Biologically Active Carbazole Alkaloids from *Murraya koenigii*

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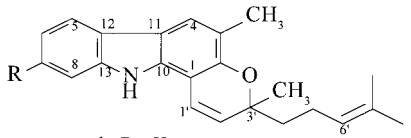
The bioassay guided fractionation of the acetone extract of the fresh leaves of *Murraya koenigii* resulted in the isolation of three bioactive carbazole alkaloids, mahanimbine (**1**), murrayanol (**2**), and mahanine (**3**), as confirmed from their ^{1}H and ^{13}C NMR spectral data. Compound **2** showed an IC_{50} of 109 μg/mL against hPGHS-1 and an IC_{50} of 218 μg/mL against hPGHS-2 in antiinflammatory assays, while compound **1** displayed antioxidant activity at 33.1 μg/mL. All three compounds were mosquitocidal and antimicrobial and exhibited topoisomerase I and II inhibition activities.

Keywords: *Murraya koenigii; carbazole alkaloid; mahanimbine; murrayanol; mahanine; mosquitocidal; antimicrobial; antiinflammatory; antioxidant*

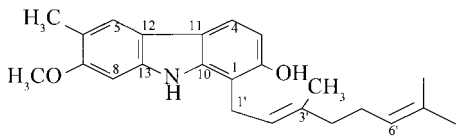
INTRODUCTION

The genus *Murraya* belongs to the family Rutaceae of the order Rutales and is in the subtribe Clauseneae, subfamily Aurantoidae (Chakraborty, 1977). It consists of shrubs or small trees distributed from southeast Asia to Australia. *Murraya koenigii* Sprang (*M. koenigii*) occurs widely in east Asia and is a small tree. It is popularly known in India as the curry leaf plant, and the leaves are widely used as a condiment. Various parts of the plant have been used in traditional or folk medicine for the treatment of head-, tooth-, and stomachaches, influenza, rheumatism, traumatic injury, and insect and snake bites, and as an antidysentric as well as an astringent (Kong et al., 1986).

Since the first report of a carbazole alkaloid, murrayanine, from the stem bark of *M. koenigii* (Chakraborty et al., 1965), a number of carbazole alkaloids have been isolated from this species, possessing C_{13}, C_{18} or C_{23} skeletons (Fiebig et al., 1985; Reisch et al., 1992; Ito et al., 1993; Chakrabarty et al., 1997). Our bioassay directed investigation of the leaves of *M. koenigii* have led to the isolation of three C_{23} carbazole alkaloids, mahanimbine (**1**) (Roy et al., 1974), murrayanol (**2**) (Reisch et al., 1992), and mahanine (**3**) (Narasimhan et al., 1970). Compounds **1** and **3** were reported earlier from the leaves of *M. koenigii* (Roy and Chakrabarty, 1974; Narasimhan et al., 1970). However, the isolation of murrayanol (**2**) is being reported for the first time from the leaves of *M. koenigii*. Compound **2** was previously isolated from the seeds of *M. koenigii* (Reisch et al., 1992). These compounds were isolated using mosquitocidal, topoisomerase I and II, and antimicrobial bioassay guided fractionation from the crude acetone extract. This is the first report of the biological activities, including antioxidant and antiinflammatory activities of these carbazole alkaloids.



1 : R = H
3 : R = OH



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MATERIALS AND METHODS

General Experimental Procedures. ^{1}H- and ^{13}C NMR and DEPT spectra were recorded on a Varian INOVA 300 MHz spectrometer. ^{1}H NMR spectra were recorded at 300 MHz, while ^{13}C NMR spectra were recorded at 75 MHz. Chemical shifts were recorded in $CDCl_3$ and the values are in δ (ppm) on the basis of the δ residual of $CHCl_3$, 7.24, and $CDCl_3$, 77.0. Coupling constants, *J*, are in hertz. The silica gel used for VLC and MPLC was Merck Silica gel 60 (35−70 μm particle size). TLC plates (GF Uniplate, Analtech, Inc., Newark, DE), after developing, were viewed under UV light (254 and 366 nm). All organic solvents used were ACS reagent grade (Aldrich Chemical Co., Inc., Milwaukee, WI).

Plant Material. Fresh leaves of *M. koenigii* were harvested from plants grown in the Michigan State University, Department of Horticulture and National Food Safety and Toxicology Center greenhouses. A voucher specimen (MSC 363917) of this plant material was deposited at the Michigan State University Herbarium in the Department of Botany and Plant Pathology.

Extraction and Isolation. The leaves (1.13 kg) were macerated with acetone (4 L) using an industrial Waring blender. After 24 h, the solvent was removed and the residue was further extracted with acetone (4 L) for 36 h. The solvent was removed and the extracts combined to yield a greenish, gummy extract (22.30 g). Fractionation of this extract (13.52

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10.1021/jf9805808 CCC: $18.00
Published on Web 01/13/1999

g) was carried out by vacuum liquid chromatography (VLC) using a polypropylene filter pump operating at a vacuum of 1.0 psi. Silica gel (250 g) was placed in a sintered glass funnel (600 mL, 10−15 μm mesh), and hexane with increasing amounts of acetone and finally methanol was used as the eluting solvents. Seven fractions, A−G, were collected: (A) 100% hexane, 800 mL, 850 mg; (B) hexane−acetone, 10:1, 300 mL, 10 mg; (C) hexane−acetone, 10:1, 900 mL, 3.31 g; (D) hexane−acetone, 4:1, 300 mL, 740 mg; (E) hexane−acetone, 4:1, 1500 mL, 2.77 g; (F) hexane−acetone, 1:1, 1200 mL, 490 mg; and (G) 100% methanol, 1200 mL, 2.99 g. Only fractions C and E were biologically active.

Medium-pressure liquid chromatography (MPLC) on silica gel (Sanki Engineering Ltd., Model LBP-V pump operating at 10−15 psi; Chemco MPLC tayperling type glass column, 55 cm in length) was performed on the bioactive fraction C (1.12 g), using hexane with increasing amounts of acetone and finally methanol, to give five fractions. Fractions I (14 mg), II (233 mg) and III (221 mg) were eluted with hexane−acetone (15:1, 150 mL each), fraction IV with hexane−acetone (1:1, 200 mL, 596 mg), and fraction V with 100% methanol (300 mL, 53 mg). The bioactive fraction, IV, was purified by preparative TLC using 10:1 hexane−acetone; and 15:1 hexane−acetone to yield compound **1** (64 mg).

MPLC was performed on fraction E (1.27 g), using hexane with increasing amounts of acetone and finally methanol to yield fractions I (27 mg), II (40 mg), III (391 mg), and IV (405 mg) eluting with hexane−acetone (3:1, 600 mL) and fraction V eluting with 100% methanol (1000 mL, 74.4 mg). The bioactive fractions were III and IV. Fraction III was further purified by preparative TLC using 3:1 hexane−acetone and 5:1 hexane−acetone to give compound **2** (98 mg). Fraction IV was further purified by preparative TLC using 3:1 hexane−acetone and 5:1 hexane−acetone to give compound **3** (95 mg). All three compounds were compared on TLC and were found to be single components as well as they were spectroscopically pure as determined from their ^{1}H and ^{13}C NMR spectra.

Compound 1. ^{1}H NMR: δ 1.45 (3H, s, 3′-Me), 1.60, 1.68 (each 3H, s, 7′-Me), 1.74−1.84 (2H, m, 4′-CH_2), 2.12−2.28 (2H, m, 5′-CH_2), 2.36 (3H, s, 3-Me), 5.12 (1H, t, J = 6.7 Hz, 6′-H), 5.64 (1H, d, J = 9.4 Hz, 2′-H), 6.63 (1H, d, J = 9.4 Hz, 1′-H), 7.19 (1H, t, J = 7.6 Hz, H6), 7.31 (1H, t, J = 7.6 Hz, H7), 7.36 (1H, d, J = 7.6 Hz, H8), 7.67 (1H, s, H4), 7.91 (1H, d, J = 7.6 Hz, H5), 8.05 (1H, s, −NH). ^{13}C NMR: δ 16.03 (3′-Me), 17.53 (7′-Me), 22.70 (C-4′), 25.63 (7′-Me), 25.79 (3-Me), 40.75 (C-5′), 78.09 (C-3′), 104.16 (C-8), 108.12 (C-7′), 110.34 (C-6′), 116.56 (C-4a), 117.35 (C-5a), 117.53 (C-4), 118.29 (C-1), 119.21 (C-5), 119.34 (C-6), 121.12 (C-7), 124.17 (C-2′), 128.37 (C-1′), 131.57 (C-3), 134.87 (C-9a), 139.46 (C-8a), 149.84 (C-2). It was identified as mahanimbine, $C_{23}H_{25}NO$, by comparison of its ^{1}H and ^{13}C NMR data with literature values (Roy and Chakraborty, 1974).

Compound 2. ^{1}H NMR: δ 1.59, 1.65 (each 3H, s, 7′-Me), 1.89 (3H, s, 3′-Me), 2.04−2.12 (4H, m, 4′ and 5′-CH_2), 2.40 (3H, s, 6-Me), 3.64 (2H, d, J = 6.4 Hz, 1′-CH_2), 3.94 (3H, s, 7-OMe), 5.10 (1H, t, J = 4.2 Hz, 6′-H), 5.35 (1H, t, J = 6.2 Hz, 2′-H), 5.39 (1H, s, −OH), 6.75 (1H, s, H8), 6.85 (1H, d, J = 8.4 Hz, H3), 7.69 (1H, s, H5), 7.73 (1H, d, J = 8.4 Hz, H4), 7.75 (1H, s, −NH). ^{13}C NMR: δ 16.11 (6-Me), 16.30 (3′-Me), 17.62 (7′-Me), 23.76 (C-5′), 25.60 (7′-Me), 26.56 (C-4′), 39.59 (C-1′), 56.71 (−OMe), 96.70 (C-8), 104.64 (C-6′), 111.24 (C-7′), 116.11 (C-1), 117.05 (C-2′), 117.62 (C-4a), 117.94 (C-4b), 121.03 (C-5), 122.33 (C-4), 123.99 (C-3), 131.54 (C-6), 136.35 (C-3′), 139.54 (C-9a), 140.16 (C-8a), 152.21 (C-2), 154.67 (C-7). It was identified as murrayanol, $C_{24}H_{29}NO_2$, and the spectral data are identical to the published data (Reisch et al., 1992).

Compound 3. ^{1}H NMR: δ 1.40 (3H, s, 3′-Me), 1.60,1.67 (each 3H, s, 7′-Me), 1.69−1.80 (2H, m, 4′-CH_2), 2.06−2.22 (2H, m, 5′-CH_2), 2.32 (3H, s, 3-Me), 5.10 (1H, t, J = 6.4 Hz, 6′-H), 5.51 (1H, d, J = 9.9 Hz, 2′-H), 6.20 (1H, br s, −OH), 6.37 (1H, d, J = 9.4 Hz, 1′-H), 6.61 (1H, s, H8), 6.66 (1H, d, J = 9.1 Hz, H5), 7.50 (1H, s, H4), 7.62 (1H, d, J = 9.1 Hz, H6), 7.65 (1H, s, −NH). ^{13}C NMR: δ 15.99 (3′-Me), 17.53 (7′-Me), 22.66 (C-4′), 25.61 (7′-Me and 3-Me), 40.60 (C-5′), 77.96 (C-3′), 97.17 (C-8), 104.36 (C-7′), 108.31 (C-6′), 116.59 (C-4a), 117.40 (C-4), 117.86 (C-5a), 118.11 (C-1), 119.92 (C-5), 120.30 (C-6), 124.20 (C-2′), 128.52 (C-1′), 131.56 (C-3), 134.73 (C-9a), 140.69 (C-8a), 148.37 (C-7), 153.29 (C-2). It was identified as mahanine, $C_{23}H_{25}NO_2$, by comparison of its ^{1}H and ^{13}C NMR data with literature values (Narasimhan et al., 1970).

Mosquitocidal Assay. Fourth instar mosquito larvae, *Aedes aegyptii*, were reared from eggs. Ten to fifteen larvae were placed in 980 μL of distilled water in test tubes and 20 μL of dimethyl sulfoxide (DMSO) solution containing test extracts or pure compounds were added. The test concentrations were 250 μg/mL for crude extracts and 100 μg/mL for pure compounds, which were then serially diluted to 0.1 μg/mL. Controls received 20 μL of DMSO in the place of test compounds. The assays were conducted in triplicate, and the mortality was recorded at 2, 4, 12, and 24 h intervals (Nair et al., 1989; Roth et al., 1998).

Topoisomerase I and II Inhibitory Assays and Antimicrobial Assays. *Saccharomyces cerevisiae* mutant cell cultures, JN271-R52, JN271−R52 $t_{-1,}$ JN271−R52 t_{-2-5}, JN394, JN394 t_{-1}, and JN394 t_{-2-5}, used for the topoisomerase assay, were cultured in Petri dishes containing YPDA medium (20 mL). The test organisms *Candida kruseii* (MSU strain), *Candida parapsilasis* (MSU strain), *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (MSU strain), and *Streptococcus pyogenes* (MHM-1645) used for the antimicrobial bioassays were cultured in Petri dishes containing YMG or PDA media (20 mL). The cells from a fully grown plate of each organism were suspended in saline solution (10 mL) and were diluted to obtain 5×10^6 CFU/mL. A 50 μL aliquot of this suspension was then used to inoculate culture tubes containing the corresponding media (930 μL). Test compounds were dissolved in DMSO and were added to the inoculated tubes (20 μL) at concentrations ranging from 100 to 0.1 μg/mL. The tubes containing cell cultures and compounds were incubated at 27 °C for 72−96 h. At the end of the incubation period, MIC_{100} values (the concentration of the test compound causing total inhibition of the test organism when compared to the control) for the test compounds were recorded for each test organism. Controls were prepared by adding DMSO (20 μL) to the inoculated tube (Chang et al., 1995; Roth et al., 1998; Nair et al., 1989).

Antiinflammatory Assay. Human prostaglandin H synthase isozymes (hPGHS-1 and -2) were expressed in *cos*-1 cells as described previously (Laneuville et al., 1994; Meade et al., 1993). Cyclooxygenase activity (COX) was measured using microsomal membranes (ca. 5 mg protein/mL in 0.1 M TrisHCL, pH 7.4) from sham-transfected *cos*-1 cells or from *cos*-1 cells transfected with either the plasmid pOSML-PGHS-1 or pOSML-PGHS-2. Cyclooxygenase assays were performed at 37 °C by monitoring the initial rate of O_2 uptake using an O_2 electrode (Yellow Spring Instruments). Each assay mixture contained 3.0 mL of 0.1 M TrisHCl, pH 8.0, 1 mmol phenol, 85 μg of hemoglobin, and 100 μmol arachidonic acid. Reactions were initiated by adding 5−25 μg of microsomal protein in a volume of 15−50 μL.

Instantaneous inhibition was determined by measuring the cyclooxygenase activity initiated by adding aliquots of microsomal suspensions of hPGHS-1 or hPGHS-2 (10 μmol O_2/min cyclooxygenase activity/aliquot) to assay mixtures containing 10 μmol of arachidonate and various concentrations of the test substances (100−600 μmol). The IC_{50} values which represent the concentrations of the test compound that gave half-maximal activity under the standard assay conditions (Laneuville et al., 1994; Meade et al., 1993) were determined.

Antioxidant Assay. Antioxidant bioassays were conducted on the purified compounds by analysis of model liposome oxidation using fluorescence spectroscopy. A mixture containing 5 μmol of 1-stearoyl-2-linoleoyl-*sn*-glycerol-3-phosphocholine (Avanti Polar Lipids, Inc., Alabaster, AL) and 5 μmol of the fluorescence probe 3-[*p*-(6-phenyl)-1,3,5-hexatrienyl]phenylpropionic acid (Molecular Probe, Inc., Eugene, OR) is dried in a rotary evaporator. The resulting lipid film was suspended in 500 μL of a solution containing 0.15 M NaCl, 0.1 mM EDTA, and 0.01 M MOPS and subjected to 10 freeze−thaw cycles using a dry ice/ethanol bath. This buffer solution was treated

with chelating resin Chelex 100 to remove trace metal ions. The lipid−buffer suspension was then extruded 29 times through a LiposoFast extruder containing a polycarbonate membrane with a pore size of 100 nm to produce large unilamellar vesicles (LUVs). A 20 μL aliquot of this liposome suspension was diluted to 2 mL in Chelex 100-treated buffer at pH 7.0, incubated for 5 min at room temperature, and followed by incubation (5 min) in a thermostated cuvette holder (23 °C) of the spectrophotometer. Peroxidation was then initiated by the addition of 20 μL of 2 mM stock $FeCl_2$ solution to achieve a final concentration of 19.6 μM of Fe^{2+} in the absence or presence of test compounds. The control sample did not contain either Fe^{2+} or the test compounds. Fluorescence intensities of these liposome solutions were measured using a spectrofluorometer (SLM Instruments, Model 4800, Urbana, IL) with data acquisition hardware and software from On-Line Instrument Systems (Bogart, GA). The samples were excited at a wavelength of 384 nm, and the emitted light was passed through optical filters (KV 418, Schott, Duryea, PA) to give an emission wavelength of 430 nm prior to detection. The intensities were measured at every 3 min interval over a period of 21 min. The decrease of relative fluorescence intensity with time indicated the rate of peroxidation (Arora and Strasburg, 1997). Commercial antioxidants *tert*-butylhydroquinone (TBHQ; Eastman Chemical Products Inc., Kingsport, TN), butylated hydroxytoluene (BHT), and butylated hydroxyanisole (BHA) were purchased from National Biochemicals Corp., Cleveland, OH, were also used as positive controls in this experiment.

RESULTS AND DISCUSSION

Fresh leaves of *M. koenigii* were extracted with acetone, and this extract was subjected to vacuum liquid chromatography (VLC), yielding two bioactive fractions (C and E). Medium-pressure liquid chromatography (MPLC) was performed on both of these fractions separately. Fraction C eluting with hexane−acetone (10:1) from the VLC gave one major fraction when purified by MPLC. It was further purified by preparative TLC to yield mahanimbine (**1**). Fraction E eluting with hexane−acetone (4:1) from the VLC was purified by MPLC and preparative TLC to yield murrayanol (**2**) and mahanine (**3**), respectively. Compounds **1**−**3** were present in the plant as 5.6, 8.7, and 8.4 mg/(100 g of fresh weight of leaves), respectively.

Mosquitocidal assays using *Aedes aegyptii* on compounds **1**−**3** indicated that they were very active. Compounds **2** and **3** had an LD_{100} (24 h) at 12.5 μg/mL concentrations, while compound **1** showed 100% mortality at 100 μg/mL. However, compound **1** showed 20% mortality at 6.25 μg/mL, while compounds **2** and **3** showed 49 and 69% mortality respectively, at 1 μg/mL.

Compounds **1**−**3** demonstrated topoisomerase I and II inhibitory activity when tested on *S. cerevisiae* mutant strains. JN 271-R52 and JN 394 are hypersensitive to topoisomerase I poisons, while JN 271-R52 t_{-1} and JN 394 t_{-1} lack the top I gene and therefore show a lack of response to topoisomerase I poisons. Both JN 271-R52 t_{-2-5} and JN 394 t_{-2-5} carry the top II gene, which is resistant to topoisomerase II poisons but responds to topoisomerase I poisons. MIC_{100} determinations of compounds **1**−**3** indicated that all three compounds completely inhibited topoisomerase I and II activity at 50 μg/mL concentrations.

The antimicrobial assay showed that compound **3** was the most active when tested on *C. kruseii* (MSU strain), *C. parapsilasis* (MSU strain), *E. coli* (ATCC 25922), *S. aureus* (MSU strain), and *S. pyogenes* (MHM-1645). Compound **1** was the least active against these test organisms. Compound **3** showed an MIC_{100} of 100 μg/mL against *C. kruseii*, *C. parapsilasis*, and *E. coli* but gave an MIC_{100} against *S. aureus* and *S. pyogenes* at 25 μg/mL. Compound **2** showed an MIC_{100} of 100 μg/mL against *C. kruseii* and *E. coli* but gave an MIC_{100} against *S. aureus* and *S. pyogenes* at 25 μg/mL. The MIC_{100} for compound **1** was 50 μg/mL against *S. aureus* and *S. pyogenes* and did not show activity on other organisms tested. Although these compounds showed weak activity toward *Candida* spp., the topoisomerase activity could be regarded as significant considering the lower MIC_{100} values. This trend in bioactivity could be attributed to the fact that compound **1** lacks a free hydroxyl group and that when this moiety is at C7 the activity is greatest as in compound **3**. Compound **2**, the second most active compound, lacked a C7 hydroxyl moiety. This suggests that an oxygenated functionality at C7 is probably important for the biological activities observed for these compounds. However, the pyran ring system or the position of the aromatic methyl group does not seem to be a major factor in exhibiting biological activity for compounds **1**−**3**.

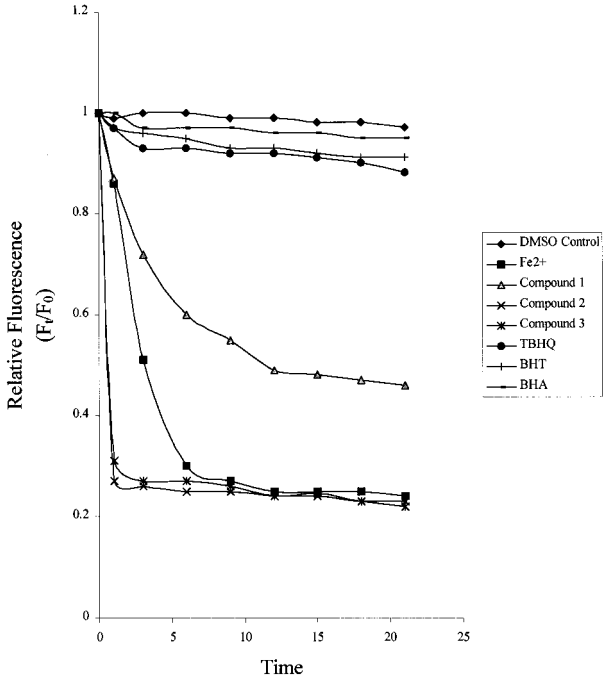

Figure 1. Antioxidant activities of compounds **1**−**3** as determined by the liposome oxidation assay using fluorescence spectroscopy.

In the antiinflammatory assays (Laneuville et al., 1994; Meade et al., 1993), which measured inhibition of the cyclooxygenase activity of the prostaglandin endoperoxide H synthase-1 and -2 isozymes (hPGHS-1 and -2), compounds **1** and **3** were not active. However, compound **2** showed an IC_{50} of 109 μg/mL against hPGHS-1 and an IC_{50} of 218 μg/mL against hPGHS-2. The positive controls used in this experiment were ibuprofen and flurbiprofen. Ibuprofen had an IC_{50} of 0.99 μg/mL against hPGHS-1 and 2.58 μg/mL against hPGHS-2. Flurbiprofen had an IC_{50} of 0.12 and 0.78 μg/mL against hPGHS-1 and hPGHS-2 enzymes, respectively.

In the antioxidant assay (Arora and Strasburg, 1997) (Figure 1) using LUVs, compounds **2** and **3** showed very weak antioxidant activity. This may be partially due to the precipitation of these compounds in the buffer solution used in the assay. However, compound **1** displayed 49% inhibition of lipid peroxidation after 21

min at 33.1 μg/mL compared to the positive controls *tert*-butylhydroquinone (TBHQ), butylated hydroxytoluene (BHT), and butylated hydroxyanisole (BHA). TBHQ showed 90%, BHT showed 94% and BHA showed 97% inhibition of lipid peroxidation after 21 min at 3.3, 4.4, and 18 μg/mL concentrations, respectively.

The leaves of *M. koenigii* are widely used as a condiment in foods, and various parts of the plant have been reported to have medicinal properties such as in the treatment of headaches, stomachaches, and rheumatism and as an astringent and an antidysenteric (Kong et al., 1986). Also, natural products possessing antioxidant activities are currently being pursued in order to try and replace synthetic antioxidant additives such as BHA and BHT. Antioxidants are added to food to prevent deterioration of food quality, which is often caused by oxidative rancidity, and hence prolong shelf life. Thus, due to the biological activities of compounds **1**−**3**, in vitro, one can deduce that the incorporation of the curry leaf (*M. koenigii*) into food or food products may impart significant health benefits to consumers as well as prolong the shelf life of the product.

ACKNOWLEDGMENT

This is a contribution from Michigan State University Agricultural Experiment Station. *A. aegyptii* eggs were supplied by Drs. A. Raikhel and A. Hays, Department of Botany and Plant Pathology, Michigan State University. Also, we thank the REF Center for Crop and Food Bioprocessing at Michigan State University.

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Received for review June 1, 1998. Revised manuscript received November 19, 1998. Accepted November 23, 1998. The NMR were obtained on instrumentation that was purchased in part with the funds from National Institutes of Health Grant 1-S10-RR04750, National Science Foundation (NSF) Grant CHE-88-00770, and NSF Grant CHE-92-13241.

JF9805808