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Insecticidal Activity of Huperzine A from the New Zealand Clubmoss, *Lycopodium varium*

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A bioactivity-directed investigation of an extract of the New Zealand clubmoss, *Lycopodium varium*, collected on subantarctic Campbell Island, has led to the isolation of the alkaloid huperzine A (**1**) as the major antifeedant and insecticidal component. Huperzine A showed insecticidal activity against the Australian carpet beetle, *Anthrenocerus australis* (LD_{50} = 110 ppm), the Australian sheep blowfly, *Lucilia cuprina* (LD_{50} = 2380 ppm), and the webbing clothes moth, *Tineola bisselliella* (LD_{50} = 630 ppm). Feeding by *A. australis* was reduced by 97% at 63 ppm.

KEYWORDS: ***Anthrenocerus australis*; *Lucilia cuprina*; *Tineola bisselliella*; *Lycopodium varium*; huperzine A; alkaloid; insecticide**

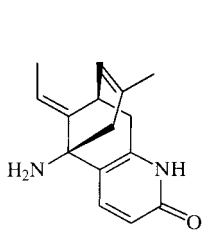
INTRODUCTION

As part of our program to screen the New Zealand flora for biologically active natural products, plant samples were collected from New Zealand's subantarctic islands (*1*). One of the species collected was *Lycopodium varium* R. Br. (family Lycopodiaceae). An extract of *L. varium* showed antifeedant activity against the beetle, *Anthrenocerus australis* Hope (Coleoptera: Dermestidae). This led us to undertake a bioactivity-directed investigation of the active component in the *L. varium* extract.

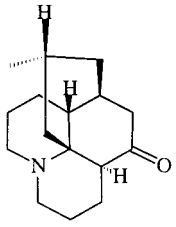
The clubmoss *L. varium* is common throughout New Zealand and the subantartic islands, occurring in lowland and montane forest or subalpine scrub, and is also found in Australia (*2*). *L. varium* exists as two well-marked forms that intergrade with each other. One is epiphytic and hangs down from high in the crown of forest trees or forms shorter plants on tree fern trunks. The other is terrestrial and grows erect in rocky ground or in scrub and open forest. This collection was from the latter form, which is more common in the lower South Island and outlying southern islands (*2*).

Literature searching revealed only one report of a chemical investigation of *L. varium*. This dealt with the taxonomic subdivisions of differing species of *Lycopodium* as related to the flavonoid glycoside components (*3*). The chemistry of a number of other species of *Lycopodium* has been studied, showing this genus to be a rich source of new alkaloid compounds (*4*, *5*), examples being lycopodine (**2**) and lycodine (**3**).

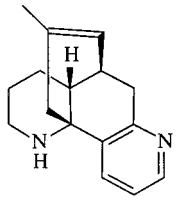
This paper describes the isolation of huperzine A (**1**) as the main antifeedant component of *L. varium*, along with dose response data for (**1**) against three insect pests. The Australian carpet beetle, *A. australis*, and the webbing clothes moth, *Tineola bisselliella* Hummel (Lepidoptera: Tineidae), are best known as important pests of woollen carpets and fabrics (*6*). The advantage of using these species as test organisms for screening for insect-active compounds is that by using wool as the test substrate difficulties associated with solvents in insect food substrates are not present. Furthermore, the assay detects



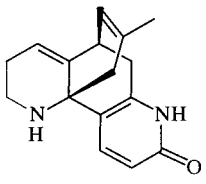
1 Huperzine A



2 Lycopodine



3 Lycodine



4 Huperzine B

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insecticidal and antifeedant activities and developmental impairment of larvae. The Australian sheep blowfly *Lucilia cuprina* Wiedemann (Diptera: Calliphoridae) is a major cause of fly strike in sheep (*7*).

MATERIALS AND METHODS

General Experimental Procedure. All solvents were distilled before use and were removed by rotary evaporation at temperatures up to 40 °C. Aldrich octadecyl-functionalized silica gel (37,763-5) was used for reversed-phase (RP) flash chromatography, Merck Kieselgel 60, 40−63 μm (9385) was used for silica gel flash chromatography, and Woelm neutral aluminum oxide was used for alumina flash chromatography. TLC was carried out using Merck DC-Plastikfolien Kieselgel 60 F_{254}, visualized with a UV lamp and then dipped in Dragendorff solution [0.85 g of $Bi(NO_3)_3$ in 40 mL of H_2O/10 mL of glacial HOAc added to 8 g of KI in 20 mL of H_2O]. Alkaloid compounds appeared as orange spots on a yellow/orange background. Mass spectra were recorded on a Kratos MS80 (electron impact, 70 eV) spectrometer. NMR spectra, of $CDCl_3$ solutions at 25 °C, were recorded at 300 MHz for ^1H and 75 MHz for ^{13}C on a Varian VXR-300 spectrometer. Chemical shifts are given in parts per million on the δ scale referenced to the solvent peaks C**H**Cl_3 at 7.25 and **C**HCl_3 at 77.00.

Collection and Extraction. *L. varium* was collected from an alpine meadow on the southern flank of Mt. Lyall on Campbell Island (52° 53′ S, 169° 10′ E) in January 1994 (voucher specimen on file at Peru Herbarium 940119-01). Air-dried (35 °C) material was ground and a subsample (5.0 g) extracted by shaking overnight in EtOH (50 mL; 95%). Initial screening was carried out using this extract.

Assay Method. The insect assays used in this work have been described elsewhere (*8*, *9*). In *T. bisselliella* and *A. australis* assays, squares of woollen cloth (2.5 cm^2, standard abradant fabric) were treated with 200 μL of test solution. The pure compound huperzine A was serially diluted to obtain six treatment rates (*T. bisselliella*, 430−4500 ppm; *A. australis*, 63−4000 ppm). Each extract, fraction, isolate, and solvent control was replicated four times, and the dilution treatments were replicated two times. Four replicates of the untreated control were included in each assay as moisture content controls. The samples were left to dry overnight at ambient temperatures. Fifteen larvae were placed onto each test sample in well-ventilated 150 mL plastic pots and the assays held at 25 °C and 50−60% relative humidity. After 14 days, larval condition, survival, and feeding damage (wool weight loss) were assessed.

For *L. cuprina*, strips of Whatman grade 1 chromotography paper (30 × 60 mm) were treated with 200 μL of test solution. Control strips were treated with the respective solvents. The pure compound huperzine A was serially diluted to obtain six treatment rates (450−5130 ppm in serum). Each extract or fraction was replicated four times, except when there was insufficient test solution, and controls had six replicates. Strips were rolled and placed in 50 × 12 mm glass vials. Newly hatched larvae (mean larvae used in assays ranged between 30 and 50/replicate) were placed in each vial along with fortified (20 g L^{-1} yeast extract and 5 g L^{-1} KH_2PO_4) sheep serum (400 μL). Vials were plugged and held for 24 h in constant light at 25 °C, after which larval mortality and development were assessed.

Assays were declared invalid if larval mortality or pupation exceeded 10%. Mortality was normally 0% for *T. bisselliella* and *L. cuprina*, but some cannibalism occurs under assay conditions with *A. australis*. Results were analyzed using regression analysis and Flexi 2.2 (*10*). Differences between means were tested using Dunnett's LSD test.

Bioactivity-Directed Isolation of Huperzine A (1). Dry plant material (60 g) was extracted by blending with EtOH (1 × 400 mL, then 3 × 200 mL) to give a crude extract [8.08 g, −81% wool weight loss (wwl) at 8 mg/mL]. This extract (8.0 g) was submitted to RP flash chromatography over C_{18} (adsorbed onto 16 g of C_{18}, loaded on a 70 g C_{18} column) with an $H_2O-CH_3CN-CHCl_3$ gradient. Activity was spread over combined fractions eluted with 1:1 H_2O/CH_3CN (−100% wwl at 10 mg/mL), 1:2 H_2O/CH_3CN (−83% wwl at 10 mg/mL), and 1:3 H_2O/CH_3CN (−82% wwl at 10 mg/mL). These fractions were combined (995 mg), dissolved in $CHCl_3$, and extracted with 1 M HCl. The aqueous extract was basified to ∼pH 9 with aqueous ammonia and then extracted into $CHCl_3$ to give the alkaloid fraction (124 mg, −93% wwl at 2 mg/mL). A sample (96 mg) was further chromatographed over neutral alumina eluting with 0.5−2.0% MeOH in $CHCl_3$. Two fractions eluted with 1% MeOH/$CHCl_3$ showed insecticidal as well as significant antifeedant activity (−97 and −99% wwl, 4.7 and 6.3% larval survival at 2 mg/mL). Combination of these two fractions gave a pure compound identified by ^1H and ^{13}C NMR data as **1**.

*Huperzine A (CAS Registry No. 102518-79-6) (**1**):* Si TLC (1:19 MeOH/$CHCl_3$), R_f 0.35 UV plus orange spot with Dragendorff dip; ^1H and ^{13}C NMR matched Zhou et al. (*12*); EIMS, *m*/*z* 242 (M^+), and CIMS, *m*/*z* 243 (MH^+), as the major peaks.

RESULTS AND DISCUSSION

A small-scale extract of *L. varium* showed strong antifeedant activity against larvae of the carpet beetle, *A. australis*, in that although no significant larval mortality was observed, only a mean of 6.6 mg of treated wool was consumed, compared with 42.7 mg on the untreated controls ($P < 0.001$). This assay was used to direct the isolation of the active component from a bulk extract. Reversed-phase chromatography gave antifeedant activity (4.1−4.6 mg of treated consumed compared with 46.0 mg on the ethanol control, $P < 0.001$) in medium-polarity fractions, as well as larval mortality (51−54% compared with 0%, $P < 0.001$). These fractions were combined and extracted with acid to give an alkaloid fraction, which concentrated the activity. Chromatography over neutral alumina gave one pure active compound. This was identified as huperzine A (**1**) on the basis of MS and ^1H and ^{13}C NMR data (*11*, *12*).

To obtain further quantities of **1**, an isolation method that takes advantage of the presence of both basic amine and acidic amide/phenol groups in **1** was used (*13*). This method gave **1** at a level of 0.02% of dry plant weight from the subantarctic collection of *L. varium*. A collection of *L. varium* from Dunedin on the main South Island of New Zealand also contained **1**. Two other New Zealand species, *Lycopodium fastigiatum* R. Br. and *Lycopodium scariosum* Forst. f., were also collected and extracted to isolate phenolic alkaloids. No trace of **1** was found in either species, which correlates well with their lack of activity against *A. australis*.

Liu et al. isolated huperzine A (**1**) from *Lycopodium serratum* Thunb. [= *Huperzia serrata* (Thunb.) Trev.] (*11*), a plant used in traditional Chinese medicine. More recently it has been shown that the alkaloids from *Lycopodium selago* L. (*14*) and *L. serratum* (*15*), originally assigned different structures, are both identical to **1** (*13*, *16*). Huperzine A (**1**) has been shown to be a strong inhibitor of acetylcholinesterase (*17*, *18*) and has been reported to increase the efficiency of learning and memory in animals (*11*, *19*). The use of **1** as a treatment of Alzheimer's disease (*17*, *20*−*22*) and myasthenia gravis is currently under investigation (*23*). Several total syntheses of huperzine A (**1**) have appeared in the literature (*24*, *25*) along with syntheses and activities of analogues (*26*).

We are not aware of any previous reports of insecticidal activity for huperzine A (**1**). We found that **1** had insecticidal activity against three species from three different orders (Coleoptera, Lepidoptera, and Diptera), that is, broad spectrum rather than specific activity. No effects on larval molting were observed.

Activity was weakest against *L. cuprina* blowfly larvae, with an LD_{50} for **1** of 2.38 mg/mL (2380 ppm) (lower 95% = 2050 ppm, upper 95% = 2750 ppm). Survival ranged from 99 ± 1% at 450 ppm to 8 ± 3% at 5130 ppm. However, this *L. cuprina* assay is not directly comparable to the assays for the other two species as it is conducted over a 24 h period using sheep serum

as the test medium, which the larvae both ingest and inhabit. The two other species are exposed to treated wool cloth for 14 days, and ingestion is the primary method by which huperzine A (**1**) could enter the insects.

Huperzine A (**1**) had a marked effect on *T. bisselliella* moth larvae, with survival ranging from 76 ± 11% at 430 ppm to 3 ± 4% at 4500 ppm. All rates except 430 ppm had significantly fewer live larvae than the untreated controls (100% survival) at $P < 0.05$ or less. The LD_{50} of **1** for this species was 630 ppm (lower 95% = 380 ppm, upper 95% = 830 ppm).

The assay of huperzine A (**1**) against *A. australis* beetle larvae had unusually high numbers of moribund larvae in all treatments except controls, which had 93 ± 7% live larvae. When *A. australis* larval survival was assessed, larvae were judged as live, moribund (affected larvae that were still live but unable to right themselves within 5 min of being turned onto their backs), and dead. Therefore, LD_{50} calculations using live or dead larvae differ greatly, and both estimates have wide upper and lower 95% limits. The LD_{50} of **1** using live larvae was 110 ppm (lower 95% = 2.5 ppm, upper 95% = 1200 ppm; percent survival ranging from 40 ± 14 at 125 ppm to 3 ± 5 at 4000 ppm), whereas with dead larvae it was 2700 ppm (lower 95% = 1100 ppm, upper 95% = 3000 ppm). *A. australis* larval feeding on cloth treated with huperzine A (**1**) was minimal even at the lowest rate tested (63 ppm), with larvae eating a mean of only 1.5 mg of treated wool compared with 55.5 mg on the controls ($P < 0.01$). This high level of antifeedant activity is likely to have given rise to the variability observed in the LD_{50} calculations.

The use of inhibitors of acetylcholinesterase (AChE) as insecticides is well-known, with organophosphates and carbamates as current examples (*27*). The carbamate insecticides can be traced back to AChE inhibitors that were originally discovered as natural products (*28*). In view of the inhibitory activity of huperzine A (**1**) against AChE in mammalian systems (*25*), this is also likely to be the mode of action in insects. In this light it is interesting that a compound closely related to **1**, huperzine B (**4**), has recently been shown to be a better inhibitor of AChE than **1** (*29*). It is therefore possible that **4** is also a more potent insecticide than **1**.

This work demonstrates the ability of natural product screening programs to discover new agents for use in the fight against agricultural pests.

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