine (**2**) in *Stenus similis*.

pyridine alkaloid by oxidation of a piperideine precursor is uncommon.[10,11] A rare example of the formation of a pyridine from a piperideine ring is the biosynthesis of α,β-dipyridyl in the tobacco plant *Nicotiana tabacum* from anatabine.[11] A comparable step has been unknown in insects so far, but these results unambiguously demonstrate that it is possible in *Stenus* beetles.

(*Z*)-3-(2-Methyl-1-butenyl)pyridine (**2**) shows higher antimicrobial activity than stenusine (**1**).[3] Furthermore alkaloid **2** occurs among others in that fraction of *Stenus* species that are missing lateral tergite borders on all segments of the abdomen, a character that can be definitely classified as phylogenetically advanced.[5] In this way the formation of **2** in addition to **1** might have been an important step in the evolution of the pygidial gland secretion in the genus *Stenus*.

EXPERIMENTAL SECTION

General Experimental Procedures. Solvents and chemicals were obtained from the declared commercial suppliers and were used without further purification. Labeled amino acids L-Ile ($CD_3CD_2CD(CD_3)CD(^{15}NH_2)COOH$) and L-Lys·2HCl ($H_2{}^{15}N(CD_2)_4CD(^{15}NH_2)COOH\cdot 2HCl$) were purchased from Cambridge Isotopes Laboratories. Standard GC/MS analyses were performed on a Finnigan MAT GCQ ion trap system equipped with a BPX5 column (SGE, length 25 m, diameter 0.22 mm) with a He (purity 5.0) gas flow of 1 mL/min; oven temperature program: 50 °C (2 min), heating rate 10 °C/min to 280 °C.

Collecting and Keeping the Beetles. *S. similis* beetles were collected in fall 2010 and spring 2011 near Bayreuth, Germany (GPS 49°55′58″ N, 11°32′13″ E). The beetles were treated as described in Lusebrink et al. (2008).[6]

To get adapted to a fruit fly (*Drosophila melanogaster*) diet, the beetles were kept for at least four weeks in plastic boxes. The boxes were lined with gypsum containing 5% charcoal to prevent them from molting or contamination by microorganisms and to achieve a constant high humidity. Dead flies as well as their remains were removed continuously.

Preparation of the Amino Acid Solutions. For [D,^{15}N]-labeling experiments a supersaturated 10% (w/w) solution of deuterated L-Ile ($CD_3CD_2CD(CD_3)CD(^{15}NH_2)COOH$) was prepared in 0.9% saline. With deuterated L-Lys·2HCl ($H_2{}^{15}N(CD_2)_4CD(^{15}NH_2)COOH\cdot 2HCl$) a 50% (w/w) solution in 0.9% saline could be achieved because of its higher solubility in water.

Feeding Experiments and Extraction. The fruit fly adapted beetles were separated into Petri dishes (90 mm diameter), which were also gypsum lined. Living *D. melanogaster* were dipped into the labeled amino acid solutions and fed daily to the beetles. After two weeks of feeding the beetles were killed by freezing to −30 °C. The pygidial glands of each beetle were dissected and transferred into conical glass vials (1 mL, Macherey-Nagel) containing 5 μL of EtOAc SupraSolv (Merk). The glands were homogenized by sonication for 5 min, and 0.5 μL of the sample was immediately injected into the GC/MS.

Determination of the Incorporation Rate. To establish the incorporation rate, single ion monitoring (SIM) analyses were performed recording only the ions at *m*/*z* 137 ([D]-labeled product) and *m*/*z* 132 (native product), and the peaks were integrated. The incorporation rate was calculated by comparing the area of the peaks.

High-Resolution LC/MS/MS of p1b. Five *S. similis* pygidial gland systems were extracted with 60 μL of ACN (Sigma)/H_2O (1:1) containing 0.1% HCOOH (Sigma). A sample of 5 μL of the extract was injected into a HPLC (Dionex Ultimate 3000; LC-column: RP-8, 3 μM, 2.1 × 150 mm, Acclaim 120 Dionex) with a solvent flow of 0.25 mL/min; program: solvent 95% A, 5% B for 5 min; in 30 min to 0% A, 100% B; for 25 min 0% A, 100% B. Solvent A: H_2O + 0.1% HCOOH; solvent B: 80% ACN/20% H_2O + 0.1% HCOOH. HRESIMS were obtained on a coupled microTOF-Q II device (Bruker Daltonics, Bremen, Germany). MS/MS: coll. energy 35 eV; collison gas N_2. t_R = 16.4 min, HRESIMS *m*/*z* $[M + H]^+$ 152.1423 (calcd for $C_{10}H_{18}N$, 152.1439).

Reduction of the Precursor Molecule p1b with $NaBD_4$. One *S. similis* pygidial gland system was dissected, transferred into 10 μL of MeOH (Roth), and homogenized by sonication for 5 min. To this

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suspension was added a small amount of $NaBD_4$ (Fluka). After one hour of shaking at 20 °C the solid parts were separated by centrifugation (3000 rpm, 20 °C). A 1 μL sample of the clear supernatant was injected into the GC/MS and analyzed in the same manner as the EtOAc gland extracts.

■ ASSOCIATED CONTENT

Supporting Information. Selected-ion retrieval chromatograms of labeled and unlabeled *S. similis* pygidial gland secretion, full-scan EIMS of stenusine (**1**), (*Z*)-3-(2-methyl-1-butenyl)pyridine (**2**), and [D,^{15}N]-labeled stenusine (**1**), and a high-resolution HPLC/ESI-MS/MS of unlabeled metabolite **p1b** are available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Tel: +49 921-552734. Fax: +49 921-552743. E-mail: andreas.schierling@uni-bayreuth.de.

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