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EXPLANATION OF BITTER TASTE OF VENOM OF PONERINE ANT, *Pachycondyla apicalis*

LEOPOLDO CRUZ LOPEZ and E. DAVID MORGAN*

Department of Chemistry
Keele University
Staffordshire, England ST5 5BG

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Abstract—The venom gland of workers of *Pachycondyla* (= *Neoponera*) *apicalis* (Hymenoptera: Formicidae) contains the bitter-tasting cyclic dipeptide of leucine and phenylalanine [cyclo-leu-phe or 3-benzyl-6-(2-methylpropyl)-2,5-piperazinedione]. The venom also contains proteins of undetermined activity. It is suggested that the function of the venom may be both defensive and offensive. The mandibular glands of *N. apicalis* contains δ-decalactone and benzaldehyde.

Key Words—Ponerine ant, venom, bitter dipeptide, diketopiperazine, mandibular gland secretion, Dufour gland secretion.

INTRODUCTION

Schmidt (1986) has pointed out that the venoms of social bees and wasps have essentially one function, i.e., defense in its broad sense, and the venom of solitary Hymenoptera have the function of prey capture, with possibly a minor function of defense, but the ant venoms have a great variety of functions. We tend to think of venoms as injectable, stinging defensive agents, but Schmidt points out that for ants they can also be topically applied defensive agents; trail, alarm, sex, queen recognition, aggregation, attractant–recruitment and recognition pheromones; as well as repellents and toxic agents for prey capture (Schmidt, 1986). In further expansion of this theme, he pointed out that some venoms are gustatory repellents. He listed, among others, several ponerine spe-

*To whom correspondence should be addressed.

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cies that have venoms with a bitter, burning taste (to humans); among these he mentioned *Pachycondyla* (= *Neoponera*)A *apicalis* (Schmidt, 1986).

We have examined the venom of *Pachycondyla apicalis* and identified a known bitter compound that is recorded here in a venom and in an ant for the first time. We have also examined the mandibular glands and postpharyngeal glands of this species.

METHODS AND MATERIALS

Maintenance of Colony. Workers of *Pachycondyla apicalis* were collected in Tapachula, Chiapas, Mexico. The ants were reared in an artificial nest made from a small plastic box partially filled with moistened plaster of Paris. The ants were kept in the laboratory on a diet of sugar solution and dead flies.

Preparation of Glands for Gas Chromatographic Analysis. The samples for injection were prepared by cooling individual ants in a refrigerator, then dissecting out the venom reservoir in distilled water under a binocular microscope. The upper part of the head capsule was used for postpharyngeal glands. Whole heads of workers were used to examine the mandibular glands. Excess water was removed by touching the gland with a fragment of filter paper and the tissue was then attached to a fragment of glass and placed individually in short glass capillaries sealed at one end; the other end was then sealed with a flame (Morgan, 1990).

Gas Chromatography–Mass Spectrometry. Chromatography was carried out with a Hewlett-Packard 5890 gas chromatograph directly coupled to a 5970B mass selective detector. The system was controlled by a Hewlett-Packard Series 300 computer with HP 59970C Chemstation.

Chromatography was performed on an immobilized polydimethylsiloxane phase (equivalent to OV-1) in a fused silica column (12 × 0.2 mm) (SGE, Milton Keynes, UK). Helium was used as carrier gas at 1 ml/min. The sample was heated in the injector to 200°C for 4 min before crushing the capillary, as described by Morgan and Wadhams (1972). The oven was programmed from 30°C (2 min) at 8°C/min to 280°C for venom and mandibular gland secretion. The identification of the compounds was confirmed by comparison of their mass spectra and retention times with those of synthesized material.

Microozonolysis, and hydrogenation of Dufour gland substances were carried out as described by Attygalle and Morgan (1983). Analysis of cuticular hydrocarbons was as described by Bagnères and Morgan (1990).

Synthesis of Cyclic Peptide. The cyclic dipeptide was first synthesized following the method of Johnstone and Povall (1975) by heating methyl phenylalanyl-leucinate (50 mg) previously prepared from phenylalanyl-leucine with diazomethane, in refluxing dimethyl formamide (3 ml) for 12 hr. The product

was obtained by evaporation of the solvent in vacuo. It was also prepared by heating phenylalanyl-leucine itself (10 mg) in dimethyl formamide (4 ml) at 100–110°C for 10 hr. The product was purified by sublimation at 160–170°C under water pump vacuum. Its purity was checked by gas chromatography and NMR spectroscopy. The reaction was carried out with both (L)-phenylalanyl-(L)-leucine and (D, L)-phenyalanyl-(D, L)-leucine (both Sigma, Gillingham, Dorset).

Cyclo-(L)-leucyl-(L)-phenylalanyl was produced by heating (L)-leucyl-(L)-phenylalanine in dimethylformamide and purifying the product for sublimation, which gave essentially one enantiomer. ^{1}H NMR spectrum (270 MHz) in CF_3CO_2D: δ 0.184 (1H ddd, J approx. 14 Hz, 10.74, 3.42 Hz), δ 0.805 (3H, d, J 6.34 Hz), δ 0.847 (3H, d, J 6.34 Hz), δ 1.200 (1H, ddd, J 13.7, 10.74, 3.42 Hz) δ 1.47 (1H, m), δ 3.206 (1H, dd, J 14.16, 4.88 Hz), δ 3.432 (1H, dd, J 14.16, 4.39 Hz) δ 4.165 (1H, dd, J 11.23, 3.42 Hz) δ 4.804 (1H, dd, J, 4.88, 4.39 Hz), δ 7.20 (2H, m), δ 7.40 (3H m). The ^{13}C NMR spectrum (67.5 MHz, in CF_3CO_2D) had absorptions at 20.7 and 23.3 ppm (q, CH_3); 25.3 ppm (t, CH_2); 40.7 (d, CH); 44.6 (t, PhCH_2); 54.8, 57.9 (d, CHCO); 130, 131, 131.8, 135 (phenyl); 172.1, 174 (s, CO).

The mass spectra were very similar for the isomers: cyclo-L-phenylalanyl-L-leucyl (and its enantiomer) M^+, 260 (30), 204 (71), 175 (4), 169 (28), 141 (54), 120 (16), 113 (59), 103 (9), 91 (100), 86 (12), 65 (7), 57 (16), 41 (22). The mixture of cyclo-D-phenylalanyl-L-leucyl and cyclo-L-phenylalanyl-D-leucyl gave M^+, 260 (30); 204 (60), 175 (5), 169 (30), 141 (47), 120 (12), 113 (52), 103 (8), 91 (100), 86 (9), 65 (14), 57 (15), 41 (18).

Electrophoresis of Venom Proteins. The venom of *P. apicalis* was collected in a Pasteur pipet, drawn out to a fine capillary, by piercing the venom sac of a dissected gland. The venom was then added to aqueous sodium dodecyl sulphate (SDS, 19 μl) and the solution heated to boiling for 10 min to denature the proteins.

The solution was placed in a channel of polyacrylamide gel, and solutions of bee venom, venom of *Dinoponera australis*, phospholipase A_2 from bee venom (Sigma, Gillingham), and molecular mass standards were placed in parallel channels and electrophoresis carried out. The protein bands were stained first with Coomassie blue and then with silver to obtain a more sensitively stained gel.

RESULTS

When venom glands of workers of *P. apicalis* were examined by gas chromatography–mass spectrometry for volatile compounds, essentially only two peaks, eluting close together, were seen, and these gave almost identical mass

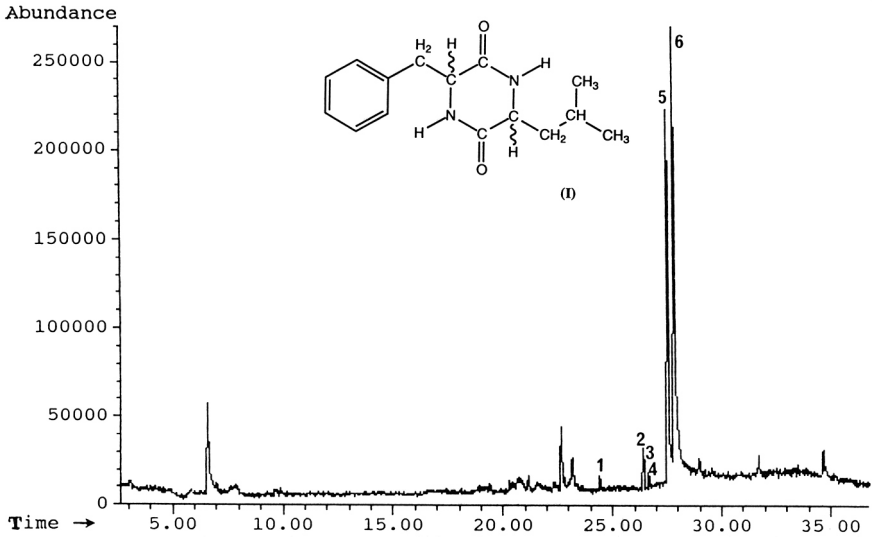

FIG. 1. Gas chromatogram of the volatile portion of the venom of *P. apicalis*. Peaks 1, 2, 3, and 4: hydrocarbons from cuticle (see Figure 2); 5: cyclo-L-leucyl-D-phenylalanyl; and 6: cyclo-L-leucyl-L-phenylalanyl, both represented by structure I.

spectra, showing they were closely similar isomers (Figure 1). Some very small amounts of linear C_{23} hydrocarbons were also evident, but these were also seen in the analysis of heads and cuticle. The mass spectra of the two major peaks showed prominent losses of benzyl and butyl groups and the presence of an ion at *m/z* 113, typical of a piperazinedione. This led us to suspect an amino-acid origin and to identification of the two substances as the diastereoisomeric forms of the cyclic dipeptide cyclo-leucyl-phenylalanyl or 3-benzyl-6-isobutylpiperazine-2,5-dione (**I**) (Figure 1). The identification was confirmed by synthesis of **I** from DL-leucyl-DL-phenylalanine, which gave two gas chromatographic peaks of equal areas and having retention times and mass spectra identical to those of the natural compounds. The mass spectrum of cyclo-leu-phe has been recorded and the fragmentation studied in detail (Szafranek et al., 1976). The spectrum recorded earlier by Heyns and Grützmacher (1966) looks unlike ours or that of Szafranek et al. (1976).

When L-leucyl-L-phenylalanine was subjected to the same cyclization process, the same compounds were formed, except that the second peak was much larger than the first, indicating that some slight racemization had occurred during cyclization, that the first-eluting peak contained cyclo-L-leu-D-phe and the second peak cyclo-L-leu-L-phe, and that the venom probably contained all four possible enantiomers. The ratio of the areas of the two peaks from the venom

(Figure 1) varied with sample but was approximately 1 : 1. When chromatography was carried out with a lower preheating and injection temperature to see if isomerization was occurring, no change was observed. There were no other volatile substances detected in the venom.

Quantification of the amount of dipeptide from three venom glands of workers was estimated by comparison with known quantities of synthetic dipeptide. Values of 2.7, 5.9, and 17.0 μg were obtained.

The ^{1}H and ^{13}C NMR spectra of the synthetic material were recorded, since these had not been available before.

The venom of *N. apicalis* was also examined by polyacrylamide gel electrophoresis (PAGE) to see if it also contained venom proteins. It was carried out in parallel with the venom of the ponerine ant *Dinoponera australis*, bee venom, and phospholipase A from bee venom. The venoms of *P. apicalis* and *D. australis* seemed identical and quite different from bee venom. The venom of *P. apicalis* gave essentially five protein bands, one of low molecular mass belonging to an unknown protein, one at ~15,000 Da, close to the phospholipase A_2 of bee venom; one at ~21,500 Da, not found in bee venom, but parallel to the soybean trypsin of the protein standards; one at ~35,000 Da, parallel to the hyaluronidase of bee venom; and one at 45,000 Da, close to the acid phosphomonoesterase of bee venom. Since the proteins of ant and bee venoms have quite different chemical structures (Leluk et al., 1989), we cannot conclude that these comigrating protein bands have corresponding enzymatic activity.

The mandibular glands contained a mixture of acetamide, benzaldehyde, and δ-decalactone in variable proportions (Figure 2). These were identified by their mass spectra and confirmed for the first two by coinjection of authentic materials. The postpharyngeal glands in the heads contained a mixture of four substances (Figure 2). The first and last eluting were readily identified as heneicosane ($C_{21}H_{44}$, M^+ 296) and tricosane ($C_{23}H_{48}$, M^+ 324) respectively. The other two (M^+ 322 and M^+ 320) eluted just before tricosane and are presumed to be a methyldocosene and a methyldocosadiene, respectively. They are not the linear C_{23} alkene and diene, because the order of elution on the column used is normally diene, then alkene. These four substances were the only ones detected when we examined a sample of cuticle using our micromethod (Bagnères and Morgan, 1990). They also appeared as contaminants in the chromatograms of the venom gland (Figure 1).

DISCUSSION

The bitter taste of some cyclic dipeptides has been known for many years, and was mentioned by Emil Fischer in 1906, but the bitter taste was studied

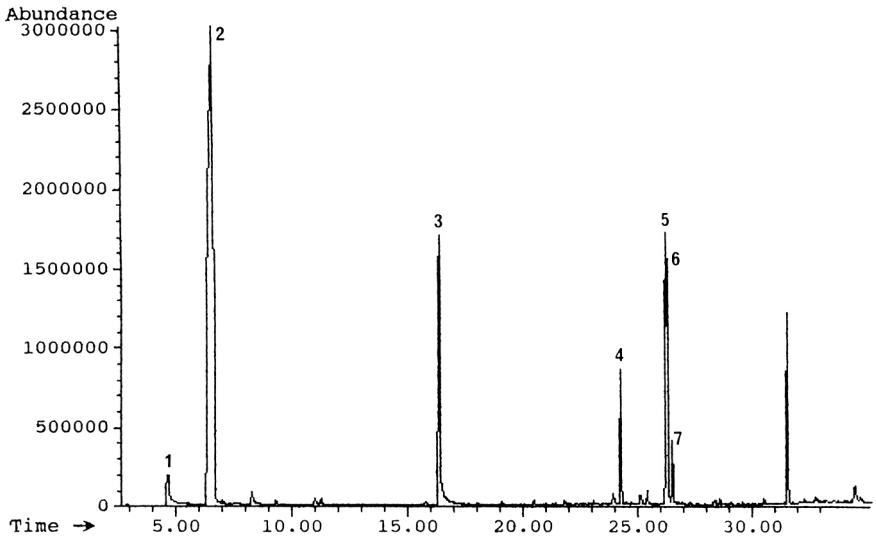

FIG. 2. Gas chromatogram of the volatile compounds in the head of a single worker of *P. apicalis*. Compounds are: 1, acetamide, 2, benzaldehyde, 3, δ-decalactone (all from the mandibular glands), 4, heneicosane, 5, unknown alkene (M^+ 222); 6, unknown alkadiene (M^+ 220); and 7, tricosane (all from the postpharyngeal gland).

systematically only much later (Matoba and Hata, 1972). It was found that there is a good correlation between bitterness and hydrophobicity of the side chains (Shiba et al., 1981) and that cyclo-leucyl-phenylalanyl is one of the most bitter compounds (Pickenhagen et al., 1975; Gardner, 1980; Ney, 1986). Moreover, the receptors for bitterness do not recognize chirality or require a strict conformation for the substrate (Shiba et al., 1981). While this information applies to human experimental subjects, generally what is bitter to us is judged to be unpleasant to other mammals by their avoidance of such materials. The presence of this cyclic dipeptide explains the bitter taste of *P. apicalis* venom reported by Schmidt (1986).

The volume of a worker venom reservoir, treated as a prolate spheroid was approximately 350 nl, and it contained on average 8.5 μg of dipeptide—a concentration of about 2.5%. We examined the venom by gel electrophoresis to see what, if any, proteins were also present. Five protein bands were detected, but we were unable to correlate them with enzymic activity.

The bitter taste suggests that the venom is a defensive agent, but Schmidt (1986) quotes this venom as having a moderate effect in causing pain. Schmidt (personal communication) also reports seeing *Pachycondyla* (= *Neoponera*) *villosa* in Costa Rica stinging wasp prey, which were then immobilized within

seconds, and the prey promptly carried off. It may well be that *Pachycondyla* venom is both offensive and, through its bitterness, defensive.

The cyclic dipeptide has been identified in insects and in venom for the first time. It is recorded as produced by the microorganism *Streptomyces noursei* (Kelley and Brown, 1966) and has been found in the skin of some amphibians (Erspamer et al., 1986), although the link with bitter taste was not apparently recognized there.

The mandibular gland secretion is noteworthy because it did not contain the alkylpyrazines that are commonly found in ponerine glands. Lactones have been identified in the secretion of cockroaches, beetles, phasmids, and bees, as well as in ants (Blum, 1981; Attygalle and Morgan, 1984). δ-Decalactone itself has been identified in the mandibular glands of stingless bees of the *Trigona carbonaria* group (Blum 1981, p. 243, quoted as Wheeler et al. 1975 but untraceable).

Blum et al. (1969) first reported benzaldehyde in the mandibular glands of a harvester ant, *Messor* (= *Veromessor*) *pergandeyi* (Myrmicinae). It was described as a defensive secretion, and a minor component of the gland was said to release alarm. Blum and Wheeler (unpublished, quoted in Blum, 1981) also found benzaldehyde in the mandibular glands of *Azteca* species (Dolichoderinae). We now have examples of benzaldehyde in the mandibular glands of ants from three subfamilies. There are several reports of benzaldehyde in the mandibular secretion of stingless bees. Blum (1981) referred to unpublished work, identifying it in *Trigona postica*, *T. xanthotricha*, and *T. tubiba*. He found benzaldehyde very attractive to workers of *T. tubiba*. Luby et al. (1973) reported it in *T. mexicana* and *T. pectoralis* but did not find any distinct behavioral response in these species, although it did enhance the alarm response of ketones also present.

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