Storage and release of hydrogen cyanide in a chelicerate (*Oribatula tibialis*)

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Cyanogenesis denotes a chemical defensive strategy where hydrogen cyanide (HCN, hydrocyanic or prussic acid) is produced, stored, and released toward an attacking enemy. The high toxicity and volatility of HCN requires both chemical stabilization for storage and prevention of accidental self-poisoning. The few known cyanogenic animals are exclusively mandibulate arthropods (certain myriapods and insects) that store HCN as cyanogenic glycosides, lipids, or cyanohydrins. Here, we show that cyanogenesis has also evolved in the speciose Chelicerata. The oribatid mite *Oribatula tibialis* uses the cyanogenic aromatic ester mandelonitrile hexanoate (MNH) for HCN storage, which degrades via two different pathways, both of which release HCN. MNH is emitted from exocrine opisthonotal oil glands, which are potent organs for chemical defense in most oribatid mites.

chemical defense | cyanogenesis | Oribatida | *Oribatula tibialis* | toxin

Chemical substances are of utmost importance in biotic interactions among plants and their herbivores/pathogens as well as among animals and their predators/parasites (1, 2). Many of these semiochemicals are emitted for defense, and one of the most deterring and toxic biogenic substances known is hydrogen cyanide (HCN, also known as hydrocyanic or prussic acid). This asphyxiant poison inhibits the cytochrome oxidase enzyme, resulting in the inability of organisms to use oxygen (3).

Biosynthesis and liberation of HCN (known as cyanogenesis) is widespread among plants, but in animals it is relatively rare. Whereas the earliest reports of HCN in arthropods are from the late 19th century (4), it was only in the early 1960s (5–8) that comprehensive chemical ecology studies began to reveal cyanogenesis as a defensive strategy of a few mandibulate arthropods, including certain species of myriapods, beetles, true bugs, and butterflies (2, 9–12). More recently, the genomic basis of cyanogenesis has been explored (13, 14).

The rarity of cyanogenesis in mandibulate arthropods (2) and its supposed absence in the other speciose arthropod subphylum, Chelicerata, may relate to the evolutionary challenge posed by using a universal toxin in defense: self-poisoning must be prevented by storing the highly volatile HCN as a safe carrier molecule or storage molecule. In case of threat or attack, the cyanogenic compounds are discharged and must be quickly degradable to release HCN. Known HCN storage compounds of mandibulate arthropods include aromatic or aliphatic glycosides, lipids, and cyanohydrins (e.g., mandelonitrile) (2, 12, 15).

Although no cyanogenic species has been known among Chelicerata, chemical defense is widespread in the group, particularly among arachnids such as whip scorpions (16, 17), harvestmen (18, 19), certain spiders (20), and mites (21, 22). Here, we demonstrate cyanogenesis in a mite of the order Oribatida, a diverse and mostly soil-dwelling group of decomposers that discharge myriad defense-related semiochemicals from a pair of large exocrine opisthonotal oil glands (22–28). The common and widespread species *Oribatula tibialis* stores HCN as the natural product mandelonitrile hexanoate (MNH) and releases HCN upon disturbance via two different chemical pathways.

Results and Discussion

Oil gland secretions of undisturbed *O. tibialis* contained β-pinene, octanoyl hexanoate, MNH, and an unknown compound of molecular weight M_r = 162 g/mol (Fig. 1*A*). Of these compounds, only the aromatic ester MNH is involved in cyanogenesis. After gentle mechanical disturbance, specimens of *O. tibialis* showed a reduced amount of MNH and the presence of an additional compound, not detected in undisturbed mites: benzoyl cyanide (Fig. 1*A*). In intensely disturbed specimens, only traces of MNH were measured, but equimolar amounts of benzoyl cyanide and a mixture of benzoic acid and hexanoic acid were detected. Direct contact with moisture resulted in hydrolysis of MNH to hexanoic acid, benzaldehyde, and HCN (Fig. 1*B*).

Release of Hydrogen Cyanide by MNH Degradation. The detected compounds indicate that HCN is released, and we propose the following two degradation pathways of mandelonitrile hexanoate (Fig. 2). ***Pathway 1.*** Mechanical disturbance of *O. tibialis* results in the active expulsion of oil gland contents, including MNH. MNH is cleaved and catalytically oxidized to benzoyl cyanide and hexanoic acid on the mite's body surface. These two products are measurable quickly after disturbance in mite whole-body extracts, indicating rapid and spontaneous chemical reactions. The mechanism of this breakdown is unknown, but it has been shown that mites possess surface-associated enzymes that contribute to previously unsuspected chemical reactions (29–31). Finally, when exposed to water in the humid environment, benzoyl cyanide hydrolyzes to benzoic acid and HCN (32, 33).

Significance

Hydrogen cyanide (HCN) is highly volatile and among the most toxic substances known, being lethal to humans at a dosage of 1–2 mg/kg body weight. HCN blocks the respiratory chain and prevents aerobic organisms from using oxygen. In nature, HCN is produced by numerous plants that store it mainly as glycosides. Among animals, cyanogenesis is a defensive strategy that has seemed restricted to a few mandibulate arthropods (certain insects, millipedes, and centipedes), which evolved ways to store HCN in the form of stable and less volatile molecules. We found an instance of cyanogenesis in the phylogenetically distant group Chelicerata ("spider-like" arthropods), involving an aromatic ester for stable HCN storage and two degradation pathways that release HCN.

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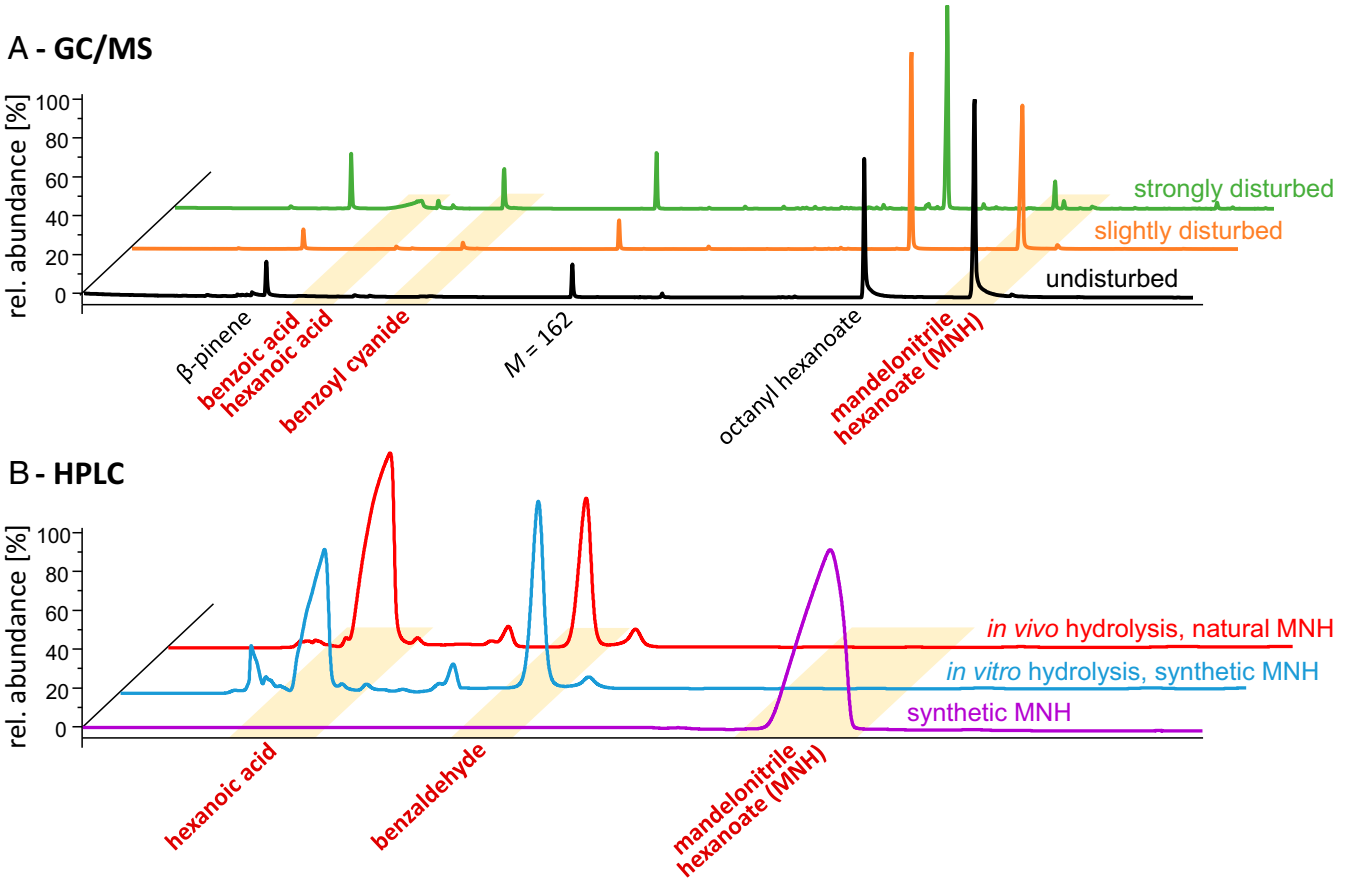

Fig. 1. GC/MS (*A*) chromatograms of oil gland secretions of *Oribatula tibialis* and HPLC (*B*) chromatograms of mandelonitrile hexanoate (MNH) hydrolysis. (*A*) Black: profile of secretions from undisturbed mites; orange: profile of slightly disturbed mites (brush stimulus); green: profile of strongly disturbed mites (vortex mixer). (*B*) Lilac: synthetic MNH; blue: in vitro hydrolysis of synthetic MNH; red: in vivo hydrolysis of natural MNH. Yellow bands highlight compounds involved in cyanogenesis.

Pathway 2. A second breakdown reaction directly releases HCN from MNH and is based on the hydrolysis of the ester bond in MNH in the presence of moisture without oxidation. MNH is spontaneously cleaved by an addition–elimination reaction resulting in the release of HCN and the residual products benzaldehyde and hexanoic acid. Compared with pathway 1 the reaction is slower, but MNH is continuously hydrolyzed to HCN and the byproducts (Fig. 1).

These results demonstrate that *O. tibialis* solved the problem of storing and releasing HCN in a previously unknown manner, using the natural cyanogenic compound MNH, which shows both low volatility and high stability in the water-free chemical environment of the oil gland reservoirs. The major constituents of this ester are mandelonitrile and hexanoic acid. Mandelonitrile (or its oxygenated form benzoyl cyanide) is probably derived from the aromatic amino acid phenylalanine (34, 35), whereas hexanoic acid is most probably synthesized de novo using acetyl–CoA in a Claisen condensation (36, 37).

HCN in Predator Defense. The large oil glands, which represent the major exocrine system in oribatid mites and which secrete MNH in *O. tibialis*, are potent organs for chemical defense, thus playing an important role in the structuring of feeding interactions in soil food webs (24–26). As with arthropod defensive compounds in general, most known oil gland secretions of oribatid mites—mainly hydrocarbons, terpenes, aromatics, esters, and alkaloids—are probably "class II compounds" (38); i.e., they irritate or repel potential predators without substantial harm (22–28). By contrast, benzoyl cyanide and HCN are "class I compounds" (38), true and harmful toxins (3, 6, 39, 40). The amount of MNH produced by *O. tibialis* is about 1.0–1.5 ng per individual mite (estimated by GC/MS), resulting in 0.1–0.15 ng HCN, if MNH is completely degraded (LD_{50}, rat, oral: 5 ng/mg (41)).

Because mites are highly derived chelicerates, and because *O. tibialis* is a member of the highly derived oribatid mite family Oribatulidae (42, 43), we believe cyanogenesis is a relatively recent defense in Chelicerata. Considering its scattered phylogenetic distribution, HCN-based chemical defense obviously evolved multiple times in arthropods. Our findings highlight how convergence on a simple, yet particularly effective, chemical defense has taken different evolutionary pathways, leaving as footprints a diversity of storage compounds for this simple, well-known poison.

Materials and Methods

Adult specimens of *Oribatula tibialis* (Nicolet) were collected from moss and litter taken from a mixed forest stand near Groß-Gerau, Hesse, Germany, using a thermal-gradient extractor over 24 h (for further methods and habitat characterization, see 44).

Oil gland exudates were extracted by submersing groups of 15–45 living mites in 20–50 μL solvent (hexane or water), a well-established method to obtain oil gland components (21, 28, 29, 45). After 3 min the solvent was separated from the mites. The two extraction solvents related to tests of two potential defense reactions—in water-free or humid conditions—and their corresponding MNH breakdown pathways. (*i*) To simulate predator attacks, without aqueous saliva contact, mites were mechanically stressed using a fine brush (equals slight disturbance) or briefly shaken in an otherwise empty GC vial using a vortex mixer (equals strong disturbance) before hexane extraction. (*ii*) To simulate a predator attack with aqueous saliva contact, mites were submerged in water. As a control, mites were directly immersed in hexane without mechanical stimulation.

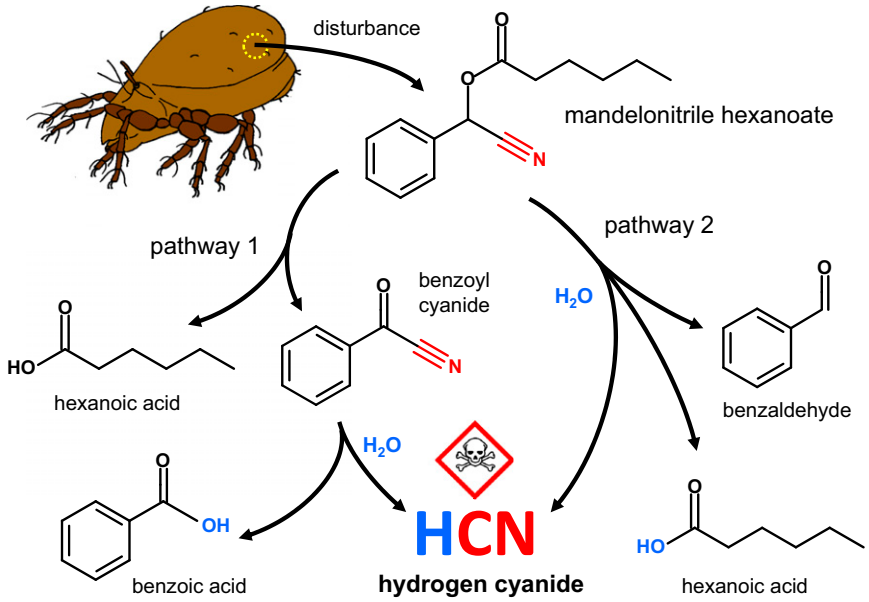

Fig. 2. Expulsion and degradation of MNH from opisthonotal oil glands in the oribatid mite, *Oribatula tibialis*. Pathway 1: MNH is cleaved by a catalytic oxidation to benzoyl cyanide and hexanoic acid on the mite´s body surface. Subsequently, benzoyl cyanide hydrolyzes to benzoic acid and HCN. Pathway 2: Direct hydrolysis of the ester bond in MNH in the presence of moisture, resulting in the release of HCN, benzaldehyde, and hexanoic acid.

In addition, synthetic MNH (see below) was directly hydrolyzed in water as an in vitro control (Fig. 1). Crude hexane sample aliquots (2–5 μL) were analyzed with a GC/MS-QP2010 Ultra gas-chromatography mass-spectrometry system (Shimadzu) equipped with a ZB-5MS column (Phenomenex) according to a protocol given elsewhere (45). Electron ionization mass spectra were recorded at 70 eV from *m/z* = 40–400. Crude aqueous extracts of the mites (in vivo) and 1 μL synthetic MNH (in 50 μL water; in vitro) were further hydrolyzed from 30 min up to 1 h. The breakdown of MNH was monitored using an Agilent 1100 HPLC system (Agilent Technologies; Fig. 1). The separation of 20-μL sample aliquots was performed using an isocratic elution with 40% phosphate-buffer (pH = 3.2) and 60% methanol:water mixture (85:15; vol/vol) for 10 min with a flow rate of 1 mL/min, on a Discovery HS C18 (15 cm × 4 mm, 5 μm) column (Sigma-Aldrich), at 25 °C. Chromatograms were recorded using a VWD-UV/VIS detector at λ = 245 nm (0–5 min) and at λ = 490 nm (5–10 min). Retention indices (46) and literature mass spectra (47, 48) for GC, as well as authentic standards for GC and HPLC, were used for compound identification.

Mandelonitrile hexanoate (MNH; IUPAC name is Cyano-(phenyl)-methyl hexanoate) was synthesized from mandelonitrile (2-hydroxy–2-phenyl-acetonitrile) and hexanoyl chloride by an Einhorn acylation (49): 1 g (0.9 mL, 7.5 mmol, 1 eq) mandelontrile was dissolved in 6 mL pyridine. Two grams (4.2 mL, 15 mmol, 2 eq) hexanoyl chloride were added drop-wise while stirring at 0 °C. After heating the mixture for 10 min to 50 °C under exclusion of moisture (using a drying tube filled with anhydrous $CaCl_2$), the reaction was terminated with ice water. HCl was added, and the mixture was extracted with hexane. The organic phase was washed three times with saturated sodium bicarbonate solution and dried with sodium sulfate. Finally, hexane was removed under a nitrogen atmosphere, resulting in 1.2 g crude, unpurified product (yield of ∼50%, containing hexane and carboxylic acid derivatives as byproducts, calculated based on NMR data; see Figs. S3 and S4). This residual product was partly redissolved in hexane and stored at −28 °C until GC/MS or HPLC analyses and further experiments. For NMR spectroscopy the product was redissolved in $CDCl_3$ and measured directly afterward. All chemicals used for synthesis were analytical GC/MS grade (≥99.9%) purchased from Merck KGaA.

MNH was characterized by combining electron ionization mass spectrometry (EI-MS; for conditions, see above) and NMR spectroscopy. NMR spectroscopy was performed on a Bruker Avance DRX 500 spectrometer (Bruker Biospin). All ^1H and ^{13}C -NMR experiments were performed with standard conditions, using $CDCl_3$ as solvent. The chloroform signals were used as internal standard for ^1H- (7.20 ppm) and ^{13}C-NMR (77.20 ppm) spectra. The reliable assignment of all ^1H and ^{13}C-signals was ascertained from 2D NMR measurements (^{13}C-DEPT, ^{13}C-HMBC und ^{13}C-HSQC spectra). (Figs. S5 and S6) Raw data were processed with the MestReNova vers. 8.0 software (Mestrelab Research).

Mass spectrometric investigation resulted in the following mass signals (and their relative abundances): 231 (M^+, 9), 133 (53), 117 (46), 116 (93), 115 (36), 105 (36), 99 (100), 89 (39), 71 (55), 43 (68). The linear retention index (46) was 1730. The ^1H and ^{13}C NMR gave the following chemical shifts: ^1H 500 MHz, $CDCl_3$: 7.44 (2H, m), 7.38 (2H, m), 7.37 (1H, m), 6.36 (1H, s), 2.32 (2H, t), 1.58 (2H, quin), 1.22 (4H, m), 0.81 (3H, t); ^{13}C 125 MHz, $CDCl_3$: 171.9 (C), 132.1 (C), 130.4 (CH), 129.4 (2CH), 127.9 (2CH), 116.3 (C), 62.8 (CH), 33.8 (CH_2), 31.2 (CH_2), 24.5 (CH_2), 22.3 (CH_2), 14.0 (CH_3). Detailed mass spectrometric data of natural and synthetic MNH, as well as NMR data of synthetic MNH, are published as supporting information (Figs. S1–S6). Synthetic MNH (equals in vitro) dissolved in water for 30–60 min readily hydrolyzed to hexanoic acid, benzaldehyde, and HCN (Fig. 1).

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