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Secondary metabolites of the chemically rich ascoglossan *Cyerce nigricans*

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Summary. Two new metabolites of an apparent propionate origin have been isolated from the organic extract of the ascoglossan mollusc *Cyerce nigricans*. The proposed structures for the new natural products are based on interpretation of their physical and spectral properties. The new compounds isolated lacked the potent ichthyodeterrent properties of the whole animal extract suggesting that other molecules are involved in the defense of this shell-less mollusc.

Key words. Ascoglossa; chemical defense; *Cyerce nigricans*; polypropionate metabolites.

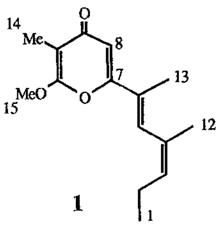
Shell-less gastropods such as nudibranchs, ascoglossans, and sea hares are known to be rich sources of unique secondary metabolites, most of which are sequestered from their chemically-rich prey[2]. Sequestering of these metabolites is widely viewed as a gastropod adaptation to acquire chemical defenses[2,3]; however, this has rarely been tested under ecologically relevant conditions[4]. In this paper we describe the structure of two new pyrones isolated from the Australian ascoglossan *Cyerce nigricans* and evaluate these metabolites as potential defenses against predatory reef fish.

Cyerce nigricans is a black and orange, aposematically colored ascoglossan that specializes on the chemically-rich green alga *Chlorodesmis fastigiata*. Previous ecological and preliminary chemical investigations on this species demonstrated that the live ascoglossan was repellant to coral reef fish as was its organic crude extract; however, the repellant nature of the extract was not due to the cytotoxic diterpenoid chlorodesmin (**3**), which is at least partially sequestered from its algal food[5]. We reasoned that other metabolites which were noted in the extract by TLC, but not identified, might be responsible for the deterrent nature of the *C. nigricans* extract. We therefore initiated additional chemical investigations of the chemistry of this ascoglossan.

Materials and methods

Twenty animals (420 mg, total dry mass) were collected from reefs near Lizard Island, Australia, and soaked in a mixture of $MeOH/CHCl_3$ (1/3). The extract was reduced in vacuo and the residue (120 mg) was fractionated on silica gel (flash chromatography). Proton NMR analysis of the column fractions revealed the presence of two unique metabolites (**1** and **2**), as well as minor quantities of chlorodesmin (**3**), fats, and sterols. The pyrones **1** (4 mg) and **2** (2 mg) were eluted from the column with 60% and 80% EtOAc in isooctane, respectively, and were further purified by HPLC (silica) using the same solvent mixtures.

The ability of these metabolites to deter feeding by a predatory reef fish was tested using the common Pacific wrasse *Thalassoma lunare* and methods that had proven successful in previous similar assays[5,6]. Each pure metabolite and a mixture of both **1** and **2** were dissolved in purified diethyl ether and injected into freeze-dried krill at concentrations ranging from approximately 2 to 23 times their natural yield. After allowing the solvent to evaporate, feeding on these krill was compared with feeding on krill injected with only solvent. Fish were held in individual aquaria and 1 to 3 pairs of treatment and control krill were offered to 2–3 fish at each concentra-



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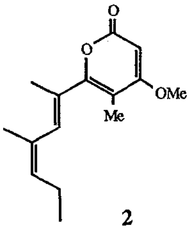

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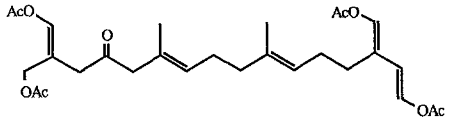

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tion of the two compounds. Each independent sample was thus composed of 1–3 separate subsamples.

Structures of pyrones **1** *and* **2**. Both compounds exhibited strong UV absorptions, at 248 nm ($\varepsilon = 6{,}700$) for **1** and at 285 nm ($\varepsilon = 11{,}800$) for **2**, indicating the presence of conjugated olefinic systems. A molecular formula of $C_{15}H_{20}O_3$ was deduced for both compounds on the basis of the EI HRMS data (248.1427 for **1** and 248.1426 for **2**). Both compounds showed similar spectral characteristics[7, 8]. In the ^1H NMR spectra, two singlets and a triplet integrating for 3 protons each were respectively assigned to two vinyl and one aliphatic methyl. Two down field singlets integrating again for 3 protons each were attributed to an aromatic methyl and an aromatic methoxy group. The presence of two olefinic and one aromatic proton, responsible for a triplet, a broad singlet, and a sharp singlet in the respective ^1H NMR spectra was obvious in the molecules. Based on these data, it was clear that the compounds did not follow terpene biosynthesis but rather possessed a carbon skeleton of an apparent polypropionate origin. Examination of the ^{13}C NMR data of the major compound (**1**) confirmed our assumptions and revealed the presence of a relatively high field carbonyl resonance. The above data, as well as the infrared absorbances, suggested a gamma-pyrone system for metabolite **1** and an alpha-pyrone system for compound **2**.

When homonuclear decoupling experiments were performed on compound **1**, it was found that irradiation of the triplet at 5.38 ppm caused, besides the simplification of the multiplet at 1.96 ppm, a profound increase in the height of the 1.60 ppm methyl resonance. A considerable increase in the intensity of the vinyl methyl at 1.67 ppm was also noted when the broad singlet at 6.48 ppm was irradiated in the same set of experiments. These results lead us to the assignments of the 6.48, 5.38, 1.67 and 1.60 ppm resonances to H-5, H-3, H-13 and H-12 protons. Similar experiments were also performed on compound **2**. The carbon NMR chemical shifts of the pyrone methines (110.5 ppm for compound **1** and 88.4 ppm for compound **2**) are characteristic for methine carbons alpha to carbonyls. The relative positions of the substituents on the pyrone ring in **1** were assigned based on the results of comprehensive nOe experiments that showed the spacial proximity of the lone pyrone proton to the H-14 vinyl methyl, of the methyl ether methyl to the H-5 olefinic proton, and finally of the methyl ether methyl to the pyrone methyl. A similar set of experiments performed with compound **2**, illustrated the spacial proximity of the lone pyrone proton and the pyrone methoxy group, as well as that of the H-5 olefinic proton and the pyrone methyl group. These assignments are in agreement with the literature values for similar compounds[9]. The configurations of the two trisubstituted olefinic bonds were established as $\Delta^3 = Z$, $\Delta^5 = E$ on the basis of their ^{13}C NMR chemical shifts (13.9 ppm and 21.9 ppm for compound **1** and 13.9 ppm and 21.7 ppm for compound **2**)[9]. The assignments of E configuration for Δ^3 are also supported by the fact that we did not observe any nOe enhancement on the vinyl methyls when the C-5 protons were irradiated.

These metabolites were not by any means the only constituents of the animals' organic extract, but were the only ones that we were able to isolate in reasonable amounts from our limited resources. Even though there is no evidence that both metabolites are derived from the same biosynthetic precursor, they both come from cyclization of linear precursors following polypropionate biosynthesis.

Effects on fish feeding. When the pure natural products were injected into krill at 1.6 and 15.7 (for compound **1**) and at 2.2 and 22 (for compound **2**) times their natural yield, these krill were eaten as readily as were control krill injected with only solvent. We saw no indication that fish found the treated food distasteful. Although sample sizes in these assays were small due to limited availability of the compounds, previous assays using these methods and natural concentrations of the organic crude extract of *Cyerce* were highly significant with the same small sample size[5]. Our results suggest that the potent deterrent effect of the crude extract could be due to unstable metabolites, minor substances not isolated in this investigation, or synergistic effects involving compounds not assayed here. The purones we isolated were clearly not significant feeding deterrents. Polypropionate-derived secondary metabolites have been isolated from at least 9 other mollusc species[6]; their ecological function, if any, has yet to be demonstrated.

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7 Compound **1** ^{1}H NMR (360 MHz in C_6D_6). 6.48, (1H, s, H-5), 6.41, (1H, s, H-8), 5.38, (1H, t, J = 6.6 Hz, H-3), 3.12, (3H, s, H-15), 2.12, (3H, s, H-14), 1.96, (2H, m, H-2), 1.67, (3H, s, H-12), 1.60, (3H, s, H-13), .90, (3H, t, J = 7.5 Hz, H-1). ^{13}C NMR (50 MHz) 180.5 (s), 162.1 (s), 159, 0 (s), 136.0 (d), 134.7 (d), 131.5 (s), 125.6 (s), 110.5 (d), 101.1 (s), 54.7 (q), 21.9, 16.5 (t), 13.9 (q), 13.9 (q), 7.1 (q). IR (cm^{-1}, film): 2960, 2920, 1650, 1610, 1460, 1315, 1260, 1170.
8 Compound **2** ^{1}H NMR (360 MHz). 5.96, (1H, s, H-5), 5.51, (1H, s, H-10), 5.33, (1H, t, J = 7.4 Hz, H-3), 2.91, (3H, s, H-15), 2.12, (3H, s, H-14), 1.91, (2H, m, H-2), 1.84, (3H, s, H-12), 1.54, (3H, s, H-13), .86, (3H, t, J = 7.5 Hz, H-1), ^{13}CMR (50 MHz, data incomplete due to limited amount of compound) 167.3 (s), 139.4 (d), 135.6 (d), 125.9 (s), 88.4 (d), 55.0 (q), 21.7 (q), 16.2 (t), 16.1 (q), 13.9 (q), 11.7 (q). IR (cm^{-1}, film): 2960, 2920, 1740, 1640, 1570, 1450, 1420, 1380.
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Conversion of phenylalanine to toluene and 2-phenylethanol by the pine engraver *Ips pini* (Say) (Coleoptera, Scolytidae)

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Summary. The pine engraver, *Ips pini* (Say), was found to produce toluene and 2-phenylethanol when boring into fresh pine logs. The hypotheses that phenylalanine is a precursor of these compounds and that beetles without their symbiotic microorganisms can perform these conversions were confirmed by treating wild and axencially-reared males and females topically with L-phenyl-d$_5$-alanine. Extracts of these beetles invariably contained deuterio-toluene, and extracts of males contained deuterio-2-phenylethanol as well.

Key words. Insecta; Scolytidae; phenylalanine; aromatics; axenic.

Phenylalanine plays a central role in insect physiology, acting as an essential nutrient[5], a major constituent of cuticle[6], and a component of structural and storage proteins[7,8]. It has also been shown to be behaviourally active, stimulating host-searching in the parasitoid *Apanteles cypris*[9], feeding behaviour in *Lygus lineolaris*[10], and positive chemotaxis in larval *Culex pipiens quinquefasciatus*[11]. Moreover, phenylalanine serves as the precursor for *p*-benzoquinone production in the defensive secretions of *Elodes longicollis*[12].

Despite the varied fates of this amino acid, only two modes of phenylalanine metabolism have been described in insects. Phenylalanine can be hydroxylated to tyrosine prior to incorporation into proteins, a common metabolic pathway. In a far less common reaction, males of the bertha armyworm, *Mamestra configurata*, convert phenylalanine into the pheromone 2-phenylethanol[13].

Volatiles captured from individual gallery systems of the pine engraver, *Ips pini*[14], contained toluene and 2-phenylethanol, aromatic volatiles without ring hydroxylation. Since the mean hourly release of 226 ng of toluene (SD = ± 195, n = 18) during 30 h of aeration could not be explained as atmospheric contamination, and insects do not produce aromatic structures themselves but receive them from their diets, we tested the hypothesis that phenylalanine is a precursor of the toluene and 2-phenylethanol found in *I. pini* volatiles. Since microbial symbionts can be involved in pheromone biochemistry and volatile production in Scolytids[15–18], and *Ips pini* carries several species of blue stain fungi and yeasts[19,20], we also tested whether the beetles can perform these conversions of phenylalanine without their symbiotic microorganisms.

Materials and methods

Experimental insects. Experiments were conducted on wild and axenically-reared male and female *I. pini*. Axenic beetles lacking culturable microorganisms were reared as previously described for *Dendroctonus ponderosae*[21], and were allowed to feed and mature for 2–6 weeks as adults. Wild beetles were allowed to emerge from logs of naturally infested lodgepole pine, *Pinus contorta* var. *latifolia*, held in screened cages at approximately 26 °C on a photocycle of 16:8 (L:D). Beetles were collected daily, separated by sex, grouped into 8–14 individuals per replicate, and used that day.

Phenylalanine treatment. 20 mg of L-phenyl-d$_5$-alanine labelled exclusively in the phenyl ring (lot No. 2250-L, Merck Frosst Canada Inc., Montreal) were dissolved in