with lymphocytes from non-anticoagulated whole blood fixed directly after venipuncture (*6*).

Thus we find, in agreement with preliminary reports (*6, 15*), that human lymphocytes can appear villous when either fixed in suspension or settled live onto substrate. It is not possible to distinguish T and B lymphocytes on the basis of surface morphology. Similar observations have been made with spleen cells in this (*16*) and other (*17*) laboratories. These findings are in contrast to those of Polliack *et al.* (*2, 3, 5*), who reported that 80 percent of human peripheral blood lymphocytes are smooth and 20 percent are villous and suggested that these differences in surface morphology can serve to distinguish T and B lymphocytes.

Of several differences in preparative techniques that might account for these divergent observations, perhaps the most important is the method of cell collection for SEM. Pollack *et al.* (*2, 3, 5*) aspirated live cells onto silver membrane filters for variable time periods [1 to 15 minutes (*18*)] before fixing them in 1 percent glutaraldehyde. It has been demonstrated (*19*) that this procedure nonspecifically smooths the surfaces of cells. Degenerating cells with phycnotic nuclei also can have smooth surfaces (*20*). In the previous investigations by Polliack *et al.* (*2, 3, 5*), observations of surface morphology were made with samples which had been subjected to preparative procedures for SEM involving large (up to 80 percent), potentially selective cell losses.

From the findings reported here, we conclude that it is not possible to identify reliably T and B lymphocytes by their surface morphology. In addition, monocytes (*6*), polymorphonuclear leukocytes (*21*), and basophils (*19, 21*) may all appear covered with short microvilli and, under certain conditions, are indistinguishable from lymphocytes. It is therefore necessary to delineate the nuclear morphology of individual cells in order to identify them in heterogeneous populations studied by SEM (*6, 21*). Furthermore we, as others (*22*), find that cell surface form can change quickly and dramatically in response to a variety of environmental modifications. Thus, rigorous monitoring of experimental parameters and preparative techniques for SEM is essential if SEM is to be meaningfully employed in cell biology and clinical immunology.

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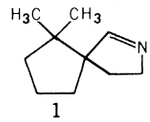
27 December 1974

Polyzonimine: A Novel Terpenoid Insect Repellent Produced by a Milliped

Abstract. *A nitrogen-containing terpene 6,6-dimethyl-2-azaspiro[4.4]non-1-ene* (polyzonimine) *was isolated from the defensive secretion of the milliped* Polyzonium rosalbum. *Polyzonimine, which is repellent to such natural enemies of the milliped as ants, acts as a topical irritant to insects (10^{-4} M induces scratching in cockroaches). Its structure was confirmed by a five-step synthesis starting from 2,2-dimethyl-7-oxabicyclo[4.1.0]heptane.*

Years ago (*1*) the presence of "camphor" was reported from the defensive secretion of the milliped *Polyzonium rosalbum*. The purported utilization of a well-known plant secondary metabolite by an arthropod was intriguing, but since the claim was not based on definitive chemical evidence, a reinvestigation of the secretion was in order. We here report on the isolation from *P. rosalbum* of a highly volatile substance, which proved not to be camphor, but rather the novel nitrogenous terpene 6,6-dimethyl-2-azaspiro[4.4]non-1-ene (**1**), for which we propose the name *polyzonimine*.



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The *Polyzonium* were collected in New York State (Tompkins and Albany counties), in dead and decaying logs, mostly in beech-hemlock forest. Their defensive glands are serially arranged, one pair per each of most body segments, with openings that are visible as small pores along the margins of the body (Fig. 1, A and B). Seizing or pinching the animals with forceps causes them to discharge their sticky whitish secretion, which can be readily wiped from their bodies with small pieces of filter paper. Several thousand *Polyzonium* (body lengths 5 to 18 mm) were "milked" in this fashion to provide secretion for chemical analysis. Most of the animals were returned to their original location after milking.

Polyzonimine, $[\alpha]_D^{20}$, + 3.26° (chloroform), was obtained in pure form by preparative gas-liquid chromatography

(GLC) (1.8-m column; 10 percent SE-30 on Chromosorb W at 200°C). High resolution mass spectrometry revealed the parent ion to have the composition $C_{10}H_{17}N$ (*m/e*: calculated 151.1360; found 151.1361), and infrared analysis showed the presence of an imine (C = N) stretching band at 1626 cm^{-1}. The apparent absence of any additional unsaturation implied a bicyclic formula. In its proton magnetic resonance (PMR) spectrum ($CDCl_3$ solution; 60 Mhz), polyzonimine showed methyl singlets at τ 9.10 and 9.07, a complex multiplet for eight protons centered at τ 8.25, a doublet of triplets (J = 7; 2.5 hz) at τ 6.20, and a triplet for the imine proton (J = 2.5 hz) centered at τ 2.60.

While polyzonimine itself is a liquid, x-ray crystallographic analysis of a closely related compound obtained by treatment of an ethereal solution of the milliped extract with perchloric acid first suggested structure **1** for the imine itself (*2*). This hypothesis incorporated all of the structural features revealed by the infrared and PMR spectral data, and we set out to confirm it by an unambiguous synthesis. The presence in structure **1** of a cyclopentane ring with two adjacent quaternary carbon atoms lent this undertaking special interest. After several unsuccessful paths were abandoned (*2*), the scheme outlined in Fig. 2 provided an efficient synthesis of the desired 2-azaspiro[4.4]non-1-ene.

2,2-Dimethyl - 7 - oxabicyclo[4.10]heptane (**2**) was readily prepared by oxidation of 3,3-dimethylcyclohexene (*3*) with *m*-chloroperoxybenzoic acid. Rearrangement of **2** to 2,2-dimethylcyclopentanecarboxaldehyde (**3**) proceeded slowly but in satisfactory yield in a refluxing benzene solution containing the epoxide, lithium bromide (three equivalents), and hexamethylphosphoramide (HMPA, three equivalents) (*4*). Formation of enamine **4** from **3** proceeded uneventfully, and the critical second quaternary center was successfully introduced by Michael addition of **4** to nitroethylene, generated in situ from 2-acetoxynitroethane (*5*). Ketalization of the resultant nitroaldehyde **5** and reduction by Raney nickel gave the cyclic imine **1** upon treatment with acid. The overall yield of **1** from **2** over these five steps was 22 percent. (±)-Polyzonimine prepared in this way was indistinguishable from the natural material on the basis of its spectral and GLC properties; it also showed the characteristic odor which was originally largely responsible for its misidentification as camphor (*1*).

Initial tests had shown *Polyzonium* to be virtually invulnerable to ants, their probable chief enemies. Individual millipeds, offered to workers of the formicine ant *Formica exsectoides* in a laboratory test enclosure, were quickly attacked, but the bites of the ants invariably prompted the millipeds to discharge, which caused the ants to disperse, and left the millipeds free to crawl away unmolested. The repellent effect could be duplicated with synthetic polyzonimine. Capillary tubes filled with the fluid, presented at close range to caged ants, feeding at a sugar source, elicited dispersal in a matter of seconds (Fig. 1, C to F).

On contact or near contact with either polyzonimine or the natural secretion, the ants commonly showed intensive and protracted cleaning activities, including a typi-

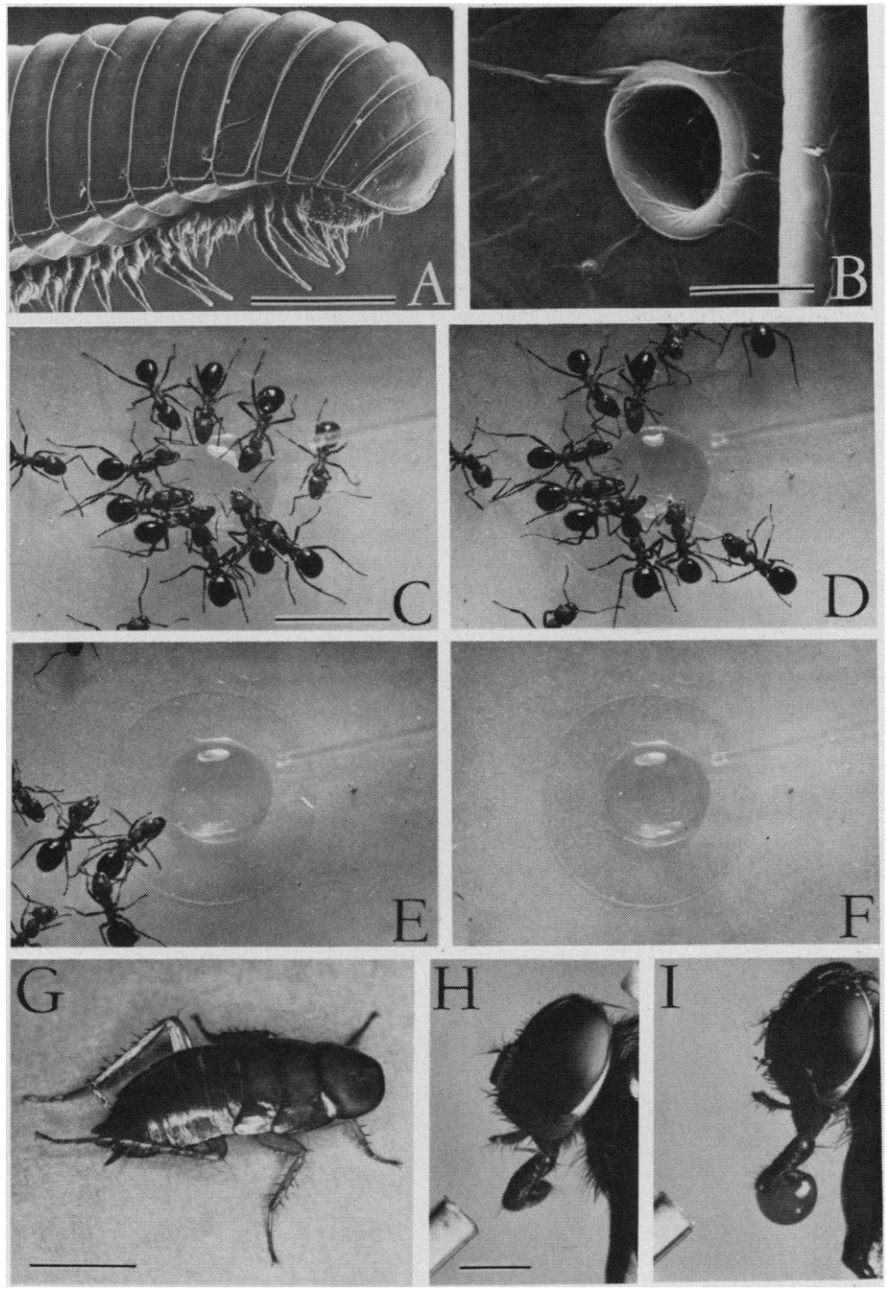

Fig. 1. (A) Scanning electron micrograph of front end of *Polyzonium*. The gland openings are visible as small pores near the outer margins of the tergites. (B) Enlarged view of a glandular pore. (C to F) Dispersal of ants (*Formica exsectoides*) in response to polyzonimine. The ants are initially seen feeding on a drop of sugar solution, and the polyzonimine is presented to them in the tip of the capillary tube introduced from the right. The time course of the photographs (motorized 35-mm camera), beginning at time zero in Fig. 1C, is: (D) 0.5 second; (E) 1.5 seconds; (F) 2.5 seconds. (G) Decapitated nymph of cockroach (*Periplaneta americana*) scratching right margin of abdominal tergite 5 after application of a topical irritant to the site. (H to I) Tethered fly (*Phormia regina*) extending its proboscis (H) and regurgitating (I) in response to close-range presentation of a capillary tube filled with volatile irritant. Reference bars: (A) 0.25 mm; (B) 0.02 mm; (C) 1 cm; (G) 1 cm; (H) 1 mm.

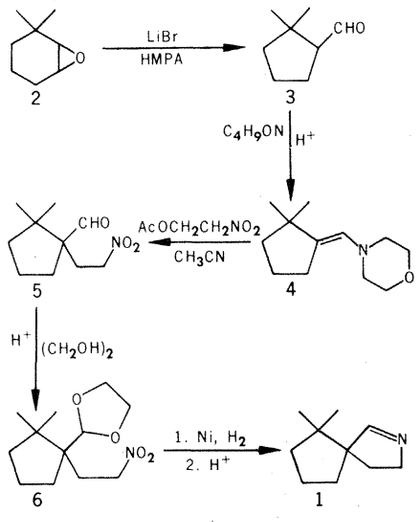

Fig. 2. Synthesis of ±-polyzonimine.

cal wiping of the antennae with the forelegs, and a dragging of the body against the substrate. These responses suggested that polyzonimine might effect its deterrency by acting as a general surface "irritant" (*6*). Two bioassays, based on the cleansing responses of a cockroach (*Periplaneta americana*) and a fly (*Phormia regina*), provided a means for quantifying this irritancy.

In nymphs of *Periplaneta*, application of a droplet of irritant to one side of abdominal tergite 4 or 5 evokes an accurately directed scratch response from the leg of the side stimulated (Fig. 1G). The response is reliable and stereotyped, and the time delay to onset of scratching can serve as an indication of the irritancy of the fluid. Tests are best carried out with nymphs rendered nonambulatory by decapitation (*7*). Polyzonimine was assayed in solution (ethylene glycol), at four concentrations ($10^{-1}M$ to $10^{-4}M$). The solvent itself was assayed as the control. From 21 to 39 roaches (last instar nymphs) were tested per sample. The droplets (0.22 μl) were administered to the tergites with a microsyringe (*8*). Onset of response was timed with a stopwatch. Failure to respond within 120 seconds was scored as no response. The roaches were tested within the period of 20 to 42 hours after decapitation. Figure 3A shows cockroach responsiveness to the various samples, plotted as a function of delay to scratching. Polyzonimine was evidently effective down to a concentration of $10^{-4}M$. Attempts were also made to stimulate roaches with higher concentrations of polyzonimine ($1M$ and undiluted), but droplet administration proved impossible because the roaches responded to the mere approach of the microsyringe, before direct topical application could be effected.

The assay with *Phormia* was based on a proboscis-cleansing response. When the flies are subjected to an irritant vapor, they evert the proboscis, regurgitate a droplet onto its tip (the labellum), and then proceed to wipe the labellum against the substrate. In tethered flies, the substrate can be made inaccessible, and proboscis extension alone, either with or without regurgitation, can serve as an indication of the response (Fig. 1, H and I). Delay to onset of extension can again be used as a measure of the irritancy of a substance. Polyzonimine was tested in undiluted form and in solution ($1M$ to $10^{-2}M$ in ethylene glycol). The samples were presented to the flies in capillary tubes (inner diameter, 0.6 mm), the replete tips of which were brought to within 0.3 to 0.6 mm of their labellum. The flies were sucrose- and water-satiated before testing, and their response times were clocked with a stopwatch. Failure to respond within 30 seconds was scored as no response. The results (Fig. 3B) show $10^{-1}M$ to be the lowest effective concentration.

Calculations based on the recovery of polyzonimine from the milliped milkings (estimated average 70 μg per animal), and on the estimated volume of secretion discharged per milliped (0.5 to 1 μl), suggest a concentration of about $0.5M$ to $1M$ for polyzonimine in the secretion. Response times of cockroaches and flies to stimulation with freshly discharged secretion tended to confirm this estimate: Cockroaches scratched in response to mere proximity of the secretion, and flies showed extension responses within 1 to 4 seconds.

Polyzonimine is an unusual natural product. To our knowledge, no monoterpene has been found to possess the carbon skeleton characteristic of polyzonimine (*9*), nor have other natural products been previously characterized (*10*) based on the 2-azaspiro[4.4]nonane ring system.

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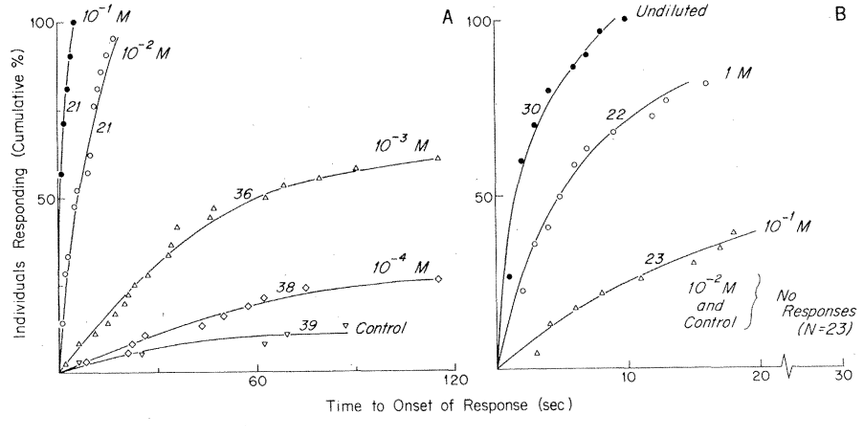

Fig. 3. (A) Sensitivity of cockroaches (*Periplaneta americana*) to topical application of polyzonimine solutions to the abdomen. Sensitivity is expressed as the delay to onset of the scratch reflex induced (Fig. 1G). (B) Sensitivity of flies (*Phormia regina*) to polyzonimine (undiluted and in solution) presented to them in capillary tubing at close range. Sensitivity is expressed as the delay to onset of the proboscis-extension response induced (Fig. 1, H and I). Numbers of insects tested per sample are indicated by numbers on the curves. The solvent used in the solutions (ethylene glycol) was tested as the control.

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