0014-4754/96/030259-05$1.50 + 0.20 © Birkhäuser Verlag Basel, 1996

Cyclohexadienones-insect growth inhibitors from the foliar surface and tissue extracts of *Senecio cannabifolius*

L. Lajide*, P. Escoubas** and J. Mizutani

Plant Ecochemicals Project, Research Development Corporation of Japan (JRDC), Eniwa RBP, Megumino Kita 3-1-1, Eniwa-Shi, Hokkaido 061-13 (Japan)
Received 27 December 1994; received after revision 31 May 1995; accepted 26 June 1995

Abstract. Dewaxed leaf surface extracts of 12 plants from Hokkaido, prepared by dipping fresh leaves in chloroform for 3 min, were used in a choice leaf-disk bioassay against larvae of the tobacco cutworm *Spodoptera litura*. Activity was found only in the extract of *Senecio cannabifolius*, a very successful weed in Hokkaido. Individual fractions of the extract, however, were not active. Incorporation of the individual fractions of the surface extracts as well as fractions of the methanolic extracts of the leaf residue into an artificial diet fed to neonate *S. litura* led to the isolation of ethyl (1-hydroxy-4-oxocyclohexa-2,5-dien-1-yl) acetate, the major surface compound, as the active principle. This compound was also present in the methanolic extract of the leaf residue together with methyl (1-hydroxy-4-oxocyclohexa-2,5-dien-1-yl) acetate, which had the same growth inhibitory effect on the larvae. The presence of these compounds in the foliar surface and tissue suggests a defensive role against herbivores.
Key words. *Senecio cannabifolius*; Compositae; foliar surface chemistry; tobacco cutworm; *Spodoptera litura*; Lepidoptera; Noctuidae growth inhibition; ethyl (1-hydroxy-4-oxocyclohexa-2,5-dien-1-yl) acetate; methyl (1-hydroxy-4-oxocyclohexa-2,5-dien-1-yl) acetate.

In recent years, much emphasis has been devoted to integrated pest management programs, whereby excessive reliance on the use of synthetic pesticides is avoided, and alternative methods of pest control involving the use of naturally occurring insect and growth regulators (IGR)[1–3] are used instead. As part of our search for botanical pesticides, we have examined the foliar surface chemistry of some Hokkaido plants for anti-insect activities.

The occurrence of surface bioactive compounds in some plants is significant for communication between the plant and its environment in growth, development and survival. Many of these substances, which are of different classes, are present in the leaf exudates extruded via trichomes or formed within the plant and then secreted at the surface. The quality and quantity of these surface compounds suggest they might be important in a defensive role in plants.

An excellent review of the relationship between insect behaviour at the leaf surface and learning has been published[4–6]. A number of studies on the chemistry and role of foliar surface chemicals including trichome exudates have been carried out, using mehtods including chemical defense allocation in wild parsnip[7], identification of defensive chemicals in surface waxed[8,9], determination and identification of leaf surface coumarins[10,11] aphid deterrence by glucose esters in glandular trichome exudate of wild tomato[12], the effect of trichome B exudate of *Solanum berthaultii* on consumption by Colorado potato beetle[13], and influence of tobacco leaf surface chemicals on germination of the fungus *Peronospora tabacina*[14].

During our survey of Hokkaido plants for anti-insect activities we found *Senecio cannabifolius*, a problematic weed to many farmers, growing luxuriantly in many parts of the island. An arsenal of biochemical defenses may contribute to the fitness of *S. cannabifolius*, since many plants are known to produce secondary compounds capable of inhibiting herbivores. In fact, close examination of *S. cannabifolius* leaf surfaces in the field revealed the presence of dead bugs and other insects. Although the chemistry of the genus *Senecio* has been studied extensively and several species of *Senecio* are known to contain the hepatotoxic pyrrolizidine alkaloids[15], terpene and shikimic acid derivatives[16,17], there have been very few literature reports on the foliar surface chemistry and insecticidal activity of this genus. This study deals with the insect growth inhibitory activity of a cyclohexadienone, isolated and identified as the major bioactive foliar surface compound in *S. cannabifolius* and the methyl ester isomer found in the leaf tissue.

* Corresponding author. Present address: Department of Chemistry, Federal University of Technology P. M. B. 704, Akure (Nigeria). ** Present address: Suntory Institute for Bioorganic Research, Wakayamadai, Shimamoto-cho, Mishima-Gun, Osaka 618 (Japan).

Materials and methods

General. Unless indicated otherwise, all chemicals were purchased from Wako Pure Chemicals Industries (Os-

aka) and Aldrick Chemical Company (Milwaukee, WI). Melting points were determined on Yamaco micromelting point apparatus and are uncorrected. ^1H and ^{13}C NMR spectra were recorded on a JEOL EX 270 (270 MHz) spectrometer. Mass spectrometry (EI and HRMS) was obtained from JEOL DB 300.

Plant materials. Fresh leaves of *Senecio cannabifolius*, *Silene alba*, *Helianthus tuberosus*, *Veronicastrum sibiricum*, *Achillea alpina*, *Cirsium kamstchaticum*, *Vitis cognetiae*, *Gymnostemma pentaphylum*, *Vicia circa*, *Actinidea arguta*, *Urtica nagabaraita* and *Fraxinus manshurica* were collected (July, 1990) at Tomakomai Experimental Forest Station and Jozankei Forest Reverse in Hokkaido.

Sweet potato (*Ipomoea batatas*) leaves for antifeedant bioassay were collected from plants grown in a controlled environmental chamber (25 °C, 16:8 LD, 60% RH) from tubers purchased at a local store.

Extraction method. For surface compounds, composite samples of fresh foliage from plants before flowering stage were dipped in chloroform (5 ml/g) for 3 min. $CHCl_3$ was removed in vacuo and the residue taken up in small volume of acetone and cooled to 0 °C overnight. The acetone solution, vacuum filtered to remove the extracted plant waxes, was concentrated in vacuo.

For the leaf tissue, the leaf residue was soaked in methanol for 24 h, after which the plant material was removed by filtration and the solvent removed in vacuo.

Insects. *Spodoptera litura* larvae from a laboratory colony were reared on an artificial diet (INSECTA LF, Nihon Nosan Kogyo Co) in a controlled enviromental chamber (27 °C, 75% RH.)

Insect choice leaf-disk bioassay. Ten sweet potato leaf disks (1 cm^2), treated with 10 ml 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 allowing five four-day-old *Spodoptera litura* larvae (beginning of third instar) to feed for one night, leaf areas were measured with a video camera interfaced with a Macintosh computer[18]. These values were transformed into an antifeedant index calculated as: $(T/(C+T)) * 100$ where T = % of treated disks consumed and C = % of control disks consumed[19]. A value of less than 20 indicated significant feeding-deterrent activity. The index varies from 0 (complete feeding inhibition) to 100 (complete feeding stimulation).

Diet incorporation bioassay. A sample solution was prepared at the appropriate concentration in acetone.

1 ml of the solution was mixed with 1 g of cellulose powder and the solvent was evaporated in vacuo for at least 1 hr in a desiccator. The cellulose powder was then thoroghtly mixed with 9 g of artifical diet (Insecta LF) in 200 ml of distilled water. The diet was equally divided into four portions and each portion was placed in a plastic Petri dish (55 mm) lined with wet filter paper. Five neonate *Spodoptera litura* larvae were placed in each dish. All dishes were then placed in a plastic container lined with wet filter paper in an incubator at 27 °C and 80% RH, in the dark. For each batch of samples, two controls were prepared: diet only (DIET), diet + cellulose powder + solvent (CONTROL). After one week, larvae were individually weighed and the mean weight (n = 20) was calculated. The weight differences between controls and samples were compared using a non-parametric statistical test (Kolmogorov-Smirnov test) to evaluate the significance.

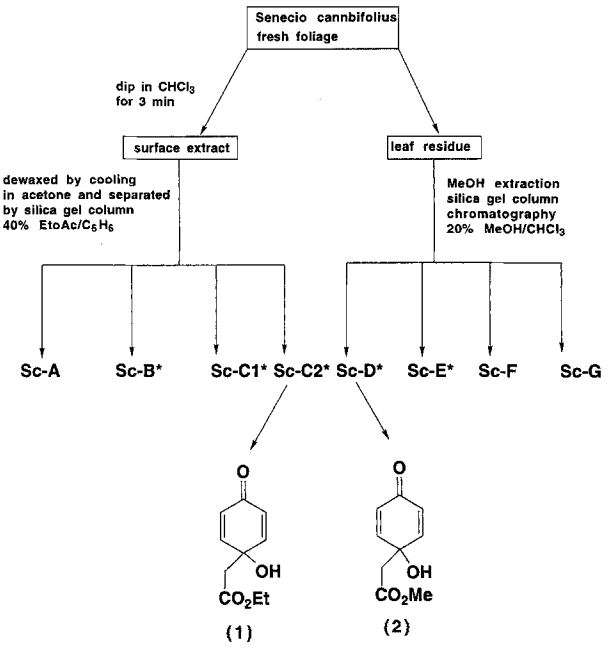


Figure 1. Bioassay directed fractionation of leaf surface and tissue extracts of *Senecio cannabifolius* and the isolation of (**1**) and (**2**). Active fractions significantly different from control are indicated by * for $P<0.05$.

Isolation and identification of growth inhibiting compounds. In a typical bioassay directed fractionation, 7.17 g of fresh leaves of *S. cannabifolius* yielded 263.3 mg of crude surface extract which was separated into four fractions, Sc-A, Sc-B, Sc-C1 and Sc-C2 in increasing order of polarity (silica gel column using ethyl acetate:benzene (40:60) as eluent), by collecting 100 ml for each fraction. The methanolic extract of the leaf residue yielded 582.8 mg of crude extract, which was separated into four fractions: Sc-D, Sc-E, Sc-F and Sc-G in increasing order of polarity (silica gel column using methanol:chloroform (20:80) as eluent), collecting 100 ml for each fraction, from rf = 0.00 – 0.50, (fig. 1). The active fractions Sc-C2 from the foliar surface extract and Sc-D from the leaf tissue extract were further purified using preparative thin layer chomatography, eluted with 40% ethyl acetate/benzene to isolate the active compounds which were then indentified using ^1H and ^{13}C, COSY (Correlation Spectroscopy), DEPT (Distortionless Enhanced Polarisation Transfer) and C-H-COSY, NMR and EI/HRMS (Electron Impact

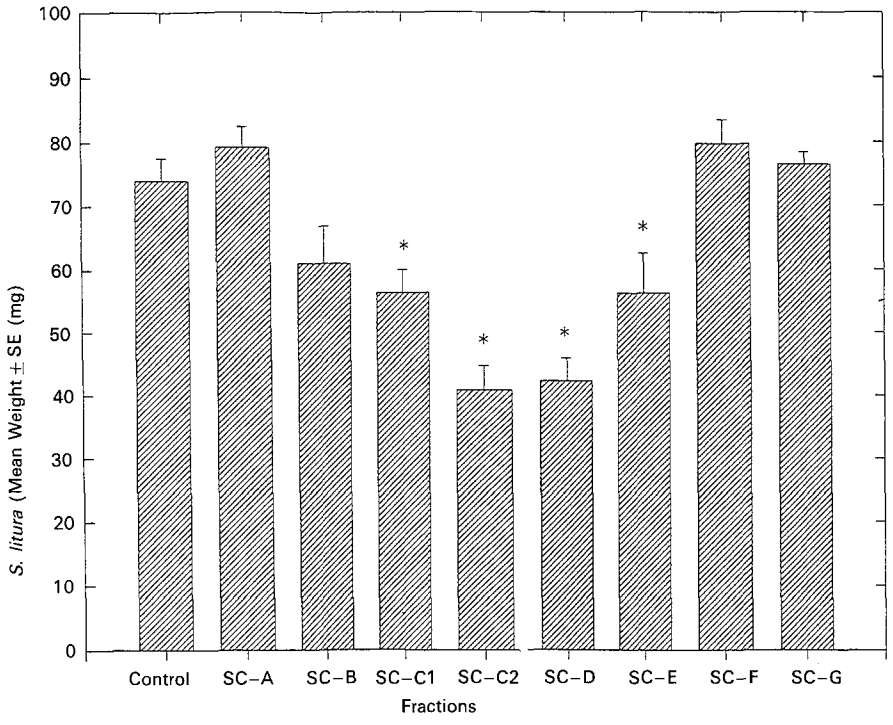
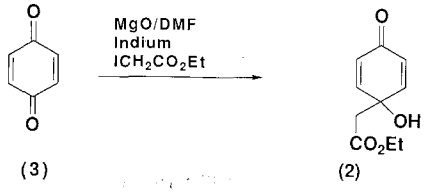


Figure 2. Effect of foliar surface extract, fractions Sc-A to Sc-C1 and leaf residue extract, fractions Sc-D to Sc-G, on the growth of *S. litura* larvae. Means of treated group significantly different from control are indicated by * for $p < 0.05$.

High Resolution Mass Spectroscopy) spectroscopy. Sc-C2 fractions gave the major surface active compound ethyl (1-hydroxy-4-oxocylohexa-2,5-dien-1-yl) acetate, (1, 23.3 mg, 0.32% fresh weight or 8.8% of total surface extract,: rf = 0.47). Recrystallization from chloroform/hexane gave the quinone ethyl ester (1) as colorless cubes, m.p. 70–71 °C. lit.[20] m.p. 67–69 °C. found M^+ 196.0741, $C_{10}H_{12}O_4$, calculated 196.0742. ^1H NMR ($CDCl_3$), δ, 1.28 (3H, t, J = 7 Hz, C-10), 2.69 (2H, s, C-7), 4.21 (2H, q, J = 7 Hz, C-9), 6.18 (2H, d, J = 10 Hz, C-3, C-5), 6.97(2H, d, J = 10 Hz, C-2, C-5); ^{13}C NMR, δ, 14.00 q, C-10), 43.84(t, C-7), 61.40 (t, C-9) 67.28 (s, C-1), 128.07 (d, C-3, C-5), 149.16 (d, C-2, C-6), 170.56 (s, C-8), 185.05 (s, C – 4); m/z 196 (3), 180 (16), 150 (14), 122 (16), 109 (52), 108(100), 88 (40), 81 (18), 60 (24), 40 (58). The same ethyl ester (1) was obtained from the leaf tissue extract (71.7 ng, 1% fr.wt.) and from Sc-D, methyl (1-hydroxy-4-oxocyclohexa-2,5-dien-1-yl) acetate, (2, 46.3 mg, 0.6% fr.wt., rf = 0.41). Recrystallization from chloroform/hexane gave the methyl ester, (2) as colorless needles, m.p. 76–77 °C, lit.[21] m.p. 77 °C, found M^+, 182.0545, $C_9H_{10}O_4$, calculated, 182.0548. ^1H NMR, ($CDCl_3$)δ, 2.71 (2H, s, C-7), 3.76 (3H, s, C-9), 6.20 (2H, d, J = 10 Hz, C-3, C-6), 6.96 (2H, d, J = 10 Hz, C-2, C-5); ^{13}C NMR, δ, 43.28 (t, C-7), 52.29 (q, C-9), 67.29 (s, C-1), 128.24 (d, C-3, C16), 148.90 (d, C-2, C-5), 171.17 (s, C-8), 184.93 (s, C-1); m/z 182 (8), 169 (5), 150 (18), 122 (17), 109 (100), 94 (7), 81 (39), 74 (64), 69 (15), 43 (38). This compound was also obtained when the extraction solvent was changed to ethanol, in order to prove that the methyl ester (2) was not an artefact of methanol extraction.

Synthesis of ethyl (1-hydroxy-4-oxocyclohexa-2,5-dien-1-yl) acetate, (1). A modified procedure of Araki et al[22] was used. Ethyl iodoacetate (25 g) was added to a stirred suspension of small pieces of indium metal (7.17 g), stabilized by 5% magnesium oxide in dimethylformamide (200 ml) under argon. The resulting mixture was stirred at room temperature until the reaction became exothermic. 1,4-benzoquinone, (3, 5.06 g) was then added in one portion and stirred for 1 hr. The reaction mixture was poured into cold dilute HCl and extracted with CH_2Cl_2 (80 ml). The organic layer was separated. The residue obtained after the removal of CH_2Cl_2 was purified on a silica gel column with 20% ethyl acetate/hexane as eluent. The major band yielded the desired quinone ethyl ester (1, 5.7 g, 72%), identical in all respects to the natural compound (scheme).



Scheme. Synthetic route to ethyl (1-hydroxy-4-oxo-2,5-cyclohexadienyl) acetate (**1**).

Results and discussion

From the foliar surface extract of 12 selected Hokkaido plants, it was evident that only the crude extracts of

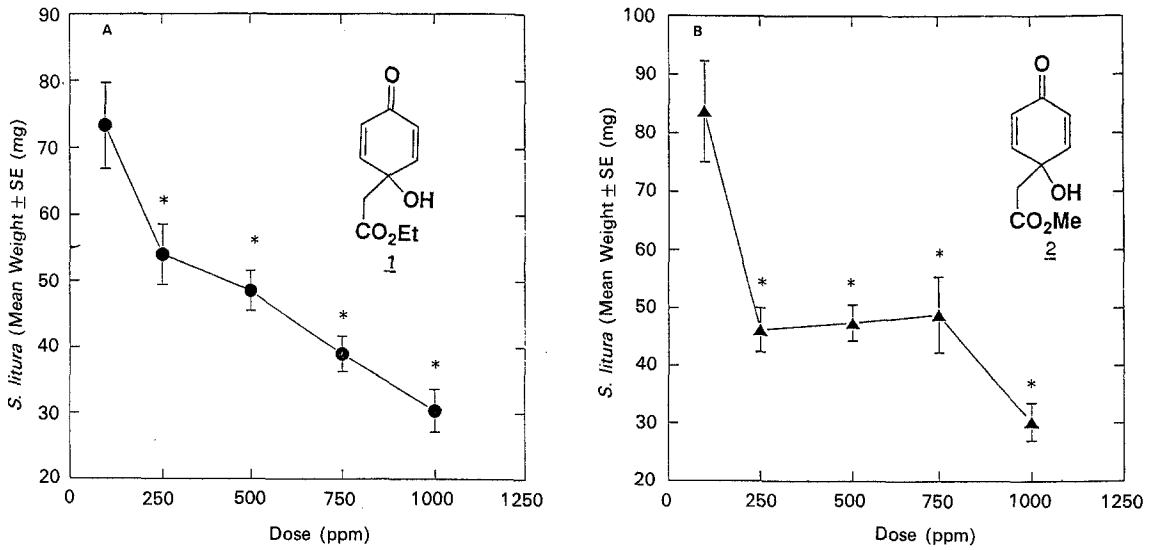


Figure 3. Dose-response growth inhibition curve of A) ethyl and B) methyl (1-hydroxy-4-oxo-2,5-cyclodhexadienyl) acetate on the weight of *S. litura* larvae. Means of treated group significantfiy different from control are indicated by * for $p < 0.01$.

S. cannabifolius demonstrated significant feeding inhibition at 10,000 ppm concentration.

The results of the diet incorporation bioassay of individual fractions of the foliar surface extract (Sc-A, Sc-B, Sc-C1, Sc-C2) and the leaf tissue extract (Sc-D, Sc-E, Sc-F and Sc-G), containing 500 ppm of plant extract with the exception of fractions Sc-A (250 ppm) and Sc-B (100 ppm), are given in fig. 2. Fractions Sc-A from the foliar surface extract and Sc-F and Sc-G from the leaf tissue extract demonstrated very little effect on the growth of *S. litura* larva. The 40% growth reduction produced by fraction Sc-B with the lowest concentration in the series was very significant, while fractions Sc-C1 and Sc-E both gave 46% growth reduction. The isolation and identification of the active principle or principles from Sc-B, Sc-C1 and Sc-E fractions was not pursued further, because of paucity of extract material from these fractions.

The fractions with the strongest growth inhibitory activity were Sc-C2 and Sc-D, which gave 60% and 59% growth reduction respectively. The results of the dose-response of the diet incorporation of the pure compounds (**1**) and (**2**) are given in fig. 3. At 1000 ppm, both compounds produced severe growth inhibition, while at the minimum concentration of 250 ppm the ethyl ester (**1**) gave 45% growth reduction and the methyl ester, (**2**), 55%.

The cyclochexadienone (**1**) has previously been isolated from several *Senecio* species[17] while the methyl ester, (**2**) known as jacaranone, has previously been isolated from *Jacaranda caucana*[23], an alga, *Delesseria sanguinea* as a metamorphosis inducer of *Pecten* lavae[21] and from *Senecio picardae*[24] (Bohlmann et al, 1990). The antineoplastic activity of (**2**) has also been described[25]. This is, to our knowledge, the first report on the insecticidal activities of these compounds on a Lepidoptera.

The growth retardation effect of these compounds on *S. litura* larva, coupled with its stability under normal light (no chemical decomposition of crystals of (**1**) after one year of exposure to normal laboratory environment), and the presence of the ethyl ester (**1**) in high concentration on the leaf surface, suggest the utilization by *S. cannabifolius* of the foliar surface and tissue chemistry for defense against herbivores. Secondary plant compounds may act as insecticides by poisoning per se or by production of toxic molecules after ingestion. The compounds may also deter or possibly also repel an insect from feeding. Although quinone, p-quinones and their methides, which are highly reactive electrophilic molecules, have been implicated as intermediates in oxidative phosphorylation and elimination reactions[26,27] they may also undergo nucleophilic attack by a cell constituent(s), for example an S-group of a key enzyme[28].

The precise reaction of the cyclohexadienone ester and the receptor sites at the tissue, cellular or biochemical level have yet to be determined. The simplicity of the structures of these compounds and the ease of synthesis offer unique advantages in there utilization potential plant protection chemicals. These compounds are not highly toxic to insects at low concentrations, but nevertheless stress the insect population thus reducing their overall fitness, and they may therefore be used in combination with low concentrations of other insecticides.

Acknowledgements. This project is funded and supported by Research and Development Corporation of Japan under the ER-ATO program. Grateful thanks to Y. Matsuzawa for technical assistance, K. Watanabe and E. Fukushi for EIMS/HRMS, and Dr. Nakai of Hokkaido University Botany Department for identification of the plants.

1 Rosenthal, G. A., Sci. Am. 258 (1985) 94. pp. 1–34.
2 Rosenthal, G. A., in: Herbivores: Their interaction with Secondary Plant Metabolites, 2nd edition, Volume I. Eds M. R. Berenbaum and G. A. Rosenthal.
3 Balandrin, M. F., Klocke, J. A., Wurtele, E. S., and Bollinger, H. W. Science *228* (1986) 1154.
4 Chapman, R. F., Bull Entomol. Res. *64* (1974) 339.
5 Chapman, R. F., and Bernays, E. A. Experientia *45* (1989) 215.
6 Bernays, E. A., and Chapman, R. F., Host-Plant Selection by Phytophagous Insects. Chapman and Hall, New York 1994.
7 Nitao, J. K., and Zangerl, A. R. Ecology *68* (1987) 521.
8 Stadler, E., and Buser, H. R. Experientia *40* (1984) 1157.
9 Eigenbrode, S. D., Espelie, K. E., and Shelton, A. M. J. chem. Ecol. *17* (1991) 1694.
10 Zobel, A. M., and Brown, S. A. J. nat. Prod. *51* (1988) 941.
11 Zobel, A. M., Wang, J., Mach, R. E., and Brown, S. A., J. chem. Ecol. *17* (1991) 1859.
12 Goffreda, J. C., Mutschler, M. A., Ave, D. A., Tingey, W. M., and Steffens, J. C., J. chem. Ecol. *15* (1989) 2135.
13 Pelletier, Y., and Smilowitz, Z., J. chem. Ecol. *16* (1990) 1847.
14 Menetrez, M. L., Spurr, H. W. Jr., Danehower, D. A., and Lawson, D. R., J. chem. Ecol. *16* (1990) 1565.
15 Roeder, E., and Lu, K., Phytochemistry *30* (1991) 1734.
16 Barrero, A. F., Sanchez, J. F., Alvarez-Manzaneda, E. J., and Alvarez-Manzaneda, R., Phytochemistry *27* (1988) 1191.
17 Bohlmann, F., Knoll, K., Zdero, C., Marhanta, P. K., Grenz, M., Suwita, A., Ehlers, D., LeVan, N., Abraham, R. W, and Natu, A. A., Phytochemistry *16* (1977) 965.
18 Escoubas, P., Lajide, L., and Mizutani, J., Entomologia exp. appl. *66* (1993) 99.
19 Alkofahi, A., Rupprecht, J. K., Anderson, J. E., McLaughlin, J. L., Mikolajczak, K. L., and Scott, B. A., in: Insectides of Plant Origin (ACS Symposium Series, vol. 387) p. 25. Eds. J. T. Arnason, B. J. R. Philogene, and P. Morand. American Chemical Society, Washington D. C. 1989.
20 Gelbaum, L. T., Zalkow, L. H., and Hamilton, D., J. nat. Prod. *45* (1988) 370.
21 Yvin, J. C., Chevolot, L., Chevolot-Magueur, A. M., and Cochard, J. C., J. nat. Prod. *48* (1985) 814.
22 Araki, S., Katsumura, N., Kawasaki, K., Butsugan, Y., J. chem Soc., Perkin Trans. 1, *3* (1991) 499.
23 Ogura, M., Cordell, G. A., and Farnsworth, N. R., J. nat. Prod. *39* (1976) 255.
24 Bohlmann, F., Dupre, S., and Nordenstam, B., Phytochemistry *29* (1990) 3163.
25 Pettit, G. R., Einck, J. J., Brown, P., Harvey, T. B. III., Ode, R. H., and Page, C. R., J. nat. Prod. *43* (1980) 609.
26 Turner, A. B., Q. Rev. chem. Soc. *18* (1964) 347.
27 Norris, D. M. in: Chemistry of Plant Protection, p. 97. Eds W. S. Bowers, W. Ebing, T. R. Fukuto, D. Martin, R. Wegler and I. Yamamoto, Springer Verlag, Heidelberg 1986.
28 Kubo, I., and Nakanishi, K, in: Host Plant Resistance to Pests, p. 165 Ed. P. A. Hedin. Am. Chem. Soc. Washington D. C. 1977.