Antifeedant Activity of Metabolites of *Aristolochia albida* against the Tobacco Cutworm, *Spodoptera litura*

Labunmi Lajide,* Pierre Escoubas, and Junya Mizutani

Research and Development Corporation of Japan, Eniwa RBP, Megumino Kita 3-1-1, Eniwa-Shi, Hokkaido 061-13, Japan

The antifeedant activity of the metabolites of *Aristolochia albida* root extracts was examined against tobacco cutworm larvae (*Spodoptera litura*, Noctuidae) using a leaf-disk choice bioassay. The antifeedant activities of the crude methanolic extract and aristolochic acid were very strong. 6-Hydroxyaristolochic acid showed moderate activity, while aristolic acid, aristolactam, and aristolone did not demonstrate any antifeedant activity at a 0.1% concentration. Methyl aristolochiate and methyl 6-hydroxyaristolochiate prepared by diazomethane treatment of the acids showed significantly lower activities when compared with the parent acids. When the carboxylic acid group of aristolochic acid was decarboxylated or reduced to the benzyl alcohol or to the aldehyde, significantly lower antifeedant activities were observed for the compounds. These results suggest that a free carboxylic acid group in close proximity to a nitro group in the aristolochic acid ring structure is important to antifeedant activity. In a comparative experiment, aristolochic acid demonstrated a level of activity comparable to azadirachtin, a potent natural antifeedant.

INTRODUCTION

The past decades have seen the desire to limit the use of synthetic chemical insecticides, and there is thus an urgent need to develop alternate means whereby crops may be protected from insect pests using new control methods. These controls include the use of pheromones and antifeedants, the breeding of resistant varieties, and the use of genetic engineering (MacLaren, 1986).

From this perspective, we have examined a variety of plant samples for their insecticidal and insect antifeedant properties, focusing on medicinal plants or following ethnobotanical information. We report here results of the investigation of the bitter root extracts of a Nigerian medicinal plant, *Aristolochia albida*, a tropical climber in the savannah grassland that has not been previously examined chemically, and the antifeedant activities of the metabolites isolated and identified so far.

Plants in the genus *Aristolochia* have been extensively studied for their medicinal activities (Shamma and Moniot, 1978). In the area of plant–insect interactions, aristolochic acids have been examined as ovipositional stimulants for several species of butterflies (Nishida and Fukami, 1989). Aristolochic acid (2) has been found to demonstrate insect chemosterilant activity (Saxena et al., 1979) as well as strong feeding-deterrent properties against the fifth instar larvae of the locusts *Locusta migratoria* and *Schistocera gregaria* (Chapman and Bernays, 1977; Bernays and Chapman, 1977). The activities of the metabolites, including the methyl esters of the acids, and synthetic aristolochic acid derivatives were examined against the tobacco cutworm, *Spodoptera litura*, using a leaf-disk choice bioassay. Bioassay-directed fractionation led to the identification of aristolochic acids as the active constituents of the root extract. Since the antifeedant activity of aristolochic acid was found to be very strong, its activity was also compared to that of neem oil, a well-known antifeedant mixture from the tree *Azadirachta indica*, and to azadirachtin, the main constituent responsible for this activity (Broughton et al., 1986).

* Author to whom correspondence should be addressed.

EXPERIMENTAL PROCEDURES

Instruments. UV absorption spectra were recorded on a Hitachi U3210 spectrophotometer; IR spectra were obtained on a JASCO IR 700 spectrophotometer. Mass spectrometry was recorded with a JEOL HMS-DX 300. ^1H and ^{13}C spectra were determined on a JEOL EX 270 (270 MHz) spectrometer. Melting points were measured with a YAMACO micromelting point apparatus and are uncorrected.

Insects. *S. litura* larvae from a laboratory colony were reared on an artificial diet (Insecta LF, Nihon Nosan Kogyo Co.) in a controlled environmental chamber (27 °C, 75% relative humidity).

Plant Material. Sweet potato (*Ipomoea batatas*) leaves were collected from plants grown in a controlled environmental chamber (25 °C, 16/8 light/dark, 60% relative humidity) from tubers purchased at a local store.

Insect Leaf-Disk Bioassay. Bioassay-guided fractionation and determination of dose–response activity of pure compounds was determined using a procedure described in detail elsewhere (Escoubas et al., 1992). Ten sweet potato leaf disks (1 cm^2), treated with 10 μL of sample solution or, in the case of control, with solvent alone, were arranged alternately in 9 cm diameter plastic Petri dishes, in three replicates. After five 4-day old *S. litura* larvae (beginning of third instar) were allowed to feed for one night, leaf areas were measured with a video camera interfaced with a Macintosh computer. An antifeedant index was calculated as $(100 \times T/C + T)$, where T is the percentage of treated disks consumed and C is the percentage of control disks consumed. An index of less than 20 is considered to indicate significant antifeeding activity (Alkofahi et al., 1989). The index varies from 0 (complete feeding inhibition) to 100 (complete feeding stimulation).

Chemicals. Diazomethane was generated using diazald obtained from Aldrich Chemical Co., Milwaukee, WI. Unless indicated otherwise, all other chemicals were purchased from Wako Pure Chemicals Industries Ltd., Osaka. Silica gel 60 used for column chromatography was obtained from Merck. For thin-layer chromatography, Merck TLC gel plates $60F_{254}$ 5744 were used.

Isolation of *A. albida* Root Metabolites. The root material of *A. albida* (140 g) collected in Billiri, northeastern Nigeria, was soaked in methanol for 72 h. The solid material was filtered and the methanol was removed in vacuo to give a crude extract (13.6 g). Partitioning between ethyl acetate and water layers afforded an ethyl acetate fraction (3.4 g). An ethyl acetate fraction was further separated into acetonitrile (1.8 g) and hexane-soluble

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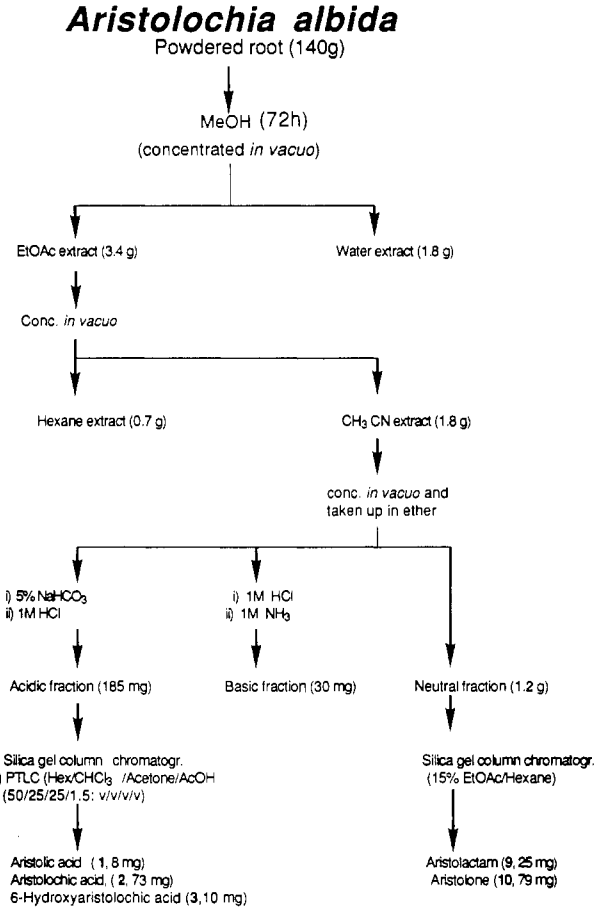
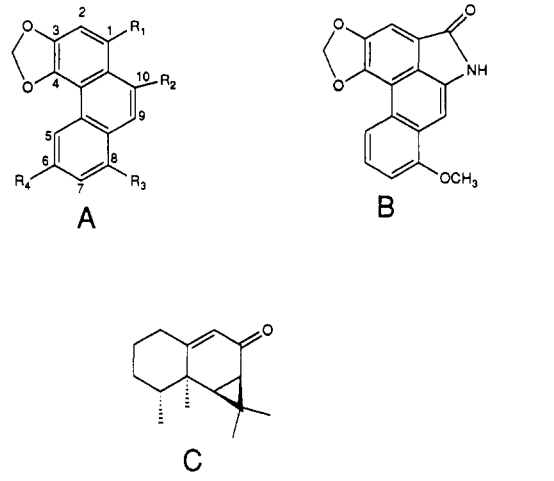


Figure 1. Isolation flow chart for metabolites of *A. albida*.



A:	R_1	R_2	R_3	R_4
Aristolic acid (1)	CO_2H	H	OCH_3	H
Aristolochic acid (2)	CO_2H	NO_2	OCH_3	H
6-hydroxyaristolochic acid (3)	CO_2H	NO_2	OCH_3	OH
Methyl aristolochiate (4)	CO_2CH_3	NO_2	OCH_3	H
Methyl 6-hydroxyaristolochiate(5)	CO_2CH_3	NO_2	OCH_3	OH
6	H	NO_2	OCH_3	H
7	CH_2OH	NO_2	OCH_3	H
8	CHO	NO_2	OCH_3	H

B: Aristolactam (9)

C: (-) Aristolone (10)

Figure 2. Structures of compounds isolated from *A. albida* and synthetic derivatives.

(0.7 g) fractions. The acetonitrile-soluble fraction was concentrated, redissolved in ether, and shaken with 5% aqueous $NaHCO_3$. The aqueous layer was separated and neutralized with 1 M HCl solution. Extraction of the acidic fraction with ether and subsequent evaporation in vacuo gave the crude acidic extract (0.185 g). The organic layer was later shaken with 1 M HCl solution to obtain the basic extracts which, on neutralization with 1 M NH_3 solution and subsequent extraction with ether followed by evaporation, gave the basic extract (0.03 g). The neutral ethereal fraction was washed with water and dried (Na_2SO_4). Evaporation of the ether in vacuo gave the neutral crude extract (1.2 g) (Figure 1).

The acidic extract, using a combination of silica gel column chromatography and final purification by preparative thin-layer chromatography using hexane/chloroform/acetone/acetic acid (50:25:25:1.5 v/v/v/v) as developing eluent, afforded the acids **1–3** (Figure 2).

Aristolic Acid (1, 8 mg, 0.006%). The physicochemical properties and analytical data coincided with the published data for the compound (Watanabe et al., 1988). Recrystallization from methanol gave colorless crystals, mp 272–275 °C. Found: 296.0665 (M^+, $C_{17}H_{12}O_5$). Calcd: 296.0943 (100).

Aristolochic Acid (2, 73 mg, 0.05%). The structure of **2** was confirmed by comparison of the data with those already published (Kupchan and Merianos, 1968). Recrystallization from methanol/chloroform gave wine red crystals of **2**, mp 275–278 °C. FDMS, 241 M^+ (100). Found: 294.0544 ($M^+ - HNO_2$; $C_{17}H_{10}O_5$). Calcd: 294.0781.

6-Hydroxyaristolochic Acid (3, 10 mg, 0.007%). Recrystallization from methanol gave wine red crystals. The physicochemical data were identical in all respects with the already published data (Kupchan and Merianos, 1968), mp 269–271 °C. FDMS, 357 M^+ (100). Found: 310.0546 ($M^+ - HNO_2$; $C_{17}H_{10}O_6$). Calcd: 310.0784.

Silica gel column chromatography of the neutral fraction using 15% ethyl acetate/hexane as eluent gave compounds **9** and **10**.

Aristolactam (9, 25 mg, 0.02%). The analytical data were in good agreement with those reported in the literature (Kupchan and Merianos, 1968). Recrystallization from methanol/chloroform gave yellow needles, mp 315–318 °C. Found: 293.0708 (M^+, $C_{17}H_{11}NO_4$). Calcd: 293.0932, 95.

(–) Aristolone (10, 79 mg 0.06%), which was the least polar in the column elution, was recrystallized from hexane to give long colorless needles, mp 100–101 °C. The analytical data agreed with the published data for the compound (Bauer et al., 1967; Berger et al., 1968; Nishida and Kumazawa, 1973).

Synthesis of Aristolochic Acid Derivatives. *Methyl Aristolochiate (4) and Methyl 6-Hydroxyaristolochiate (5).* These esters were prepared by adding excess ethereal diazomethane solution to the methanolic solution of the respective acid followed by quenching with acetic acid. Evaporation of the solvent followed by recrystallization from methanol gave **4** (96%) as orange crystals, mp 285–286 °C, identical in all respects with the data published for the compound (Pakrashi et al., 1977) and **5** (95%) as yellow crystals, mp 249–253 °C, identical with the data published in the literature for the compound (Nakanishi et al., 1987).

8-Methoxy-3,4-(methylenedioxy)-10-nitrophenanthrene (6). A solution of aristolochic acid (**1**, 30 mg), copper(I) oxide (13 mg), and 2,2′-bipyridyl (85 mg) in anhydrous dimethylformamide (20 mL) was gently refluxed in an atmosphere of nitrogen for 2 h. The cooled reaction mixture was poured into cold, dilute hydrochloric acid (1 M) and the precipitate filtered. The precipitate and the filtrate were extracted with ethyl acetate, and the combined ethyl acetate solution was washed with saturated sodium hydrogen carbonate solution. The residue obtained on evaporation of the ethyl acetate was chromatographed on a short silica gel column with 20% ethyl acetate/hexane as eluent. The major band which developed yielded the decarboxylated nitro compound (**6**, 20 mg, 77%), which crystallized from chloroform/hexane as yellow needles, mp 199–200 °C. EIMS. Found: 297.0679 (M^+, $C_{16}H_{11}NO_5$). Calcd: 297.0900 (100), 257 (6), 221 (11), 193 (27), 165 (28), 163 (32), 150 (29), 82 (14), 75 (11), 40 (24). ^1H NMR ($CDCl_3$) δ 8.76 (1 H, s, C9-H), 8.68

(1 H, d, J = 8 Hz, C5-H), 8.04 (1 H, d, J = 9 Hz, C1-H), 7.68 (1 H, t, J = 8 Hz, C6-H), 7.33 (1 H, d, J = 9 Hz, C2-H), 7.07 (1 H, d, J = 8 Hz, C7-H) 6.32 (2 H, s, OCH_2O), 4.05 (3 H, s, OCH_3).

8-Methoxy-3,4-(methylenedioxy)-1-(hydroxymethyl)-10-nitrophenanthrene (7). Five milligrams of $LiAlH_4$ was added to a solution of methyl aristolochiate (4) (50 mg) in 1,4-dioxane (25 mL). The reaction mixture was stirred at 0 °C for 10 min, after which time it was quenched with 2% HCl (80 mL) and then diluted with ethyl acetate (30 mL). The ethyl acetate layer was separated, washed with water, and dried (Na_2SO_4). The residue obtained on evaporation of ethyl acetate was chromatographed on short silica gel column using 30% ethyl acetate/hexane as eluent. The major slower moving band gave the alcohol (7, 35 mg, 76%), which crystallized from chloroform as pale yellow needles, mp 250–255 °C (dec). EIMS: 309 ($M^+ - H_2O$, 11), 294 (7). Found: 280.0725 ($M^+ - HNO_2$, $C_{16}H_{12}NO_4$). Calcd: 280.0920 (24), 266 (36), 252 (35), 236 (100), 222 (9), 207 (10), 193 (23), 178 (21), 165 (36), 151 (16), 118 (16), 88 (27), 76 (23), 44 (16). ^1H NMR ($CDCl_3$) δ 8.83 (1 H, s, C9-H), 8.69 (1 H, d, J = 8 Hz, C5-H), 7.62 (1 H, s, C2-H), 7.56 (1 H, t, J = 8 Hz, C6-H), 7.15 (1 H, d, J = 8 Hz, C7-H), 6.25 (2 H, s, OCH_2O), 5.05 (2 H, s, CH_2O), 4.01 (3 H, s, OCH_3).

8-Methoxy-3,4-(methylenedioxy)-10-nitrophenanthrene-9-carboxaldehyde (8). The alcohol (7, 60 mg) was dissolved in anhydrous dichloromethane (20 mL) and stirred at room temperature as pyridinium chlorochromate (75 mg) was added in portions. The mixture was stirred for a further 3 h and then diluted with ethyl acetate and the reaction mixture filtered through a Celite pad. The residue obtained on evaporation of the solvent was chromatographed on a short silica gel column with 10% ethyl acetate/hexane as eluent. The major band gave the aldehyde (8, 45 mg, 75%), which crystallized from hexane/chloroform mixture as yellow needles, mp 276–278 °C (dec). EIMS: No M^+. Found: 297.0655 ($M^+ - CO$, $C_{16}H_{11}NO_5$). Calcd: 297.0850 (20), 280 (100), 279 (14), 265 (25), 237 (17), 193 (10), 163 (10), 150 (22), 111 (23), 97 (35), 83 (36), 71 (45), 69 (41), 57 (73), 55 (46), 43 (52). ^1H NMR ($CDCl_3$) δ 10.05 (1 H, s, CHO), 8.89 (1 H, s, C9-H), 8.73 (1 H, d, J = 8 Hz, C5-H), 7.78 (1 H, s, C2-H), 7.52 (1 H, t, J = 8 Hz, C6-H), 7.09 (1 H, d, J = 8 Hz, C7-H), 6.39 (2 H, s, OCH_2O), 4.05 (3 H, s, $-OCH_3$).

RESULTS AND DISCUSSION

The isolation of aristolochic acid metabolites (Figure 1) and the syntheses of aristolochic acid derivatives 4–8 (Figure 3) were very straightforward as described under Experimental Procedures. The results of antifeedant activity of metabolites of the root extracts of *A. albida* are given in Table I. The antifeedant activity is greatest with aristolochic acid (2), whereas the naturally occurring derivative (3), possessing a hydroxy group in the 6-position, was less active. The decrease in activity of 3 is consistent with observations reported in some phenanthrene-based alkaloids that an undissociated phenolic group leads to a marked decrease in activity (Evidente et al., 1986). Furthermore, the esterification of the two acids to afford compounds 4 and 5 resulted in a very significant loss of antifeedant activity against the larvae of *S. litura*. Only weak activity could be observed at a maximum concentration of 1000 ppm for 4, and no activity was detected for 5 at the same concentration. This observation might suggest that the free carboxyl (COOH), in close proximity to the nitro (NO_2), is necessary for antifeedant activity. It is possible that the bonding arrangement between the two functional groups contributes a great deal to the volatility of aristolochic acid (2) and its bitter taste. Aristolactam (9), on the other hand, had no antifeedant activity when compared to 2; the free COOH and NO_2 groups in 2 have been transformed into a lactam-type ring in 9. Aristolic acid (1), lacking the NO_2 group, and aristolone (10) did not show any antifeedant activity at all under our experimental conditions. In an attempt to further clarify the structure–activity relationship of 2,

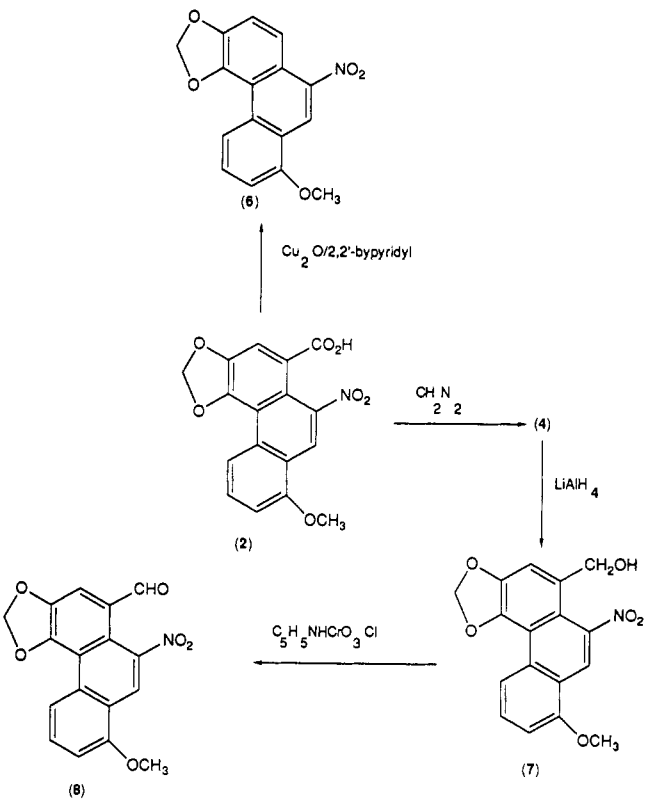


Figure 3. Synthetic scheme for compounds 4 and 6–8.

compounds 6–8 were synthesized and tested for antifeedant activity using the same experimental conditions but at 10 000 ppm, to detect even weak activity. The results obtained showed that modification of the carboxylic acid group in 2 by decarboxylation to give 6 or reduction to 7 and 8 significantly decreased the antifeedant activity even at a very high concentration. It is possible that an electrostatic interaction occurs between the carboxylate ion and a positively charged group of the receptor; this interaction may be enhanced by the close proximity of the nitro group in 2. The very high activity of 2 observed is consistent with previous observations on the antifeedant activity of aristolochic acid (2), in which 0.00001% of 2 reduced feeding by 50% against the fifth instar larvae of the locusts *L. migratoria* and *S. gregaria* (Chapman and Bernays, 1977; Bernays and Chapman, 1977). However, to our knowledge, this is the first report of activity against Lepidoptera.

However, compound 2 was present in the leaf extracts in trace amount only at the time of collection. Seasonal variations due to climatic conditions and/or the absence of herbivory predation at the time of collection may be evoked to explain this observation. Another hypothesis is that the root may function as a storage organ and that compounds could be mobilized and transported to the upper parts of the plant, upon attack by herbivores. Mobilization of plant defensive chemicals in response to stress by insects is a well-known phenomenon and has been described in several other plant families (Tuomi et al., 1990). Further investigation of the chemical composition of *A. albida* leaves in relation to the ecological situation will be needed to validate these conjectures.

A preliminary investigation of the effect of 2 on the growth and survival of *S. litura* larvae revealed significant growth inhibition activity when the compound was incorporated in an artificial diet fed to the insects (Escoubas, 1992, unpublished data). Whether the growth inhibition was due to direct feeding repellency or stomach poisoning

Table I. Activity of *A. albida* Metabolites and Synthetic Derivatives, in the Leaf-Disk Bioassay, against Third Instar Larvae of *S. litura*[a]

dose, ppm	antifeedant index (mean ± SE)											
	1	2	3	4	5	6	7	8	9	10	NO	AZA
10000						45.61 ± 7.89	36.20 ± 9.51	33.68 ± 10.93				
1000	68.09 ± 10.07	0.00 ± 0.00	0.00 ± 0.00	30.86 ± 2.35	49.70 ± 0.30				48.05 ± 5.47	49.92 ± 3.24	0.00 ± 0.00	0.00 ± 0.00
500		0.00 ± 0.00	21.42 ± 6.64	41.12 ± 2.58							0.00 ± 0.00	0.00 ± 0.00
100		3.73 ± 1.86	53.67 ± 17.37	51.04 ± 1.86							0.00 ± 0.00	0.00 ± 0.00
50		19.96 ± 0.93									0.00 ± 0.00	0.00 ± 0.00
10		48.56 ± 12.52									7.47 ± 5.37	4.28 ± 4.27
5											25.27 ± 6.87	18.59 ± 1.80
1											39.38 ± 11.55	29.54 ± 10.86
0.5											36.50 ± 8.37	40.25 ± 2.76
0.1											42.96 ± 6.98	68.57 ± 10.00
0 (control)	54.25 ± 1.80	54.25 ± 1.80	54.25 ± 1.80	54.25 ± 1.80	54.25 ± 1.80	41.88 ± 6.86	41.88 ± 6.86	41.88 ± 6.86	54.25 ± 1.80	54.25 ± 1.80	45.22 ± 2.75	45.22 ± 2.75

[a] Mean ± SE. NO, neem oil; AZA, pure azadirachtin.

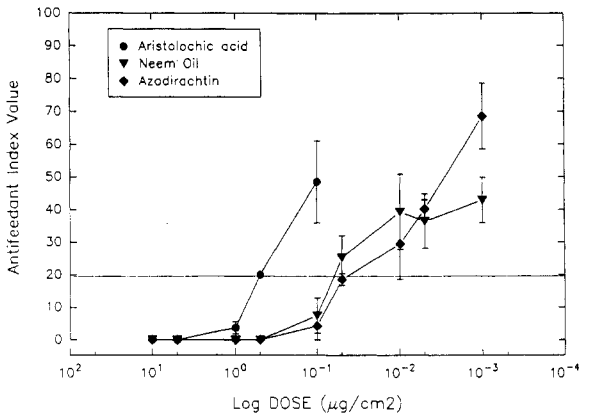


Figure 4. Comparative dose–response activity of aristolochic acid (**2**), azadirachtin, and neem oil against third instar *S. litura* in the leaf-disk bioassay.

by **2** is one of the aspects we are currently trying to clarify with the antifeedant and growth inhibition activity study of several related structures.

The potency of **2** as an antifeedant could make this compound a potentially interesting lead for the development of new plant protection chemicals. To evaluate its activity against a known standard, we have realized a comparative study using neem oil, a well-known antifeedant plant extract, and azadirachtin, one of the most potent feeding-deterrent compounds isolated so far from plants. When compared, the activity of the neem oil sample containing ca. 10% azadirachtin (Isman, 1992, personal communication) was very similar to that of pure azadirachtin. Compound **2** had a lower activity against *S. litura*, but only within 1 order of magnitude (Figure 4). Very few compounds can display such a strong activity against a generalist feeder, and this result clearly demonstrates both the importance of aristolochic acid as a potential plant defensive agent and its potential for the development of agronomically useful substances.

As mentioned previously, the synthesis and structure–activity study of a series of structurally related compounds will enable us to better understand which part of the structure plays the most important role in the interaction with the insects' taste receptors.

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