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Isolation, Identification, and Insecticidal Properties of *Piper nigrum* Amides

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Three amides were isolated from *Piper nigrum* L. and identified from their spectral characteristics as (*E,E*)-*N*-(2-methylpropyl)-2,4-decadienamide (I), (*E,E,E*)-13-(1,3-benzodioxol-5-yl)-*N*-(2-methylpropyl)-2,4,12-tridecatrienamide (II), and (*E,E,E*)-11-(1,3-benzodioxol-5-yl)-*N*-(2-methylpropyl)-2,4,10-undecatrienamide (III). The topical LD_{50} values of compounds I, II, and III against *Callosobruchus maculatus* (F.) were 2.18, 0.25, and 0.84 μg/insect for males (weight 3.8–5.7 mg) and 6.70, 1.43, and 3.88 μg/insect for females (weight 5.4–7.9 mg), respectively.

Black pepper, *Piper nigrum* L., has been reported to have contact and oral toxicity against stored-product insects (Lathrop and Keirstead, 1946; Su, 1977, 1978). It has been reported to have biological activity on other insects either as a toxicant (Harvill et al., 1943; Synerholm et al., 1945; Scott and McKibben, 1978) or as a repellent (Freeborn and Wymore, 1929). Piperine [(*E,E*)-1-[5-(1,3-benzodioxol-5-yl)-1-oxo-2,4-pentadienyl]piperidine], an alkaloidal amide of oleoresin of pepper, has been shown to be a synergist to pyrethrins (Nakayama, 1950; Ono, 1950; Gersdorff and Piquett, 1957; Matsubara and Tanimura, 1966). However, Su (1977) showed that piperine was not the constituent in black pepper that was responsible for contact toxicity to the insects. In an effort to elucidate the responsible toxic components of black pepper, we isolated and identified three amides that are highly toxic to adult cowpea weevils, *Callosobruchus maculatus* (F.).

MATERIALS AND METHODS

Extraction of Black Pepper. Dry fruits of black pepper (distributed by McCormick & Co., Inc.) were purchased from the local supermarket and ground in a high-speed micromill into fine powder of less than 250 μm. The powder (140 g) in acetone (500 mL) was stirred at 40–50 °C for 30 min and filtered. The residue was extracted 3 more times. The filtrates were combined and concentrated under reduced pressure to a small volume and then lyophilized to give the crude acetone extract.

Chemicals and Reagents. Piperine was purchased from Pfaltz and Bauer, Inc. Pyrethrin (21.5% solution) was obtained from the Pyrethrum Marketing Board, Nakuru, Kenya. High-performance LC methanol (Fisher Scientific Co.) was filtered through a Waters Associates solvent clarification kit with a 0.5-μm Millipore organic filtration system. All other solvents were the reagent grade.

Liquid Chromatographic Fractionation of Acetone Extract. Each 1-g portion of the crude extract was placed on a column (40 × 2.0 cm i.d.) of silica gel (70–230 mesh; EM Reagents) and eluted with carbon tetrachloride–ethyl acetate (10:1 by volume). After the first 800 mL of effluent was discarded, the next 600 mL was collected. This effluent was then concentrated under reduced pressure to obtain the toxic material for further separation of individual components.

Thin-Layer Chromatographic Separation of the Toxic Material. For TLC separation, Brinkman EM reagent, precoated silica gel G F_{254}, 0.25 mm, 20 × 20 cm chromatoplates were used. About 3–4 mg of the material was applied to each plate in a straight line 2.5 cm above the lower edge. A total of 95 plates was prepared. Each plate was developed twice in cyclohexane–ethyl acetate (3:2 by volume) and then examined under UV at 254 nm. Three fractions in bands of R_f 0.55–0.60 (I), 0.52–0.55 (II), and 0.46–0.51 (III) were collected. Each fraction was extracted with acetone, and the extracts were concentrated and lyophilized.

High-Performance Liquid Chromatograph Purification and Analysis. A Waters Associates Model ALC/GPC 244 high-pressure liquid chromatograph with a Model 6000A pump, an U6K injector, a R401 differential

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refractometer, and a Model 440 UV detector with a 300 × 7.8 mm i.d. μBondapak C_{18} column (octadecyltrichlorosilane covalently bonded to 10-μm μPorasil packing) was used. Methanol–water (9:1 by volume, degassed) was used as the eluting system. The column effluent was monitored at 254 nm (2.0 AUFS), and the response was recorded on a Houston Omniscribe Model B-5217-1 recorder.

Each of the TLC fractions of I, II, and III was dissolved separately in methanol to obtain the concentration of 4–5 μg/μL; then each was filtered through a Waters Associates sample clarification kit with 0.5-μm Millipore organic filter system. For each fractionation, a 25-μL aliquot of the stock solution was injected onto the column with the flow rate of the eluting solvent at 2 mL/min. The effluents were collected at the retention times of 7.10 (I), 10.90 (II), and 8.65 (III) min, respectively. The collected effluents were concentrated under reduced pressure and lyophilized. The solidified materials were then recrystallized from methanol–water.

For analysis, a 300 × 3.9 mm i.d. μBondapak C_{18} column was used. An eluting system of methanol–water (9:1 at a flow rate of 0.8 mL/min or 8:2 at a flow rate of 1 mL/min) was used. The results were monitored at 254 nm.

Additional Instrumental Analyses. A Du Pont Model 21-490B mass spectrometer was used with a direct insertion probe at a temperature of 150–250 °C. Other conditions used were as follows: ion source temperature, 150 °C; ionizing voltage, 70 eV; ion source pressure, 4 × 10^{-6} torr; scan rate, 100 s/decade from 15–500 amu.

NMR spectra were obtained from a JEOL Model FX 60Q NMR spectrometer at ambient temperature. The samples were prepared in $CDCl_3$ with tetramethylsilane as the zero reference. Infrared spectra were determined in KBr pellets with a Beckman 4230 spectrophotometer. The UV spectra were obtained with a Beckman Acta III spectrophotometer. The samples were prepared in 95% ethanol solution by using cells with 1-cm paths, with wavelengths in nanometers.

The melting points were determined on a Mettler FP1 thermal analyzer. Three determinations were obtained for each compound to give the reported values.

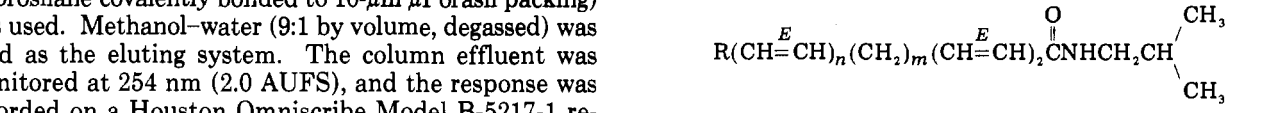
Biological Evaluation Techniques. Stock solutions of fractionated and purified materials of black pepper (16 mg/mL), piperine (100 mg/mL), and pyrethrins (5 mg/mL) were obtained by dissolving the required amount of material in acetone. Lower concentrations were obtained by further dilution of the stock solutions with acetone.

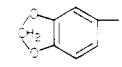
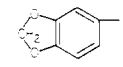
Topical treatments were applied to adult cowpea weevils (<5 h old from colonies maintained at the Stored-Product Insects Research and Development Laboratory, Savannah, GA) by anesthetizing the insects briefly with CO_2, picking them up individually with a vacuum tweezer, and applying 0.5 μL (except 1 μL for the dose in piperine) of the solution to the dorsal thorax with an ISCO microapplicator. Control insects were anesthetized and treated with 0.5 μL of acetone. Forty insects (20 males and 20 females) were treated with each dose. After treatment, the insects were held in 100 mm diameter Petri dishes (5 males and 5 females per dish) and kept in a room maintained at 27 ± 1 °C and 60 ± 5% RH. The insects were examined for mortality every 24 h for 7 days. Insects that did not move when touched gently with a small pointed object were considered dead. The results were evaluated by probit analysis of doses vs. mortalities.

RESULTS AND DISCUSSION

Acetone extraction of the pulverized black pepper yielded 11.3% of a dark syrupy crude material. When this crude extract was fractionated by liquid column chromatography, it yielded 5.2–5.7% of a light brown soft solid. Thin-layer chromatographic separation of this soft solid gave three compounds (I, II, and III) in 13.5, 12.8, and 14.7% yields, respectively.

Table I. Chemical Structures of Compounds I, II, and III Isolated from Acetone Extract of *P. nigrum*



compd	M^+	R	n	m	empirical formula
I	223	$-CH_3$	0	4	$C_{14}H_{25}NO$
II	383		1	6	$C_{24}H_{33}NO_3$
III	355		1	4	$C_{22}H_{29}NO_3$

Compound I, from high-performance LC purification (75% recovery), was obtained as an ivory, waxy solid with a mp of 86–87 °C. Its UV absorption showed 259 nm (ε 34 000). Strong infrared absorption was observed in cm^{-1} at 3305 and 3290 (NH), 2920 (CH_2), 1660 and 1610 (C=C), 1615 (amide C=O), 1540 (unsaturated amide), and 998 (*trans*-CH=CH). The mass spectrum showed the following peaks (relative peak intensity in parentheses): 223 (M^+, 42), 208 (20), 180 (9), 152 (56), 151 (base peak 100), 96 (58), 95 (26), 81 (85), 69 (42), 67 (40), 57 (68), 55 (54), 43 (66), and 41 (77). The ^1H NMR spectrum showed δ ($CDCl_3$) 0.89 (s, 3), 0.99 (s, 6), 1.20 (b, 6), 2.08 (b, 3), 3.20 (bd, 2, J = 6 Hz), 3.75 (b, 1), 5.63–6.16 (m, 3), and 7.26 (s, 1).

The UV and MS spectral data of I are consistent with those reported for (*E,E*)-*N*-(2-methylpropyl)-2,4-decadienamide isolated from wood of *Piper novae-hollandiae* Miq. by Loder et al. (1969). The NMR spectral data of I are in agreement with those of the amide that was isolated from the seeds of *Piper sylvaticum* Roxb. by Banerji et al. (1974) and from the roots of *Anacyclus pyrethrum* DC. by Burden and Crombie (1969). We observed δ at 7.26 for the amide H. It was reported at 6.57–6.98 by Banerji et al. and at 7.80 by Burden and Crombie.

Amide I has also been isolated from the fruits of *Piper longum* L. and *Piper peepuloides* Roxb. (Dhar and Atal, 1967), from the stems of *Piper nepalense* Miq. (Gupta et al., 1972), from the roots and stems of *P. sylvaticum* and *Piper boehmerifolium* Wall (Mahanta et al., 1974), and from some Compositae and Rutaceae (Jacobson, 1953; Crombie, 1955; Bowden and Ross, 1963). This amide showed the biological activities of a paralyzing effect to house flies, *Musca domestica* L. (Jacobson, 1953; Crombie, 1955), a local anesthetic effect when applied to the tongue (Bowden and Ross, 1963), and tumor inhibitory activity against Lewis lung carcinoma in mice (Loder et al., 1969). In addition, many of these *Piper* plants have also been used for medicinal purposes by the natives in Africa.

Compound II, from high-performance LC purification (80% recovery), was obtained as colorless needles with a mp of 114–116 °C. Its UV absorption showed 260 nm (ε 66 400) and 302 nm (ε 7300). Strong infrared absorption was observed in cm^{-1} at 3310 and 3290 (NH), 2920 (CH_2), 1655 and 1620 (C=C), 1630 (amide C=O), 1545 (unsaturated amide), 998 (*trans*-CH=CH), and also the presence of 920 (—OCH_2O—) was observed. The mass spectrum showed the following peaks (relative peak intensity in parentheses): 383 (M^+, 85), 248 (54), 161 (63), 135 (base peak 100), 131 (92), 115 (54), 103 (68), 57 (62), 55 (78), 43

Table II. Probit Analysis Data for Contact Toxicity 48 h after Topical Application of Compounds I, II, and III to Cowpea Weevils

material	LD_{50}, μg/insect		LD_{95}, μg/insect		slope[a]	
	male	female	male	female	male	female
I	2.18	6.70	28.86	50.12	1.47	1.88
II	0.25	1.43	1.99	13.07	1.85	1.71
III	0.84	3.88	2.54	16.19	3.41	2.65
piperine	>100	>100	>100	>100		
pyrethrins	0.08	0.16	0.28	0.59	3.12	2.98

[a] Statistically significant from zero at 95% confidence level.

(62), and 41 (68). The ^1H NMR analysis showed δ ($CDCl_3$) 0.94 (d, 6, J = 6 Hz), 1.38 (b, 8), 2.14–2.22 (b, 4), 2.61 (b, 1), 3.18 (bd, 2, J = 6.5 Hz), 5.93 (s, 2), 6.04–6.22 (m, 4), 6.75–6.89 (d, 3), and 7.26 (s, 3).

II was identified as (*E,E,E,*)-13-(1,3-benzodioxol-5-yl)-*N*-(2-methylpropyl)-2,4,12-tridecatrienamide, which was isolated from *Piper guineense* Schumach & Thonn. by Okogun and Ekong (1974) and named "guineensine" and from *Piper retrofractum* (=*officinarum*) Vahl by Gupta et al. (1976). Su (1979) found that the hexane extract of *P. guineense* (extract furnished by B. L. Sondengam, University of Yaoundé, Yaoundé, Cameroon) was very toxic to several species of stored-product insects. Compound II in *P. guineense* is probably the component responsible for its insect toxicity.

Compound III, from high-performance LC purification (80% recovery), was obtained as colorless plates with a mp of 120–122 °C. its UV absorption showed 260 nm (ε 64000) and 302 nm (ε 5200). The infrared spectrum has strong absorption at 3300 and 3285 (NH), 2920 (CH_2), 1650 and 1610 (C=C), 1620 (amide C=O), 1540 (unsaturated amide), and 990 (*trans*-CH=CH) and also weak absorption at 925 (—OCH_2O—). The mass spectrum showed the following peaks (relative peak intensity in parentheses): 355 (M^+, 53), 220 (61), 161 (32), 135 (base peak 100), 131 (53), 115 (30), 103 (55), 57 (72), 55 (62), 43 (26), and 41 (51). The ^1H NMR analysis showed δ ($CDCl_3$) 0.95 (d, 6, J = 6 Hz), 1.25 (s, 2), 1.47 (b, 2), 2.16–2.25 (b, 4), 3.25 (bd, 2, J = 6.5 Hz), 5.93 (s, 2), 6.00–6.21 (m, 5), 6.75 (s, 2), 6.88 (s, 1), and 7.26 (s, 3).

III was identified as a homologue of II with two less carbons. Okogun and Ekong (1974) found an impure sample of guineensine with m/e of 355 (M^+) and 220 and suggested that this impurity was the lower homologue of II. Recently, Miyakado et al. (1979) reported the isolation of III from black pepper with a mp of 114–115 °C and named it "pipercide". The materal we isolated from the TLC fractionation process in R_f 0.46–0.51 also had a mp of 113–115 °C. However, on high-performance LC analysis, this material showed contamination by II and several other unidentified impurities, so pipercide may, in fact, be a mixture.

Compounds I, II, and III isolated from black pepper were identified as the amides of (*E,E*)-*N*-(2-methylpropyl)-2,4-decadienamide, (*E,E,E*)-13-(1,3-benzodioxol-5-yl)-*N*-(2-methylpropyl)-2,4,12-tridecatrienamide, and (*E,E,E*)-11-(1,3-benzodioxol-5-yl)-*N*-(2-methylpropyl)-2,4,10-undecatrienamide, respectively; their structures are indicated in Table I.

Toxicological effects on cowpea weevils 48 h after treatment with compounds I, II, and III are shown in Table II. The amide piperine and pyrethrins are included for comparison. Compound II was the most toxic to the insects of the three amides. All three compounds had a paralyzing effect on the legs of the insects, which were thus immobilized. The differences in LD_{50} and LD_{95} values between males and females were probably reflected the differences in body weights since doses were calculated on the basis of weight of material per insect and not on the basis of weight of material per unit of body weight. The average body weight of cowpea weevils in this study was 4.7 mg (range 3.8–5.7 mg) for males and 6.5 mg (range 5.4–7.9 mg) for females.

The relatively high contact toxicity of compounds I, II, and III against cowpea weevils (Table II), the previous report of high contact and/or oral toxicity of black pepper crude extract and its TLC-purified extract to other species of stored-product insects (Su, 1977, 1978), and the contact toxicity to boll weevils, *Anthonomus grandis* Boheman reported by Scott and McKibben (1978) indicate the insecticidal properties of black pepper, its extract, and its amides I, II, and III. The wide occurrence of these three compounds in various parts of many *Piper* plants, which are widely used for condiment and medicinal purposes, makes it very desirable for further development in using them as safe and effective insect control agents.

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A Rapid Colorimetric Method for Analysis of Carbaryl Spray Deposits on Fruit Tree Foliage

Mikio Chiba

A rapid colorimetric method was developed to determine deposits of carbaryl insecticide on fruit tree foliage. Analyses take less than 3 min/sample when 50 or more samples are processed at a time. A 5-cm^2 disk punched from a leaf is used for the determination. Carbaryl is extracted and hydrolyzed by methanolic NaOH (0.03% w/v) and then coupled with *p*-nitrobenzenediazonium tetrafluoroborate which produces a spectrum of colors ranging from red to blue. Within a concentration range of 0.5–10 μg/cm^2 of leaf surface or 0.25–5 μg/mL of alkaline solution in a test tube, the absorbance of color obeyed Beer's law when measured at 580 nm. Little, if any, interference was observed from other commonly used pesticides, such as dicofol, tetradifon, azinphosmethyl, phosmet, captan, and folpet. If a spectrophotometer is not available or when a rapid field test is required, a semiquantitative determination is also possible.

Methodology for pesticide residue analysis has tended to emphasize greater sensitivity and specificity by using sophisticated techniques and instruments. Automated analysis is one technique well suited for analyzing large numbers of samples, especially on a routine basis (Gunther and Ott, 1966). In many laboratories, however, automated systems are not available because they are costly and much experience is needed to set up complete analytical systems.

To date, methods for determining spray deposits are generally those used for the measuring of pesticide residues in foodstuffs. Without exception, these analyses are based on "adequate sampling in a large quantity", sufficient to give reliable average values. This approach is essential in ordinary residue analyses. However, for assessment of the performance of sprayers, relative to uniformity of distribution and amount of chemical applied to the target, analyses of individual leaves are essential. The importance of individual analyses was explained and a special extraction apparatus was developed by Pielou et al. (1962). Chiba (1973) and Chiba et al. (1973) demonstrated that without individual analyses, differences in amount and distribution of deposit between leaves within a limited area and in different locations of a tree cannot be identified. Individual values, rather than a single average value, can provide answers to questions that were previously impossible to answer. Ordinary residue methods are not usually suitable for handling large numbers of samples in a limited period of time. The method described in this paper was developed to permit rapid determination of carbaryl (1-naphthyl methylcarbamate) spray deposits on leaves of fruit trees and grapevines. The original request came from field entomologists who needed an unsophisticated method that could be used by a nonchemist. Such a method could be used to judge whether another spray application is necessary after a heavy rainfall or to assess insect damage relative to the distribution of deposits on target trees or vines. Although this concept is different from the usual approach to pesticide residue analysis, the color reaction employed is basically the same as that in the TLC method described by Chiba and Morley (1964).

With this method residues on individual leaf disks may be measured by a relatively inexperienced person in less than 3 min/sample when 50 or more samples are processed at a time. In contrast, the official Association of Official Agricultural Chemists (1965) method in the hands of an experienced operator requires more than 1 h/sample when six (or eight) samples are processed together.

This new method requires only a leaf punch and a colorimeter or a spectrophotometer in a laboratory for accurate measurement. A simple semiquantitative determination can be made anywhere, however, by matching the color with a series of color standards. This is possible because the reaction product yields a spectrum ranging from red to blue, depending on concentration. The effective range of concentration is 0.25–5 μg/mL in alkaline solution.

MATERIALS AND METHODS

Apparatus Employed. *Spectrophotometers*: Spectronic 20 (Bausch & Lomb, Inc., Rochester, NY 14625) and DK-2A (Beckman Instruments, Inc., Fullerton, CA 92634). *Pour-out dispensers*: 20-mL capacity for 0.03% NaOH in methanol solution; 1-mL capacity for the chromogenic reagent (Arthur H. Thomas Co., Philadelphia, PA 19105). *Test tubes*: disposable culture tube, 18 × 150 mm (Kimble Products, Toledo, OH 43601). *Test tube caps*: 2030 cap, 17 mm (Falcon Plastics, Oxnard, CA 93030). *Leaf punch*: 2.523-cm diameter (5.00 cm^2 disk). Manufactured locally; an improved version of the unit reported previously (Gordon and Little, 1954).

Reagents. *Carbaryl*: 99% plus, analytical standard (Union Carbide Corp., Salinas CA 93901); Sevin 50% WP (wettable powder) (Niagara Chemicals, Burlington, Ontario L7S 1W6). *p-Nitrobenzenediazonium tetrafluoroborate*

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