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CHEMICAL DEFENSE OF GIANT SPRINGTAIL *Tetrodontophora bielanensis* (WAGA) (INSECTA: COLLEMBOLA)

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Abstract—The giant springtail, *Tetrodontophora bielanensis* (Waga), is characterized by integumental openings (pseudocells) from which small droplets of a sticky defensive fluid are secreted after molestation. The secretion originates initially from secretory cells below the pseudocellae; subsequent irritations result in release of hemolymph, which was identified by both chemical and microscopical methods as well as by scanning electron microscopy. Bioassays with topically treated ground beetles *Nebria brevicollis* showed that the pseudocellular fluid evokes a total disorientation and cleansing behavior of the beetle. The main constituents were identified as the following pyridopyrazines: 2,3-dimethoxypyrido[2,3-b]pyrazine (**1**), 3-isopropyl-2-methoxypyrido[2,3-b]pyrazine (**2**), and 2-methoxy-4*H*-pyrido[2,3-b]pyrazine-3-one (**3**). These alkaloids are mainly present in the pseudocellar fluids of female and male springtails but are absent in their food or feces. Minor amounts are found in the hemolymph of adults, while larvae contain traces of **2** only. All compounds were synthesized and tested for activity. In natural concentrations, the synthetic alkaloids elicited the same effects from the ground beetles as the pseudocellar fluid.

Key Words—Chemical defense, alkaloids, pyridopyrazines; Collembola, *Tetrodontophora bielanensis*, pseudocells, cleansing behavior, disorientation, 2,3-dimethoxypyrido[2,3-b]pyrazine, 3-isopropyl-2-methoxypyrido[2,3-b]pyrazine, 2-methoxy-4*H*-pyrido[2,3-b]pyrazine-3-one.

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INTRODUCTION

Apterygote insects represent an ecologically important group of mainly soil-, bark-, litter-, and moss-inhabiting arthropods that occur in a wide range of moist environments. With maximal densities of 53,000 individuals m^2, springtails (Collembola) in particular are often exceedingly abundant and are among the most important consumers in many soil ecosystems. Springtails and other apterygotes also represent important and readily available prey for many predatory mites, spiders, beetles, bugs, or hymenopteran species. According to the primitive character of this large group of mainly entognathous hexapods, apterygote insects were recently considered to be devoid of chemical defenses (Berenbaum and Seigler 1992). However, a careful literature survey amply reveals that chemical defense is widespread in both ento- and ectognathous hexapods. The weakly sclerotized Protura contain voluminous abdominal defensive glands and, like staphylinid beetles, can bend their abdominal tips toward the aggressor to emit an acidic sticky gland material (Berlese, 1909; Francois and Dallai, 1986). When molested springtails escape from potential predators by suddenly releasing their furca, which is held under tension beneath the abdomen, thus propelling themselves through the air. Many species of the eyeless soil-inhabiting Onychiuridae have lost their jumping ability for escape and instead may release sticky droplets of unknown chemical composition from small integumental openings called pseudocells (Hale and Smith, 1966; Koncek, 1924). These pseudocellar fluids may mechanically repel predators by contaminating their body and appendages. In *Onychiurus* species these fluids exhibit paralyzing activities on predatory mites (Karg, 1961) and effectively repel other springtails and millipedes (Simon, 1961). Aromatic alkaloids, 2,3-dimethoxypyrido[2,3-b]pyrazine (**1**) and 3-isopropyl-2-methoxypyrido[2,3-b]pyrazine (**2**), were identified in the extracts of whole *Tetrodontophora bielanensis* (Budesinsky et al., 1986). Since it has not yet been clarified whether the pseudocellar fluid of onychiuran Collembola represents a true secretion or simply hemolymph, we want to report here on the origin and depletion of these droplets in the giant (length 5–9 mm) collembolan species *Tetrodontophora bielanensis* (Waga). Moreover, unambiguous structural assignments of biologically active constituents of this fluid will be presented as well as bioassays using pseudocellar fluids and synthetic compounds in order to demonstrate the defensive activity of the constituents.

METHODS AND MATERIALS

Individuals (630) of the giant collembolan species *Tetrodontophora bielanensis* (Waga) were collected and sieved either from a beech-forest litter of the Czech Elbsandsteingebirge near Hrensko (altitude 250 m) or from litter of a pine–beech forest near Cierny Balog (Tatra, Slovakia; altitude 900 m). In the

laboratory most of the specimens were frozen or kept alive in small plastic boxes supplied with moistened plaster bottoms and soil (constant temperature control 12°C; light–dark 8:16). The springtails were fed with dried dog food and granulated pollen.

Whole specimens or dissected body parts of *T. bielanensis* were transfered to Gisin's fixation mixture (Gisin, 1960), dehydrated in a graded series of acetone, critical-point dried with carbon dioxide, and sputter coated with gold. Scanning electron microscopy (SEM) was carried out by using a Cambridge Stereoscan 90. For examination of the cuticular parts, fragments of *T. bielanensis* were previously treated with 5% potassium hydroxide for 24 hr.

Tiny droplets of pseudocellar fluid were secreted after slight molestations of springtails with a forceps, and the droplets were collected with microcapillaries. In order to obtain hemolymph, the collophore of previously frozen springtails was dissected and hemolymph samples were taken with a microcapillary. Collected fluids were used for microscopical examination (photomicroscope Olympus BH-2), Wright-staining (Clark, 1973), electrophoresis, GC-MS examination, and bioassays. One microliter of pseudocellar fluid or hemolymph of *T. bielanensis* was employed for each electrophoretic separation. For native disk PAGE the sample was mixed with 20 μl buffer (10 mM Tris HCl, 1 mM EDTA, 0.01% bromophenolblue), centrifuged at 13,000*g* for 30 min, and the supernatant diluted with buffer to give a sample–buffer ratio of 1:80 prior to electrophoresis. Electrophoresis was carried out at 10°C using the PhastSystem and PhastGel Gradient 8-25 (both Pharmacia). The gel was developed by silver staining (method 3 according to PhastGel Silver Kit instruction manual). For SDS disk PAGE samples were mixed with SDS-buffer (2.5% SDS, 5% mercaptoethanol), heated for 5 min at 80°C, centrifuged, diluted with SDS buffer, separated, and stained as above.

The ^{1}H and ^{13}C NMR spectra were obtained with Bruker WM 400 and AC 250P instruments with TMS as an internal standard. Mass spectra (70 eV) were obtained with a VG 70/250 S mass spectrometer coupled to a Hewlett-Packard HP 5890 A gas chromatograph. Gas chromatography was carried out with a Carlo-Erba Fractovap 2101 instrument equipped with a flame ionization detector and on-column injection. Separations were performed using a 30-m DB-5 (0.32 mm internal diameter (ID), thickness of decoding film (d_f) = 0.25 μm) fused silica column with hydrogen as the carrier gas. Melting points (uncorrected) were measured with an Ernst Leitz instrument. Column and thin-layer chromatography were performed on silica gel (70-230 mesh, Merck).

Syntheses of Pyridopyrazines 1–3

*1H,4H-pyrido[2,3-b]pyrazine-2,3-dione (**B**).* This alkaloid was prepared from 2,3-diaminopyridine (**A**) according to the method of Winterfield and Wildersohn (1970).

2,3-Dichloro-pyrido[2,3-b]pyrazine (E). A mixture of 320 mg (2 mmol) **B,** 1 ml $POCl_3$, and 200 mg triethyl amine was heated under reflux for 2.5 hr. The mixture was treated with ice water, neutralized with aq. NaOH solution and extracted with chloroform. After drying over $MgSO_4$ and removal of the solvent, the product was purified by column chromatography (silica) to yield 320 mg (80%) of the pure product. mp: 149°C [Lit. 144–145°C (Winterfeld and Wildersohn, 1970)]. ^{1}H NMR (250 MHz, $CDCl_3$): δ = 7.79 (dd, 1H, H-7, $J_{6,7}$ = 4.2 Hz, $J_{7,8}$ = 8.4 Hz), 8.42 (dd, 1H, H-8, $J_{6,8}$ = 1.8 Hz), 9.29 (dd, 1H, H-6). ^{13}C NMR (62.9 MHz, $CDCl_3$): δ = 126.29 (C-7), 130.18 (C-8a), 137.20 (C-8), 146.50 (C-4a), 148.75 (C-3), 154.13 (C-2), 154.92 (C-6).

2,3-Dimethoxy-pyrido[2,3-b]pyrazine (1): A mixture of 800 mg **E** and 5 ml 2 M sodium methoxide solution was heated under reflux for 1 hr. After cooling, the mixture was treated with water and extracted three times with chloroform. The combined organic phases were dried over $MgSO_4$ and the solvent removed. Column chromatography (silica, ethyl acetate–methanol 1 : 1) yielded the pure alkaloid **1** in 53% yield (400 mg). mp: 125°C. ^{1}H NMR (250 MHz, $CDCl_3$): δ = 4.18 (s, 3H, CH_3), 4.26 (s, 3H, CH_3), 7.48 (dd, 1H, H-7, $J_{6,7}$ = 4.4 Hz, $J_{7,8}$ = 8.0 Hz), 8.15 (dd, 1H, H-8, $J_{6,8}$ = 1.8 Hz), 8.78 (dd, 1H, H-6). ^{13}C NMR (62.9 MHz, $CDCl_3$): δ = 54.76 (q, CH_3, J = 147.8 Hz), 55.10 (q, CH_3, J = 147.8 Hz), 122.31 (C-7, J = 165.0 Hz, J = 9.0 Hz), 132.06 (C-8a, J = 8.6 Hz), 135.07 (C-8, J = 164.0 Hz, J = 6.4 Hz), 147.89 (C-4a, J = 13.0 Hz, J = 5.2 Hz), 149.06 (C-6, J = 179.3 Hz, J = 9.0 Hz, J = 3.3 Hz), 150.39 (C-3, J = 3.8 Hz), 152.39 (C-2, J = 3.8 Hz). The mass spectrum and formula for compound **1** are presented in Figure 5 below.

3-Isopropyl-1H-pyrido[2,3-b]pyrazine-2-one (F). A mixture of 1 g (9.3 mmol) **A** and 2 g (10.4 mmol) methyl 3-methyl-2-oxobutyrate (3 : 1 mixture with diethyl oxalate) (Weinstock et al., 1981) were heated under nitrogen for 1 hr to 100°C. After cooling, the residue was washed successively with pentane, chloroform and ethanol. A mixture of the regioisomeric ketones 3-isopropyl-1*H*-pyrido[2,3-b]pyrazine-2-one (**F**) and 2-isopropyl-4*H*-pyrido[2,3-b]pyrazine-3-one (**G**) was present in the chloroform and ethanol phases; the insoluble residue represents the dione **B.** After combining the chloroform and ethanol phases and removing the solvents, the ketones were separated by column chromatography (silica, 0.5% NH_4OH, ethyl acetate). 3-Isopropyl-1*H*-pyrido[2,3-b]pyrazine-2-one (**F**) R_f = 0.09 (ether), mp: 254–256°C, yield: 540 mg (31%). ^{1}H NMR (250 MHz, $CDCl_3$): δ = 1.37 (d, 6H CH_3, J = 6,8 Hz), 3.70 (sept, 1H, CH), 7.46 (dd, 1H, H-7, $J_{6,7}$ = 4.6 Hz, $J_{7,8}$ = 8.4 Hz), 7.75 (dd, 1H, H-8, $J_{6,8}$ = 1.6 Hz), 8.67 (dd, 1H, H-6), 12.5 (bs, 1H, NH). MS (70 eV): m/z(%) = 39 (18), 64 (14), 65 (17), 78 (6), 91 (12), 92 (12), 93 (12), 119 (13), 120 (14), 133 (10), 146 (100), 160 (27), 161 (50), 174 (78), 189 (62, M^+).

2-Isopropyl-4*H*-pyrido[2,3-b]pyrazine-3-one (**G**) R_f = 0.67 (ether), mp: 193–194°C, yield: 720 mg (41%). ^{1}H NMR (250 MHz, $CDCl_3$): δ = 1.34 (d, 6H, CH_3, J = 6,8 Hz), 3.64 (sept, 1H, CH), 7.33 (dd, 1H, H-7, $J_{6,7}$ = 4.8 Hz, $J_{7,8}$ = 8.0 Hz), 8.17 (dd, 1H, H-8, $J_{6,8}$ = 1.6 Hz), 8.59 (dd, 1H, H-6), 12.5 (bs, 1H, NH). MS (70 eV): m/z(%) = 39 (18), 64 (10), 66 (12), 78 (8), 93 (14), 120 (22), 133 (8), 146 (100), 161 (87), 174 (58), 189 (75, M^+).

3-Isopropyl-2-methoxy-pyrido[2,3-b]pyrazine (2). As described above in the synthesis of **1,** the ketone **F** was converted into 2-chloro-3-isopropyl-pyrido[2,3-b]pyrazine [**H,** 85% yield; ^{1}H NMR (250 MHz, $CDCl_3$): δ = 1.49 (d, 6H, CH_3, J = 6,6 Hz), 3.77 (sept, 1H, CH), 7.89 (dd, 1H, H-7, $J_{6,7}$ = 4.4 Hz, $J_{7,8}$ = 8.4 Hz), 8.56 (dd, 1H, H-8, $J_{6,8}$ = 1.6 Hz), 9.26 (dd, 1H, H-6)], which was subsequently converted to **2.** ^{1}H NMR (250 MHz, $CDCl_3$): δ = 1.41 (d, 6H, CH_3, J = 6.8 Hz), 3.55 (sept, 1H, CH), 4.14 (s, 3H, CH_3), 7.54 (dd, 1H, H-7, $J_{6,7}$ = 4.4 Hz, $J_{7,8}$ = 8.2 Hz), 8.16 (dd, 1H, H-8, $J_{6,8}$ = 1.8 Hz), 8.87 (dd, 1H, H-6). ^{13}C NMR (62.9 MHz, $CDCl_3$): δ = 20.30 (2 CH_3), 30.78 (CH), 54.14 (OCH_3), 124.16 (C-7), 134.41 (C-8a), 135.51 (C-8), 148.57 (C-4a), 149.29 (C-6), 156.13 (C-2), 159.43 (C-3). The mass spectrum (70 eV) and formula for compound **2** are presented in Figure 5 below.

2-Methoxy-4H-pyrido[2,3-b]pyrazine-3-one (3). A mixture of 1 g (9.1 mmol) **A,** 1.85 g (18.3 mmol) triethyl amine, 1.56 g (8.3 mmol) methyl 2,2-dichloro-2-methoxyacetate and 20 ml absolute toluene was heated under nitrogen to 100°C. After 1 hr the cooled mixture was filtrated and the residue subsequently washed with chloroform and water. The brownish residue contained a 9:1 mixture of the two regioisomers 2-methoxy-4*H*-pyrido[2,3-b]pyrazine-3-one (**I** = **3**) and 3-methoxy-1*H*-pyrido[2,3-b]pyrazine-2-one (**II**), which were separated by column chromatography (silica, methanol–triethyl amine, 99:1). The products were insoluble in most organic solvents with the exception of DMSO and pyridine. Structural assignments of the products was achieved by the use of ^{1}H, ^{13}C NMR coupling obtained with gated-decoupled ^{13}C NMR-spectra (see below). 2-Methoxy-4*H*-pyrido[2,3-b]pyrazine-3-one (**3**) yield: 420 mg, 34%. ^{1}H NMR (400 MHz, [D_5]-pyridine): δ = 4.02 (s, 3H CH_3), 7.19 (dd, 1H, H-7, $J_{6,7}$ = 5.0 Hz, $J_{7,8}$ = 8.0 Hz), 7.97 (dd, 1H, H-8, $J_{6,8}$ = 1.6 Hz), 8.50 (dd, 1H, H-6). ^{13}C NMR (100 MHz, [D_5]-pyridine): δ = 54.65 (OCH_3), 119.58 (C-7), 126.79 (C-8a), 134.08 (C-8), 144.32 (C-4a), 146.69 (C-6), 152.81 (C-2), 156.64 (C-3). The mass spectrum of compound **3** is shown in Figure 5 below. 3-Methoxy-1*H*-pyrido[2,3-b]pyrazine-2-one (**II**) yield: 50 mg, 4%. ^{1}H NMR (400 MHz, [D_5]-pyridine): δ = 4.09 (s, 3H, CH_3), 7.28 (dd, 1H, H-7, $J_{6,7}$ = 4.6 Hz, $J_{7,8}$ = 8.0 Hz), 7.70 (dd, 1H, H-8, $J_{6,8}$ = 1.6 Hz), 8.63 (dd, 1H, H-6).

1,4-Dimethyl-1H,4H-pyrido[2,3-b]pyrazine-2,3-dione (C). To a solution of 100 mg (0.63 mmol) **B** in 20 ml absolute DMSO were added 40 mg (1.66 mmol) sodium hydride. A whitish precipitate was formed with hydrogen release.

After the gas formation had ceased, 200 mg (1.4 mmol) methyl iodide were added. The precipitate dissolved, and the solution became reddish. The reaction mixture was then treated with 30 ml brine. After extraction with methylene chloride the combined organic phases were dried with $MgSO_4$. The residue obtained by filtration and removal of the solvent was subjected to column chromatography to yield 60 mg (50%) of a pure product (silica, ethyl acetate/0.5% NH_4OH). ^1H NMR (400 MHz, $CDCl_3$): δ = 3.67 (s, 3H, CH_3), 3.77 (s, 3H, CH_3), 7.28 (dd, 1H, H-7, $J_{6,7}$ = 4.2 Hz, $J_{7,8}$ = 8.0 Hz), 7.59 (dd, 1H, H-8, $J_{6,8}$ = 1.6 Hz), 8.30 (dd, 1H, H-6). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 28.33 (q, NCH_3, J = 143.0 Hz), 29.21 (q, NCH_3, J = 140.8 Hz), 119.04 (C-7, J = 166.0 Hz, J = 9.0 Hz), 121.56 (C-8, J = 164.1 Hz, J = 6.7 Hz, J = 1.0 Hz), 123.39 (m, C-8a), 138.63 (m, C-4a), 142.16 (C-6, J = 182.2 Hz, J = 7.6 Hz, J = 2.9 Hz), 153.33 (C-2), 154.50 (C-3). MS (70 eV): m/z (%) = 52 (12), 66 (8), 78 (15), 91 (9), 107 (8), 119 (6), 120 (12), 135 (8), 134 (82), 148 (12), 162 (28), 163 (22), 191 (100, M^+).

2-Methoxy-4-methyl-4H-pyrido[2,3-b]pyrazine-3-one (D). The *N*-methylated ketone **D** was obtained from **3** in an identical synthesis as described for **C**. Yield 63%. ^1H NMR (400 MHz, $CDCl_3$): δ = 3.85 (s, 3H, CH_3), 4.15 (s, 3H, CH_3), 7.26 (dd, 1H, H-7, $J_{6,7}$ = 4.2 Hz, $J_{7,8}$ = 8.0 Hz), 7.92 (dd, 1H, H-8, $J_{6,8}$ = 1.6 Hz), 8.43 (dd, 1H, H-6). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 28.17 (q, NCH_3, J = 142.8 Hz), 54.90 (q, NCH_3, J = 147.8 Hz), 119.73 (C-7, J = 164.0 Hz, J = 8.6 Hz), 126.76 (C-8a, J = 7.6 Hz), 134.54 (C-8, J = 163.0 Hz, J = 7.0 Hz), 143.17 (m, C-4a), 145.97 (C-6, J = 179.1 Hz, J = 7.6 Hz, J = 2.4 Hz), 152.02 (C-3), 154.77 (C-2). MS (70 eV): m/z(%) = 52 (11), 64 (14), 78 (12), 91 (28), 105 (8), 119 (28), 120 (23), 132 (14), 133 (10), 134 (14), 148 (29), 162 (55), 176 (17), 191 (100, M^+).

2-Quinoxalinole in acetone was used as an internal standard to quantify the total amount of pyridopyrazines in pseudocellar fluids and hemolymph samples (6 samples each) of three specimens of *T. bielanensis* by GC. The presence/absence and semiquantitative determination of the three pyridopyrazines within various compartments, feces, and food of *T. bielanensis* was tested by GC-MS (four samples in each case). Percentage of total peak areas of the pyridopyrazines (total ion chromatogram) reflect their proportion either within the hemolymph or pseudocellar fluids of females, males and larvae.

In order to test the biological significance of *T. bielanensis* pseudocellar fluid on natural predators, interactions of *Nebria brevicollis* ground beetles and the giant springtails were observed and analyzed qualitatively and quantitatively using a videocamera (JVC, GX-N8E). Single individuals of each species were placed within a light-exposed 6 × 6 plastic arena (moistened calcium sulfate bottom) and 20 interactions were recorded qualitatively by using a Panasonic NV-180EG recorder. Quantitation of deterrency was measured by placing 1 μl of pseudocellar fluid (*T. bielanensis*), hemolymph (*T. bielanensis*), or hemo-

lymph of darkling beetle, *Tenebrio molitor* (control), respectively at the mandible underside of a *N. brevicollis* beetle. Of the various behavioral reactions of topically treated ground beetles (10 specimens per tested fluid), disorientation behavior, cleansing behavior, and elytral movements were selected and quantified for 10 min after application of test fluids. The duration of a particular behavior was expressed as a percentage of the observation time with 10 min of video monitoring equal to 100%.

In the same way the percentage of time spent in cleansing behavior and disorientation was measured from mixed groups of carabid beetles (four specimens of *Nebria brevicollis*, three specimens of *Pterostichus oblongopunctatus*) within 10 min after having been topically treated with 1 μl of test fluids of authentic pyridopyrazines. Solutions (0.05, 0.1, and 0.5 M) of 2-methoxy-4*H*-pyrido[2,3-b]pyrazine-3-one, 2,3-dimethoxypyrido[2,3-b]pyrazine, and 3-isopropyl-2-methoxypyrido[2,3-b]pyrazine in DMSO were used.

RESULTS

Origin and Depletion of Droplets of T. bielanensis

After a slight mechanical molestation with forceps, specimens of *T. bielanensis* release sticky whitish droplets from integumental openings called pseudocells (Figure 1A,B). Compared to these pseudocellar fluids, hemolymph samples show a slight violet to whitish coloration. Pseudocellar droplets appear especially at the rear border of the tergites. A careful examination of this springtail species reveals the presence of numerous pseudocells on both its dorsal and ventral sclerites. The total number of dorsal pseudocells is 48, and they are located at antennal bases (three per half) and rear borders of the head (2), thoracic (3 × 2), and abdominal tergites (5 × 2; 1 × 3; Figure 1A,B). The 36 ventral pseudocells are associated with head, coxae, collophore, and abdominal sternites. Numbers and arrangement of pseudocells represent taxonomically important species-specific characters in onychophoran springtails.

The integument and pseudocells of *T. bielanensis* are surrounded by macrotubercles (Figure 2g–i), which are composed of numerous apically fused microtubercles (Figure 2a,b). Pseudocells show an oval shape (diameter about 20 μm; Figure 2a,e) and represent integumental depressions. Externally they are marked by 15–20 longitudinally interrupted ribs that are arranged on the surface of a longitudinally divided cap, also seen from the interior view (Figure 2e). Obviously the external ribs are composed of several fused microtubercles (Figure 2b). SEM examination of shedded old *T. bielanensis* cuticles revealed that the complete pseudocell is molted (deformated caps and surrounding macrotubercles).

Both cap halves and ribs do not survive treatment with 5% potassium

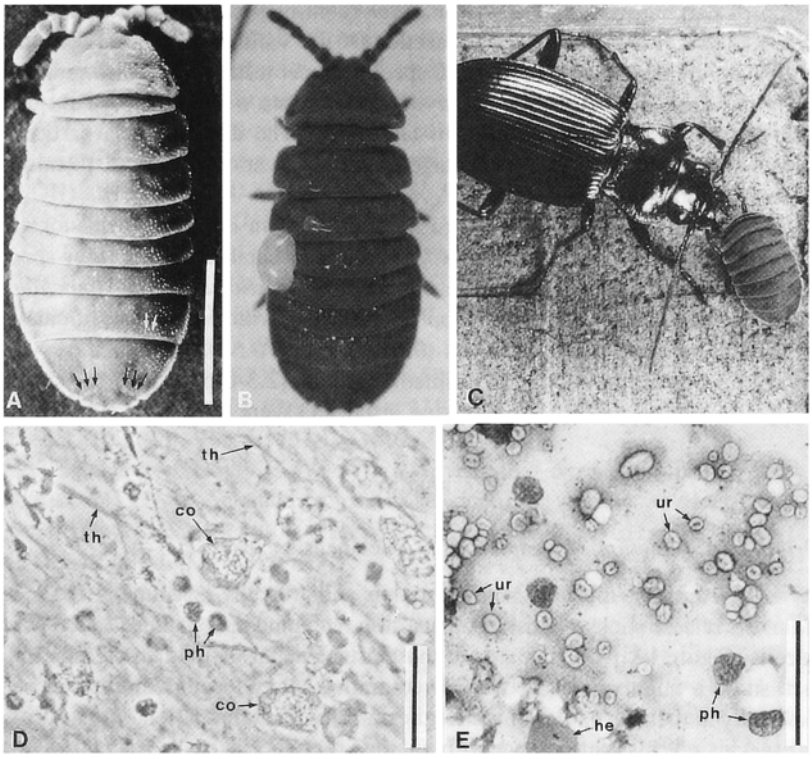

FIG. 1. Position of pseudocells in *T. bielanensis* (arrows) along posterior borders of tergites IV (two pseudocells marked) and V (six pseudocells marked) (A). Depletion of pseudocell secretion, and hemolymph at left posterior border of tergite I (B). Rolling-up behavior of *T. bielanensis* (right) after a slight contact with ground beetle *nebria brevicollis* (left) (C). Microscopic view of pseudocell secretion (D: phase contrast) and hemolymph (E: Wright stain). Abbreviations: co: coagulocytes, th: protein threads; ph: prohemocytes; ur: urate crystals; he: hemocyte. Bars in A B: 2 mm; in D, E: 20 μm.

hydroxide, which also destroys integumental macrotubercles (Figure 2c). Macerated pseudocells only are left as oval-shaped stable rings (Figure 2c–e), which serve as an entrenchment zone for the two cap halves in nonmacerated pseudocells. Specimens of *T. bielanensis* that were killed on Dry Ice exhibited pseudocells with a complete opening sequence (Figure 2a,f–i). At first the two cap halves broke longitudinally (Figure 2f), then pseudocellar fluid was squeezed out and both cap halves were pressed outward (Figure 2g). Later pseudocellar droplets may cover not only the external pseudocell area (Figure 2h) but are

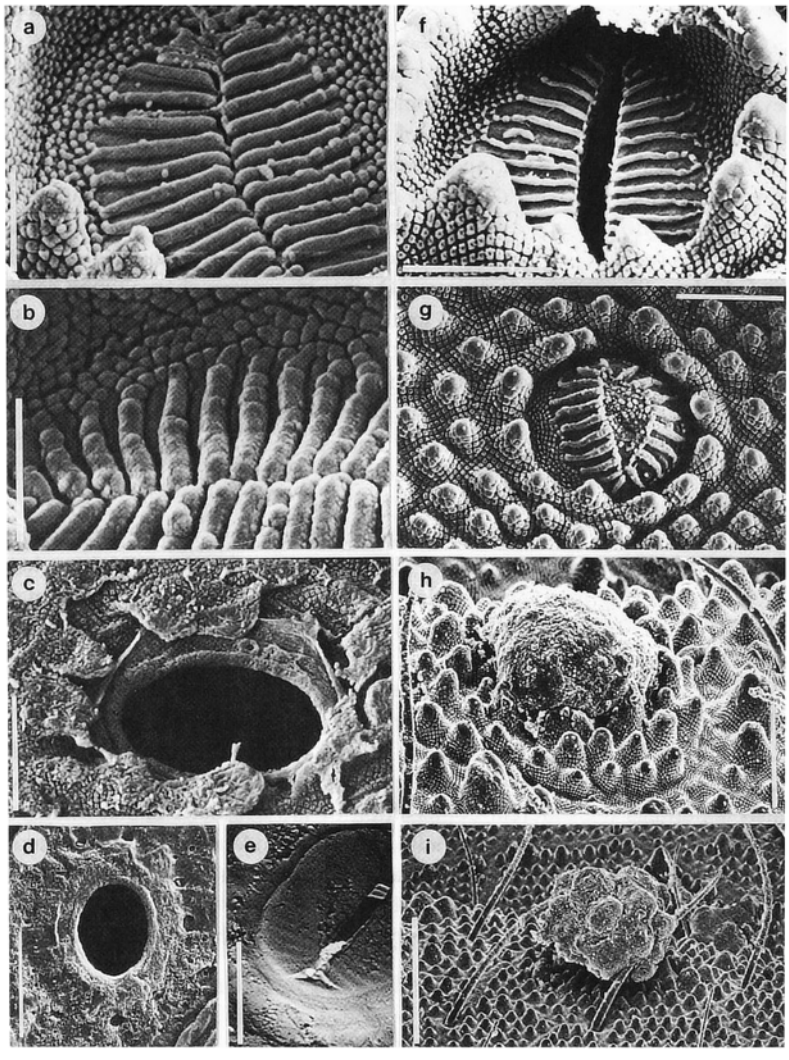

FIG. 2. SEM views of *T. bielanensis* pseudocells (a–i). Oval-shaped pseudocells are externally surrounded by microtubercles which fuse to produce 15–20 longitudinally interrupted ribs arranged on the surface of a longitudinally divided cap (a,b). External (c) and internal (d,e) view of a pseudocell without (e) and after (c,d) treatment with 5% KOH. Macerated pseudocells only remain as oval-shaped stable rings (c,d), which serve as an entrenchment zone for the two cap halves in nonmacerated pseudocells (e). Opening sequence (a,f–i) of a pseudocell (external view) with longitudinally breaking (f) and outward pressing (g) of cap halves and squeezing of pseudocellar secretion (h). Residue of previously squeezed pseudocellar droplets remain adhering at setae and macrotubercles of the collembolan integument (i).

even found side by side along the rear border of a sclerite. The remains of previously squeezed pseudocellar droplets may be found as amorphous material adhering to setae and macrotubercles of the collembolan integument (Figure 2i).

A microscopic investigation of hemolymph samples and pseudocellar fluid shows remarkable differences. Freshly collected pseudocellar fluid contains various prohemocytes (Figure 1D; ph: 7 μm diameter) and several irregular shaped coagulocytes (co: 20 μm diameter), which may quickly burst and thereby release clouded, threadlike filaments (Figure 1D, th). In contrast, hemolymph samples are characterized by many ellipsoid urate crystals (Figure 1E, ur: 2–5 μm diameter), lipid droplets, prohemocytes, and a few hemocytes (he) including coagulocytes. After repeated mechanical molestations, pseudocellar fluid contains urate crystals whereas coagulocytes are almost missing. This observation favors the idea that the composition of initially squeezed pseudocellar fluid differs from hemolymph samples. Only after repeated molestations do pseudocells seemingly exude droplets of hemolymph.

In order to test this hypothesis, peptide patterns of originally squeezed pseudocellar fluids were electrophoretically compared with collophore hemolymph droplets (Figure 3). Native disk PAGE exhibits about six common peptides (51, 68, 230, 240, 430, 770 kDa) in both fluids, whereas several peptides are exclusively present in one type of fluid only (Hemolymph: 85, 125, 190, 285, 290, 350, 510, 630 kDa; pseudocellar fluid: 66, 127, 560, 680 kDa). SDS disk PAGE results in nine common peptides (18, 23, 33, 42, 44, 48, 54, 58, 115). However, pseudocellar fluid (13.5, 16, 25, 68, 70, 77, 127, 135, 145) and hemolymph (22, 38, 39, 65, 73) exclusively contain five to nine peptides, which underlines the different composition of the two collembolan fluids. We conclude, therefore, that the initially emitted pseudocellar fluid represents a secretion distinctly different from hemolymph; removing this secretion with a piece of filter paper and subsequent molestation of the *Tetrodontophora* specimen results in emission of tiny hemolymph droplets through the pseudocellar orifice.

Chemical Constituents

A GC-MS investigation of the pseudocellar fluid revealed the presence of three alkaloids and common C_{16} and C_{18} fatty acids (Figure 4). According to their mass spectra, two of the alkaloids were identified as 2,3-dimethoxypyrido[2,3-b]pyrazine (**1**) and 3-isopropyl-2-methoxypyrido[2,3-b]pyrazine (**2**), both of which had been previously isolated from whole body extracts of *T. bielanensis* (Budesinsky et al., 1986). The third, previously unknown alkaloid, exhibited the mass spectrum shown in Figure 5 with a molecular ion at m/z = 177 (14 mass units less than **1**). A high-resolution mass spectrum proved its composition to be $C_8H_7N_3O_2$.

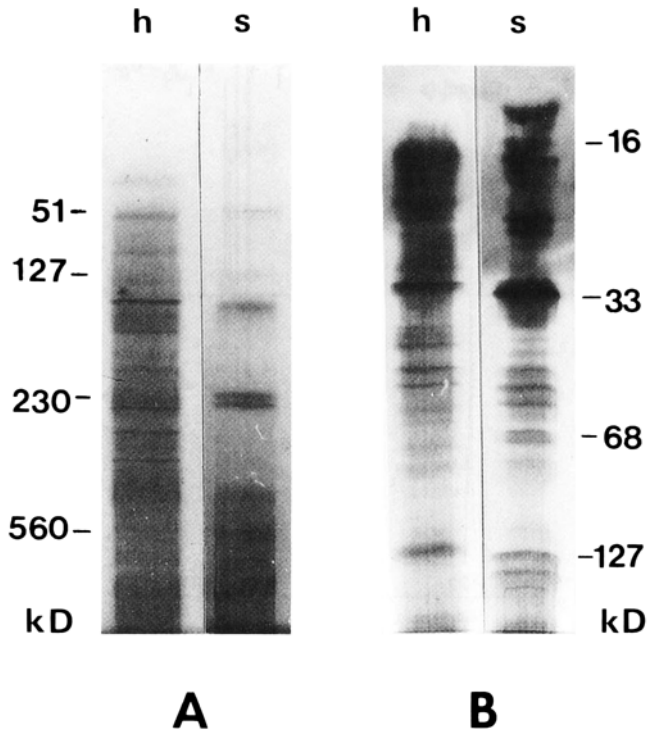

FIG. 3. Native disk PAGE (A) and SDS-PAGE (B) of *Tetrodontophora bielanensis* hemolymph samples (h) and pseudocellar fluid (s). Dilution of each body fluid 1:80 (applied volume 1 μl). Inserted molecular masses of proteins were determined by using high- (A) and low-molecular-weight reference proteins.

Apart from the singlets of the methoxy groups of **1** and **2**, the ^{1}H NMR spectrum of the pseudocellar fluid showed an additional singlet at 4.06 ppm that could also be attributed to a methoxy group. This led to the conclusion that the unknown alkaloid was a demethylated analog of **1**. To elucidate the position of the remaining methoxy group in **3** and to prove its structure, a synthesis of the two possible methoxypyrido[2,3-b]pyrazinones was carried out. According to the method of Anschütz (1906) for the synthesis of imidates, ethyl dichloromethoxyacetate was heated with 2,3-diaminopyridine (**A**) in the presence of triethyl amine in toluene. The product proved to be a 9:1 mixture of the two regioisomers **I** and **II** of methoxypyrido[2,3-b]pyrazinone, of which the major compound **I** was identical with the unknown natural product **3**. Structural assignment of the synthetic products was achieved by use of gated-decoupled ^{13}C

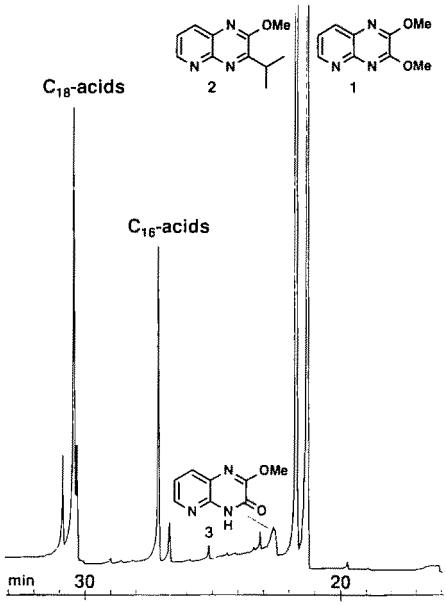

FIG. 4. Gas chromatogram of pseudocellar fluid from *Tetrodontophora bielanensis*, 30-m DB-5, temperature programmed from 60°C to 300°C at 5°C/min, on-column injection.

NMR spectroscopy of *N*-methylated compounds. These derivatives were obtained by reaction of NaH/DMSO/MeI with **I** or 1*H*,4*H*-pyrido[2,3-b]pyrazine-2,3-dione [**B**, obtained by reaction of 2,3-diaminopyridine (**A**) with diethyl oxalate according to the procedure of Weinstock et al. (1981)]. The bridging C atoms are found between 123 and 133 ppm for C-8a and 140 and 150 ppm for C-4a in the ^{13}C NMR spectra of pyrido[2,3-b]pyrazines synthesized by us and other authors (Nagel et al., 1979). The signal for C-4a in the dimethoxy compound **1** shows a quartet with $J_{C,H}$ = 5 and 11 Hz while C-8a is a doublet with $J_{C,H}$ = 8 Hz (see Figure 6A). On the other hand, the C-4a and C-8a signals of 1,4-dimethyl-1*H*,4*H*-pyrido[2,3-b]pyrazine-2,3-dione (**C**) are degenerated to broad multiplets due to coupling to the H-atoms of the *N*-methyl groups (see Figure 6C).

By *N*-methylation of **I** the derivative **D** is obtained, and its spectrum shows signals of C-8a as a doublet with J = 8 Hz and of C-4a a broad multiplet (see Figure 6B). This reveals that C-4a is coupling to the *N*-methyl group, which is thus located at N-4. Therefore, the alkaloid **I** is 2-methoxy-4*H*-pyrido[2,3-b]pyrazine-3-one, the natural alkaloid **3.** This is in accordance with the finding of **I** as the main product in the synthesis, since the more nucleophilic 2-amino

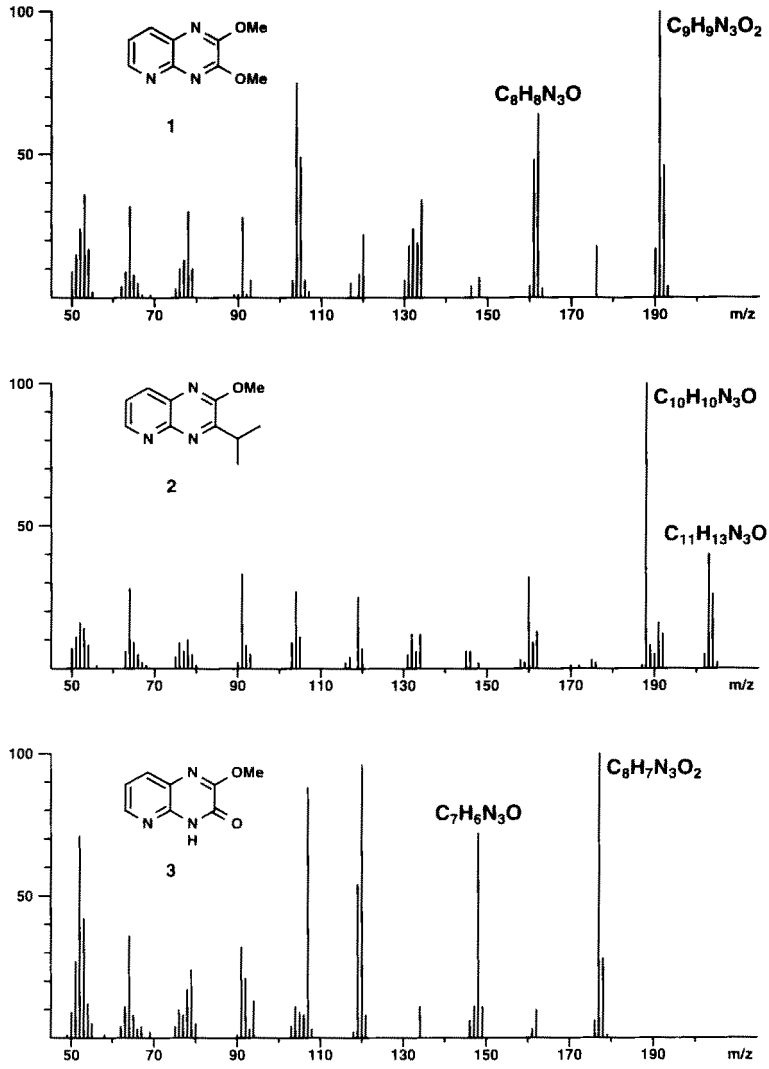

FIG. 5. Mass spectra of the pyridopyrazines **1**, **2**, and **3**.

group of 2,3-diaminopyridine reacts preferentially with the more reactive dichloromethoxymethyl group of the condensing acetate. For bioassays, the two alkaloids **1** and **2** were prepared for the first time starting from 2,3-diaminopyridine (**A**, see Figure 7). Condensation with diethyl oxalate yields the dione **B**, which could not be selectively *O*-methylated using diazomethane, trimethyl-

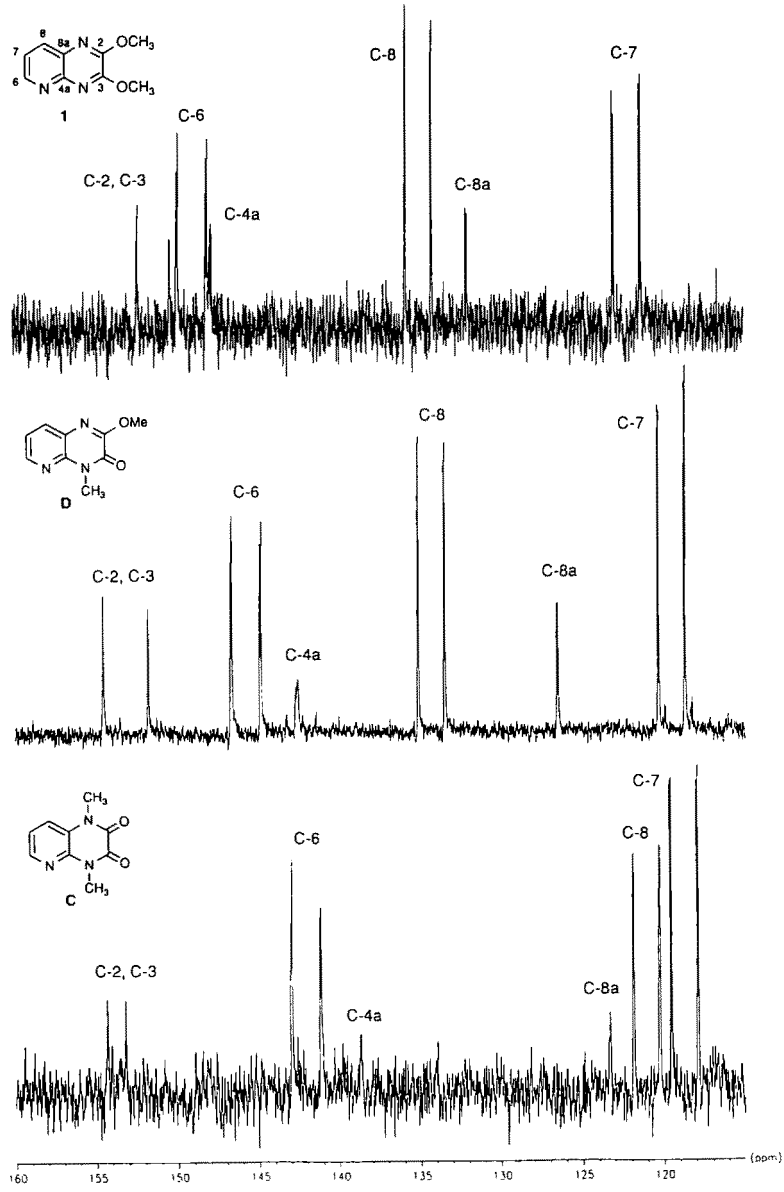

FIG. 6. Gated-decoupled ^{13}C NMR spectra of: 2,3-dimethoxypyrido[2,3-b]pyrazine (**1**), 4-methyl-2-methoxy-4*H*-pyrido[2,3-b]pyrazine-3-one (**D**), and 1,4-dimethyl-1*H*,4*H*-pyrido[2,3-b]pyrazine-2,3-dione (**C**).

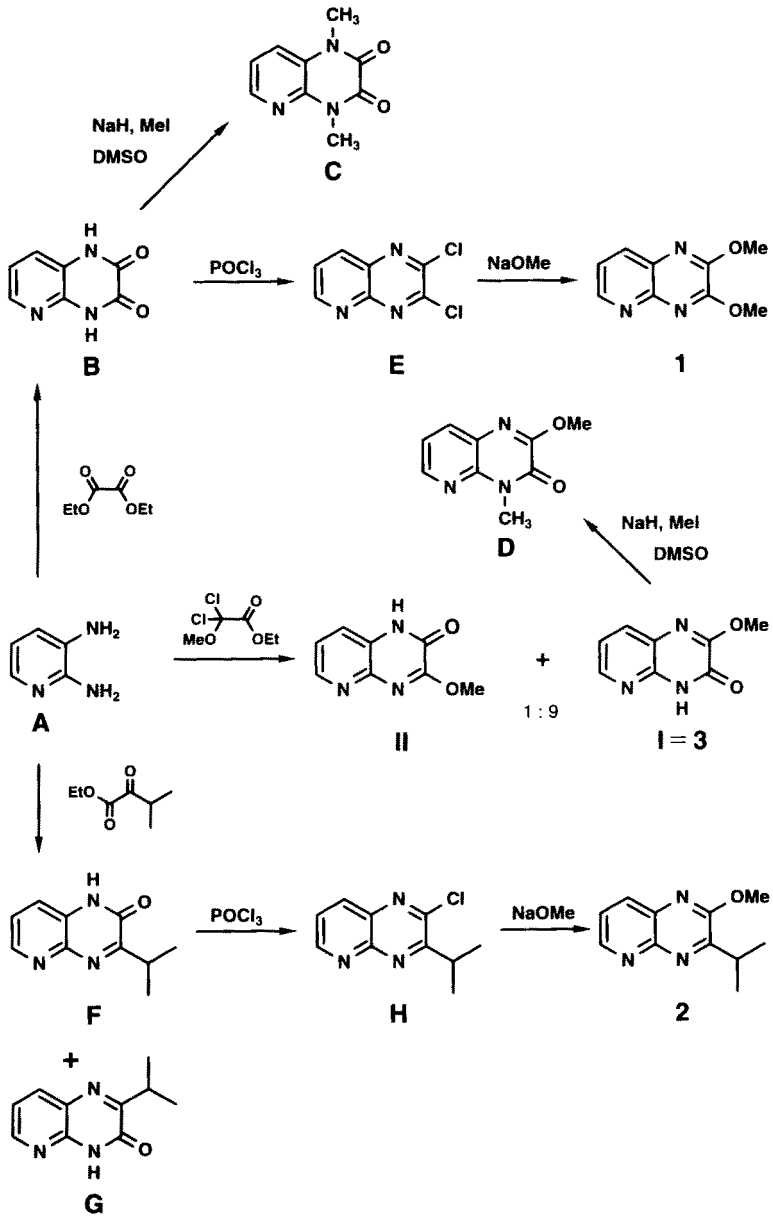

FIG. 7. Synthesis of the pyridopyrazines **1**, **2**, **3**, **C**, and **D**.

oxonium tetrafluoroborate, or Ag_2O/MeI. The results of the methylations are similar to those obtained by Cheeseman in the methylation of hydroxyquinoxalines (Cheeseman, 1955). Instead, chlorination with $POCl_3$ to the dichloride **E** followed by nucleophilic displacement with sodium methoxide yielded the alkaloid **1.** In a similar reaction, methyl 3-methyl-2-oxobutyrate (Weinstock et al., 1981) was condensed with **A** to yield a mixture of 3-isopropyl-1*H*-pyrido[2,3-b]pyrazine-2-one (**F**) and 2-isopropyl-4*H*-pyrido[2,3-b]pyrazine-3-one (**G**), which were separated by column chromatography. Chlorination of **F** to **H** and reaction with sodium methoxide yielded the desired alkaloid **2.** The spectral data of **1** and **2** were identical to the values reported by Budesinsky et al. (1986).

The total concentration of the three pyridopyrazines in adult specimens is about 0.1 mol/liter within pseudocellar fluid and up to 0.05 mol/liter in hemolymph samples. 2,3-Dimethoxypyrido[2,3-b]pyrazine (**1**), 3-isopropyl-2-methoxypyrido[2,3-b]pyrazine (**2**), and 2-methoxy-4*H*-pyrido[2,3-b]pyrazine-3-one (**3**) could be found within pseudocellar fluids and hemolymph samples of both males and females of *T. bielanensis* (Table 1). Pseudocellar secretions of the springtail larvae contain approximately equal amounts of **1** and **2;** the 3-isopropyl-2-methoxy-derivative represents the only pyridopyrazine of the larval hemolymph. Minor amounts of **1** and **2** are also present in the guts of both sexes, the ovaries and the male fat body; however, neither feces nor food of *T. bielanensis* contain even traces of these alkaloids.

Biological Significance of Reflex Bleeding

The target animals, *Nebria brevicollis*, are nocturnal polyphagous carabids that clearly prefer springtails as prey (Pollet and Desender, 1988). When there was a contact of *T. bielanensis* with antennae of the carabid beetle, the beetle's forelegs jerked forward and the springtail showed curling behavior (Figure 1C). If the beetle tried to grasp the springtail with its mandibles, *T. bielanensis* exuded a droplet from a pseudocell. Aggressive behavior of *Nebria* was immediately interrupted, and the carabid beetle tried to clean its mandibles. In cleansing behavior the mandibles are spread out and dug into the ground (Carrel and Eisner, 1974). The beetles further showed uncoordinated movements, fell on their back (disorientation), and usually secreted a clear fluid from their mouth. After a contact with pseudocellar fluid, the beetles usually extended their legs, slightly lifted their abdomens, and spread their elytra (elytra movement). A second attack of the carabid beetle was never observed.

Topical treatment of carabid mandibles and quantitative analyses of subsequent disorientation, cleansing behavior, and elytral movement revealed distinctly different results depending on the kind of fluids used in the experiment (Figure 8). Cleansing behavior was observed with all three test fluids but was only significantly increased ($P < 0.01$, nonparametric STP test; Sokal and

TABLE 1. PERCENTAGE OF PYRIDOPYRAZINES IN VARIOUS BODY COMPARTMENTS OF *T. bielanensis*

	Percentage		
	2,3-Dimethoxy-pyrido[2,3-b]-pyrazine (**1**)	3-Isopropyl-2-methoxy-pyrido[2,3-b]-pyrazine (**2**)	2-Methoxy-4*H*-pyrido[2,3-b]pyrazine-3-one (**3**)
Female			
Pseudocellar fluid	23.5	39.0	37.5
Hemolymph	48.7	18.3	33.0
Gut	x	x	–
Fat body	–	–	–
Ovaries	x	x	–
Male			
Pseudocellar fluid	25.5	31.5	43.0
Hemolymph	65.2	28.8	6.0
Gut	x	x	–
Fat body	x	x	–
Larva			
Pseudocellar fluid	42.6	57.4	–
Hemolymph	–	x	–
Feces (adults	–	–	–
Food (wood particles)	–	–	–
Food (granulated pollen)	–	–	–

[a] x: present in small amounts, but not quantified; –: absent.

Rohlf, 1981) if pseudocellar fluid had been topically applied previously on mandibles of the ground beetle. Disorientation of carabid beetles resulted after contact with pseudocellar fluid only, whereas elytral movement was observed exclusively after testing of pseudocellar fluid and *T. bielanensis* hemolymph (Figure 8). Distinct cleansing behavior was especially released after a topical application of 1 μl of 0.5 M DMSO solutions of **3** and **1** and even a 0.1 M DMSO solution of **2**. As compared to the DMSO solvent, which did not harm the beetles, strong disorientation could be observed only if 0.5, 0.1, and 0.05 M solutions of **2** were applied. Both cleansing behavior and disorientation of carabids increased with increasing concentrations of the three authentic pyridopyrazines (see Discussion). The pronounced activity of **2** (Table 1) is interesting in three respects: (1) Compound **2** quantitatively represents the main pyrazine in pseudocellar fluids and hemolymph of males and females of *T. bielanensis*. (2) It constitutes the only pyridopyrazine in the hemolymph samples

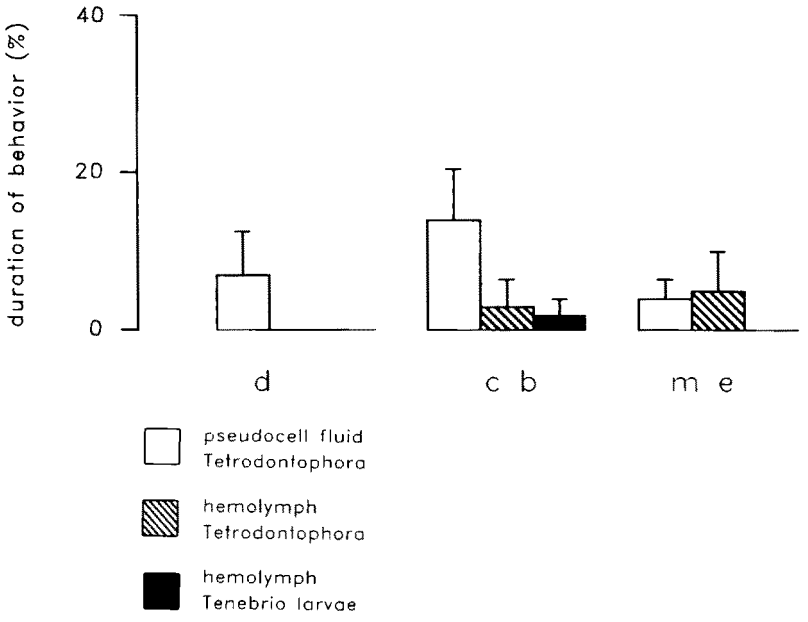

FIG. 8. Percentage duration of disorientation (d), cleansing behavior (cb), and elytral movements (me) of carabid beetles *Nebria brevicollis* within 10 min after having been topically treated with 1 μl test fluids. Pseudocellar fluid (light bar) and hemolymph of *T. bielanensis* (shaded bar) as well as hemolymph of *T. molitor* (black bar; controls) were used for tests. Ten carabid beetle specimens were used per test fluid (95% confidence values are denoted). Hemolymph samples of both *Tetrodontophora* and *Tenebrio* caused no disorientation behavior.

of the *Tetrodontophora* larvae. (3) The total concentration of the three pyridopyrazines in adult specimens amounts to about 0.1 mol/liter within pseudocellar fluids and up to 0.05 mol/liter in hemolymph samples.

DISCUSSION

Origin and Depletion of Pseudocellar Droplets of T. bielanensis

Several springtail species devoid of pseudocells show reflex bleeding behavior after nonspecific irritations such as mechanical contact or vibration (Paclt, 1956). Reflex bleeding at soft integumental areas usually is connected to rolling-up behavior and seems to be due to an increase in internal body pressure. Pseudocells are seemingly restricted to Tullbergiinae, and Pachytullbergiinae, Onychiurinae, and Tetrodontophorinae and probably represent an

advanced condition as compared to reflex bleeding. The primitive type of pseudocells is externally located at the trochanters of legs of *Onychiurus* species and is not distinctly differentiated from the surrounding integument. Furthermore, it is considerably smaller and shows no circular ring as observed in *T. bielanensis* (Rusek, 1984). The normal advanced type of pseudocell, however, is enlarged and corresponds to pseudocells of *T. bielanensis* or *Onychiurus armatus* (Rusek and Weyda, 1981). Depending on the species, the number, distribution, and external gross and fine structure of advanced springtail pseudocells may vary considerably (Hale and Smith, 1966; Rusek and Weyda, 1981). Morphologically there is a remarkable congruency between pseudocells of *T. bielanensis* and *Onychiurus armatus* that becomes evident after SEM and TEM studies (this paper; Rusek and Weyda, 1981). Pseudocellar caps are epicuticular structures that do not survive KOH maceration. On the contrary, the KOH-resistant stable pseudocellar ring represents a part of the endocuticle. One to several secretory cells are distinctly associated with the pseudocell. These cells adhere at the interior border of pseudocellar caps and are distally floating within hemolymph. The secretory cells of *O. armatus* contain many voluminous secretory vesicles (Rusek and Weyda, 1981), which clearly indicates that pseudocells are secretory structures par excellence. After slight molestation, rupture of the epicuticular caps occurs, which simultaneously destroys the secretory cells and liberates their contents. Subsequent irritations result in liberation of hemolymph (reflex bleeding) through the pseudocellar ring. Whether the closure of the longitudinal pseudocellar slit after molestation is due to elasticity of certain structures of the pseudocell is not yet clear. However, the suggestion of Koncek (1924), that secretory cells under the pseudocell would be brushed aside during its opening and hemolymph-outflow may be rejected.

As was shown by native disk PAGE, hemolymph samples contain more exclusive peptides as compared to pseudocellar secretion. However, SDS-PAGE produced more exclusive peptides in the pseudocellar secretion as compared to hemolymph samples. Due to the decreased pore diameter of PhastGel gradient 8-25 after SDS treatment, high-molecular-weight plasma proteins probably cannot diffuse into the gel, which may be responsible for this effect. Special peptides of pseudocellar secretions probably originate primarily from pseudocell coagulocytes and may principally represent clotting proteins, toxic peptides, or enzymes involved in pyridopyrazine biosynthesis. However, clotting proteins should be also present in hemolymph samples because coagulocytes are also present. Moreover, bioassays with authentic pyridopyrazines produced similar effects as the crude pseudocellar secretion. Therefore, the presence of toxic proteins within the pseudocellar coagulocytes seems unlikely.

The distribution of the three pyridopyrazines in different compartments of *T. bielanensis* (Table 1) cannot reveal structures or tissues where these unusual compounds are synthesized. However, it seems possible that pyridopyrazines are

synthesized within secretory cells located below the pseudocells. From there pyridopyrazines might be transfered into hemolymph and subsequently distributed interiorly within the body of *T. bielanensis*. Three arguments would favor this suggestion: As compared to hemolymph, the highest total amounts of pyridopyrazines are found in pseudocellar fluids (1). The complete series of pyridopyrazines is only present in the pseudocellar fluid, not in other compartments of larvae or adults (2). If the 3-isopropyl-2-methoxy-derivative **2** should represent the precursor for **1** and **3,** it is remarkable that the percentage of **2** decreases from pseudocellar fluid to hemolymph (3, Table 1). On the other hand, it may not be excluded that one or two pyridopyrazines might also be synthesized somewhere in the interior body of the springtail and then subsequently enriched within the pseudocellar secretory cells.

Biologically Active Constituents

Budesinsky et al. (1986) isolated **1** and **2** from total body extracts of 7300 *T. bielanensis*. Both pyridopyrazines and the newly identified compound **3** have been isolated for the first time from their natural glandular sources. Comparable nitrogen-containing aromatic heterocycles from arthropods contain either fewer [e.g., methyl-8-hydroxyquinoline-2-carboxylate; 1-methyl-2-quinolone; glomerin; homoglomerin (Dettner 1987, Numata and Ibuka, 1987)] or more nitrogen atoms [various pteridines (Numata and Ibuka, 1987)]. The biosynthetic pathway leading to the pyridopyrazines is unknown. Our HPLC analyses of body extracts of males and females of *T. bielanensis* showed that this species contained considerable amounts of uric acid, the granules of which are also found in hemolymph samples. This is reasonable since Collembola have no Malpighian tubules as excretory organs (Feustel, 1958). Uric acid therefore, might be an available pool for nitrogen-containing compounds in all collembolan species.

Intermediates for the biosynthesis of pyridopyrazines are unknown, although purines (and uric acid) might serve as precursors for related pteridines. Nevertheless, we regard **2** to be the precursor of the other two pyridopyrazines. An oxidative degradation of the isopropyl side chain would produce **3,** which could be methylated to produce **1.** This pathway is supported in three respects: The pyridopyrazine **2** is the only compound of this type present in the larval hemolymph, which supports the suggestion that larvae may exhibit more primitive characteristics than the adults (Table 1). At the same time this compound shows the maximal deterrent activity as compared to the two other analogs (Figure 9). In addition, **3** as the final product of the biogenetic sequence could not be detected in various body compartments (Table 1). A precursor for the pyridopyrazine **2** might be valine or its amide, as was suggested for the biosynthesis of 3-alkyl-2,6-dimethylpyrazines in ants (Numata and Ibuka, 1987).

Synthetic derivatives of pyrido[2,3-b]pyrazines, which have also been named 1,4,5-triazanaphthalenes or 5-azaquinoxalines, are well known as anti-

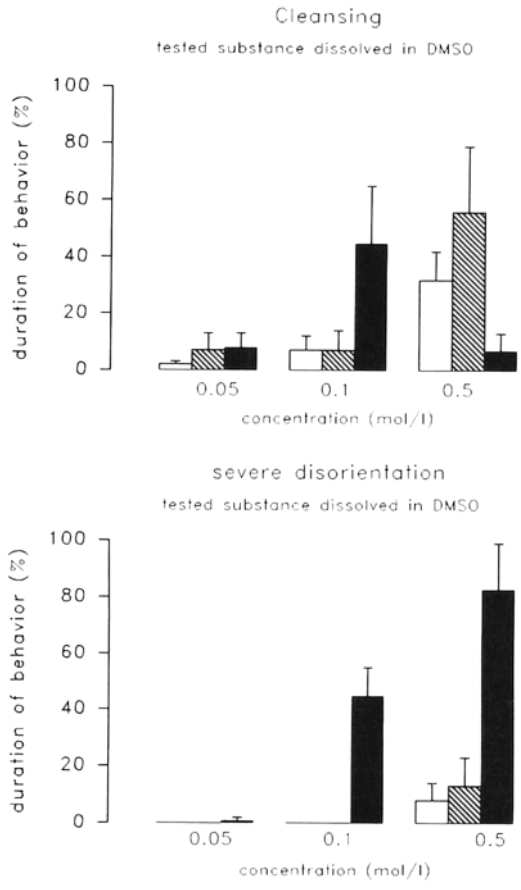

FIG. 9. Percentages duration of cleansing behavior (above) and disorientation (below) of mixed groups of carabid beetles (*Nebria brevicollis*, *Pterostichus oblongopunctatus*) within 10 min after having been topically treated with 1 μl of test fluids. Test fluids were 0.05, 0.1, and 0.5 M solutions of authentic 2-methoxy-4*H*-pyrido[2,3-b]pyrazine-3-one (light bar), 2,3-dimethoxypyrido[2,3-b]pyrazine (shaded bar), and 3-isopropyl-2-methoxypyrido[2,3-b]pyrazine (black bar) dissolved in DMSO. With each test fluid seven carabid beetles (four specimens of *N. b.* and three specimens of *P. o.*) were used (95% confidence values are noted).

metabolites and folic acid antagonists. Whereas various 8-substituted amino derivatives of pyrido[2,3-b]pyrazines show antibacterial activities, a number of 3,6-diamino-derivatives exhibits antiviral, diuretic, and antiinflammatory properties (Cheeseman and Cookson, 1979). Our results clearly show that these unique pyrazines are not directly derived from food. They must be synthesized

within internal tissues of Collembola. The distribution pattern may indicate that coagulocytes contribute to the complete set of pyridopyrazines present, because **3** is exclusively found within this compartment.

Biological Significance of Reflex Bleeding

By comparing results from Figure 9, it is evident that biological activity of the three pyridopyrazines increases with lipophilicity. The significant decrease of cleansing behavior after application of a 1-μl droplet of a 0.5 M solution of 3-isopropyl-2-methoxypyrido[2,3-b]pyrazine on a beetle mandible is a consequence of the fact that these beetles were disorientated during almost the whole observation period. Cleansing behavior, which was expected to be increase after treatment with a 0.5 M solution, decreased at the expense of a strong disorientation behavior. Another preliminary experiment demonstrated that the three compounds with different biological activities might also act synergistically in the laboratory and in the field. When 1 μl of a DMSO solution (0.1 M in total pyridopyrazines: mixture of **1, 2,** and **3** in a ratio of 3 : 3 : 1) was applied topically to a mandible of a carabid beetle, the duration of cleansing behavior and disorientation was comparable to 1 μl of a pure 0.1 M solution of the most active compound **2.** This synergism indicates that compounds **1** and **3,** with weaker activities when tested alone, exhibit increased biological activities when tested together with **2.**

Many possibilities exist for springtails to avoid contacts with predators or to escape after being discovered. Primary defenses may include slow movements or cryptic colorations (Bauer and Pfeifer, 1991). Secondary defenses of springtails are their jumping ability, the presence of integumental hairs and scales, and a fast unpredictable stampede (Bauer and Pfeifer, 1991). In the first case, after a mechanical stimulation, Collembola escape from a predator by jumping away within a few milliseconds. The second defensive mechanism is also effective, since such scales and hairs (present in only few families of Collembola) are easily removed and are always found sticking to the mouth parts of arthropods that feed on springtails (Bauer and Pfeifer, 1991).

Concerning the secondary defenses, especially chemical defenses, all important mechanisms as observed in other insects are also found in springtail species. Southeast Asian and Pacific species of the genera *Piroides* and *Corynephora* (Dicyrtomidae) show warning coloration and have a clublike defensive gland located dorsally at the abdominal tip (Greenslade and Suhardjone, 1992; Bauer, personal communication). Many springtail species, especially the eyeless soil-inhabiting Onychiuridae, do not have jumping ability and instead may release defensive compounds from pseudocells or toxic hemolymph chemicals by reflex bleeding (Paclt, 1956). It is probable that these insects may represent a rich source of biologically active new defensive compounds. This is supported by

observations of Weinreich (1968) and Bauer and Pfeifer (1991), who observed hunting behavior of the springtail-feeding rove beetle, *Stenus comma*, which has a protrusible labium to catch collembolan prey. This visually orienting beetle ignores certain collembolan species of a genera *Folsomia*, *Xenyella*, and *Tomocerus* and never feeds on *Onychiurus armatus* or *Podura aquatica*. Since *Podura aquatica* neither possesses pseudocells nor shows reflex bleeding, the presence of internal toxins or epicuticular deterrents might be suggested. Specimens of *Podura aquatica* were caught in 75% of all attacks by *Stenus comma*, but 88% of the seized collembolans were released within a few seconds and about 50% of them survived the attack (Bauer and Pfeifer, 1991). After these interactions, the beetles cleaned their mouthparts and rubbed them in the soil.

In other Collembola the use of alarm pheromones, which may act even across families, has been described (Purrington et al., 1991). The chemical nature of these alarm compounds has not been elucidated, but pseudocellar compounds and hemolymph toxins such as the pyridopyrazines may play a role in alarm behavior. The smell of *Tetrodontophora bielanensis* strongly resembles the smell of toxic coccinellid beetles that exhibit reflex bleeding (unpublished observation). Some 2-methoxy-3-alkylpyrazines have been described as warning odors (Moore et al., 1990; Dettner and Liepert, 1994). Whether *T. bielanensis* contains similar odor signals remains to be clarified, but synthetic alkaloids **1–3** are almost odorless.

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