DOI:10.1021/jf102087c

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

Phytotoxicity of Sarmentine Isolated from Long Pepper (*Piper longum*) Fruit

Huazhang Huang,* Christy M. Morgan, Ratnakar N. Asolkar, Marja E. Koivunen, and Pamela G. Marrone

Marrone Bio Innovations, 2121 Second Street, Suite B-107, Davis, California 95618

Discovery of novel natural herbicides has become crucial to overcome increasing weed resistance and environmental issues. In this article, we describe the finding that a methanol extract of dry long pepper (*Piper longum* L.) fruits is phytotoxic to lettuce (*Lactuca sativa* L.) seedlings. The bioassay-guided fractionation and purification of the crude extract led to isolation of sarmentine (**1**), a known compound, as the active principle. Phytotoxicity of **1** was examined with a variety of seedlings of field crops and weeds. Results indicated that **1** was a contact herbicide and possessed broad-spectrum herbicidal activity. Moreover, a series of sarmentine analogues were then synthesized to study the structure−activity relationship (SAR). SAR studies suggested that phytotoxicity of sarmentine and its analogues was specific due to chemical structures, i.e., the analogues of the acid moiety of **1** were active, but the amine and its analogues were inactive; the ester analogues and amide analogues with a primary amine of **1** were also inactive. In addition, quantification of **1** from different resources of the dry *P. longum* fruits using liquid chromatography−mass spectrometry showed a wide variation, ranging from almost zero to 0.57%. This study suggests that **1** has potential as an active lead molecule for synthesized herbicides as well as for bioherbicides derived from natural resources.

KEYWORDS: ***Piper longum*; sarmentine; phytotoxicity**

INTRODUCTION

Utilization of synthetic herbicides not only prevents economic loss in food production but also improves quality of crop products (*1*). However, the use of synthetic herbicides may cause adverse effects on the environment and human health (*2*,*3*), and it has led to increasing resistance among many weed species (*4*). Therefore, it is necessary to develop alternative means for weed management that are ecofriendly, economical and efficacious (*5*).

Application of natural phytochemicals as weed management provides an alternative to chemical herbicides (*6*). In nature, many natural compounds have allelopathic properties. When released in air or soil, they kill neighboring weeds or inhibit their germination and/or growth. Moreover, many of these compounds are easily biodegradable due to environmental factors such as light, oxygen, temperature and/or biological metabolic enzymes. These phytotoxic chemicals include phenolic compounds (e.g., catechin, ellagic acid, sorgoleone, juglone, ceratiolin, usnic acid), terpenoids (e.g., 1,8-cineole, geranial, neral, cinmethylin, solstitiolide), quassinoids (e.g., ailanthone, chaparrine, ailanthinol B), benzoxazinoids (e.g., hydroxamic acids), glucoinolates (e.g., glucohirsutin, hirsutin, arabin), and some amino acids such as meta-tyrosine (*7*−*9*). Some of the phytotoxic compounds such as clove oil (Matratec), *d*-limonene (GreenMatch and Nature's Avenger) and lemongrass oil (GreenMatchEX) have been commercialized as bioherbicides in the US for weed management. However, due to the high cost and high use rates of the raw materials, exploration of other phytochemicals as bioherbicides or herbicide leads is necessary.

Long pepper, *Piper longum* L. (Piperaceae), is a slender aromatic climber with perennial woody roots. It grows primarily in tropical regions. The fruits, commonly known as "pippali" in India and "Bi Bo" in China, are used as a spice and also as a preservative in pickles. Whole pepper plants are also used as cattle feed. In traditional medicinal practice, *P. longum* fruits have been advocated to be beneficial in the treatment of diseases and ailments such as gonorrhea, menstrual pain, tuberculosis, sleeping problems, respiratory tract infections, chronic intestinal pain, and certain forms of arthritis (*10*−*13*). Other reported beneficial effects of *P. longum* include analgesic and diuretic effects, relaxation of muscle tension, and alleviation of anxiety (*14*, *15*). In addition, pipernonaline from *P. longum* has been found to possess mosquito larvicidal activity (*16*). To our knowledge, phytotoxic compounds from *Piper* species have never been reported before.

Herewith we are describing the isolation and structure elucidation of sarmentine (**1**), a phytotoxic compound from *P. longum*, and the phytotoxic spectrum of this compound against field crops and weeds. The structure−activity relationship of sarmentine (**1**) was preliminarily studied by synthesizing a series of its analogues. Finally, the percent content of sarmentine (**1**) in different dry *P. longum* fruit samples obtained from different sources was then investigated.

MATERIALS AND METHODS

Chemical. Sepra C18-E (50 μM and 60 Å) and silica gel sorbent (70−230 mesh size) were purchased from Phenomenex (Torrance, CA)

*Corresponding author. Fax: 530-750-2808. E-mail: hhuang@marronebio.com.

 Published on Web 08/17/2010

and Fisher Scientific (Fair Lawn, NJ), respectively. Polyoxyethylene (20) sorbitan monolaurate (i.e., Glycosperse O-20 KFG) and sodium lauryl sulfate (i.e., SLS) were obtained from Lonza Inc. (Allendale, NJ) and Spectrum Chemical Mfg. Corp. (New Brunswick, NJ), respectively. Cyclopentylamine, *trans*-cinnamic acid, ethyl *trans*-2-*cis*-4-decadienoate, hexamethyleneimine, *trans*-2-decenoic acid, decanoic acid, and 4-dimethylaminopyridine were purchased from ACROS Organics (Morris Plains, NJ). 5-Biphenyl-4-ylmethyl-tetrazole-1-carboxylic dimethylamide (LY2183240) was purchased from Sigma-Aldrich. All other chemicals were reagent grade.

Fruits of *P. longum* and Pretreatment. Four samples of dry *P. longum* fruits were purchased from Chinese medicinal herb stores. Two of them were purchased from WAN FUNG Chinese Herb Shop in Richmond, CA, on May 25, 2008, and March 23, 2009, respectively. The other two samples were purchased from WAH TSUN Chinese Herb Co. in Sacramento, CA, on June 2, 2008, and March 4, 2009, respectively. The fruits were completely ground with a coffee grinder (Toastmaster Inc., Boonville, MI). The freshly ground powder of the fruits was extracted with appropriate solvents as described below.

Weed and Crop Seedlings. All seedlings of weeds and crop plants were planted in 8 × 8 × 7.2 cm or 10 × 10 × 9 cm plastic pots. All pots were stored in a greenhouse with 28 °C temperature and 60% humidity. Seedlings including pigweed (*Amaranthus retroflexus* L.), barnyard grass (*Echinochloa crus-galli* L.), bindweed (*Convolvulus arvensis* L.), crabgrass (*Digitaria sanguinalis* L.), dandelion (*Taraxacum officinale* F.), lambsquarter (*Chenopodium album* L.), annual bluegrass (*Poa annua* L.), wild mustard (*Brassica kaber* L.), black nightshade (*Solanum nigrum* L.), curly dock (*Rumex crispus* L.), horseweed (*Conyza canadensis* L.), sweet corn (*Zea mays* L.) and wheat PR 1404 (*Triticum aestivum* L.) were planted in potting soil mixture. Seedlings including rice M-104 (*Oryza sativa* L.), sedge (*Cyperus difformis* L.) and sprangletop (*Leptochloa fascicularis* Lam) were planted in mud which was collected adjacent to a rice field (Woodland, CA). When treated, all seedlings were 15 days old except for rice (10 days), wheat (20 days), corn (20 days), sprangletop (20 days), sedge (20 days) and horseweed (70 days).

Bioassay-Guided Fractionation and Isolation. The active compound was isolated by four major steps described as follows: (1) The methanol extract of freshly ground *P. longum* fruit powder was screened in a 96-well plate bioassay with Bibb lettuce (*Lactuca sativa* L.) seedlings, and positive hits were obtained (i.e., death at 144 h after treatment). (2) The methanol extract (0.5 g) was then subjected to separation through a reverse phase C18 column and was eluted with 20%, 40%, 60%, 80% and 100% methanol in water. Fractions were dried under vacuum and efficacy was re-evaluated by 96-well plate bioassay with Bibb lettuce seedlings. The active fraction, the most hydrophobic fraction, was used to guide the next step for separation. (3) Ethyl acetate extract (17.6 g) was loaded into a flash column. The column was sequentially eluted with hexane (1 L), hexane/ethyl acetate (3:1, 1 L), hexane/ethyl acetate (1:1, 1 L), ethyl acetate (1 L) and acetone (1 L). Based on indication of thin layer chromatography (TLC), nine fractions were collected. The efficacy of each fraction was evaluated by foliar spraying of barnyard grass. The concentration of each fraction was 5 mg/mL with a carrier solution consisting of 4% ethanol and 0.2% glycosperse O-20 KFG. The active fractions (3.4 g) were combined together and subjected to the next step. (4) A secondary silica column separation was performed with a combination of hexane and ethyl acetate (3:1) as an elution solvent to obtain the active ingredient (0.96 g). This active ingredient (0.96 g) was recrystallized at −20 °C in a mixture of hexane and ethyl acetate which yielded a crystal compound (0.83 g), colorless oil at room temperature. Purity was examined by liquid chromatography and mass spectrometry (LC/MS). Detailed conditions for LC/MS are described below in the later section of Materials and Methods.

Structural Analysis. Structural identification of the active compound was based on data from both nuclear magnetic resonance (NMR) spectra and high resolution mass spectrometry. NMR spectra including ^{1}H, ^{13}C, DEPT, COSY, HMQC and HMBC were acquired from a Bruker Avance 600 spectrometer (Bruker BioSpin Corporation, Billerica, MA). Chemical shift values are given in ppm downfield from an internal standard (trimethylsilane). Signal multiplicities are represented as singlet (s), doublet (d), double doublet (dd), triplet (t), quartet (q), quintet (quint) and multiplet (m). Exact mass of the active compound was determined by high-performance liquid chromatography−tandem mass spectroscopy from Superfund Analytical Core at Mass Spectrometry Laboratory in the University of California at Davis.

Synthesis of Sarmentine Analogues. To the ice-cooled carboxylic acid (3 mmol) solution in dichloromethane (20 mL) were sequentially added 1-ethyl-3-(3′-dimethylaminopropyl)carbodiimide (3.3 mmol) and 4-dimethylaminopyridine (3 mmol). After 5 min, amine (3.3 mmol) was added to the reaction solution. The reaction was slowly warmed to room temperature and continued overnight. The reaction was extracted with ethyl acetate (200 mL), and the organic phase was dried with anhydrous sodium sulfate. After evaporation under vacuum, the residue was run through a silica gel column with an appropriate ratio of ethyl acetate in hexane (i.e., from 1:10 to 1:4 = ethyl acetate: hexane). The yields of the final products ranged from 85% to 90%. The final products (see **Table 3**) were characterized with proton NMR, mass spectrum and melting point analyses. Melting point was measured by OptiMelt (automated melting point system: SRS, Stanford Research Systems, Sunnyvale, CA). The ramp was set up at 1 °C/min.

***N*-Cyclopentyldecanamide (3):** white solid, mp 47.3−48.5 °C; ^{1}H NMR ($CDCl_3$) δ (ppm) 5.35 (br, 1H), 4.22 (m, J=7.00, 1H), 2.12 (t, J=7.20, 2H), 1.98 (m, 2H), 1.59−1.67 (m, 6H), 1.26−1.36 (m, 14H), 0.88 (t, J=7.00, 3H); ESI$^+$ m/z=$[M + H]^+$=240.

***N*-Cyclopentyldecen-2-amide (4):** white solid, mp 71.8−72.5 °C; ^{1}H NMR ($CDCl_3$) δ (ppm) 6.82 (dt, J_1 = 15.20, J_2 = 7.20, 1H), 5.71 (d, J = 15.20, 1H), 5.33 (br, 1H), 4.27 (m, J=7.00, 1H), 2.15 (m, 2H), 2.10 (m, 2H), 1.67 (m, 2H), 1.60 (m, 2H), 1.40 (m, 4H), 1.28 (m, 8H), 0.88 (t, J = 7.00, 3H); ESI$^+$ m/z=$[M + H]^+$=238.

***N*-Cyclopentyl 2*E*,4*Z*-decadienamide (5):** ^{1}H NMR ($CDCl_3$) δ (ppm) 7.55 (dd, J_1 = 14.80, J_2 = 11.70, 1H), 6.06 (t, J = 11.70, 1H), 5.79 (d, J_1 = 14.80, 1H), 5.75 (m, 1H), 5.50 (br, 1H), 4.30 (m, J = 7.00, 1H), 2.29 (q, J = 8.20, 2H), 2.01 (m, 2H), 1.68 (m, 2H), 1.61 (m, 2H), 1.40 (m, 2H), 1.28 (m, 6H), 0.88 (t, J = 7.00, 3H); ESI$^+$ m/z=$[M + H]^+$=236.

***N*-Cyclopentyl, *trans*-cinnamamide (6):** white solid, mp 144−145 °C; ^{1}H NMR ($CDCl_3$) δ (ppm) 7.62 (d, J = 15.6 Hz, 1H), 7.50 (d, J = 7.0 Hz, 2H), 7.35 (m, 3H), 6.37 (d, J = 15.6, 1H), 5.61 (d, J = 5.0, Hz, 1H, NH), 4.35 (m, J = 7.0, 1H), 2.06 (m, 2H), 1.71 (m, 2H), 1.64 (m, 2H), 1.46 (m, 2H); ESI$^+$ m/z = $[M + H]^+$ = 216.

***N*-(Decanoyl)pyrrolidine (7):** ^{1}H NMR ($CDCl_3$) δ (ppm) 3.45 (t, J= 6.80, 2H), 3.40 (t, J = 6.80, 2H), 2.24 (t, J = 7.20, 2H), 1.94 (quint, J= 6.80, 2H), 1.84 (quint, J = 6.80, 2H), 1.62 (quint, J = 7.20, 2H), 1.25−1.30 (m, 12H), 0.87 (t, J=7.20, 3H); ESI$^+$ m/z=$[M + H]^+$=226.

***N*-(2-Decenoyl)pyrrolidine (8):** ^{1}H NMR ($CDCl_3$) δ (ppm) 6.90 (dt, J_1 = 15.20, J_2 = 7.00, 1H), 6.07 (d, J = 15.20, 1H), 3.52 (t, J = 6.30, 2H), 3.50 (t, J = 6.30, 2H), 2.19 (m, 2H), 1.96 (quint, J=7.00, 2H), 1.85 (quint, J = 7.00, 2H), 1.44 (m, 2H), 1.28 (m, 8H), 0.88 (t, J = 7.00, 3H); ESI$^+$ m/z=$[M + H]^+$=224.

(2*E*,4*Z*-Decadienoyl)pyrrolidine (9): ^{1}H NMR ($CDCl_3$) δ (ppm) 7.62 (dd, J_1 = 14.60, J_2 = 11.70, 1H), 6.17 (d, J = 14.60, 1H), 6.13 (t, J = 11.70, 1H), 5.78 (m, 1H), 3.55 (t, J=7.00, 2H), 3.52 (t, J=7.00, 2H), 2.30 (q, J = 7.40, 2H), 1.97 (quint, J = 7.40, 2H), 1.87 (quint, J = 7.40, 2H), 1.40 (quint, J = 7.40, 2H), 1.29 (m, 4H), 0.88 (t, J = 7.00, 3H); ESI$^+$ m/z = $[M + H]^+$ = 222.

***N*-(*trans*-Cinnamoyl)pyrrolidine (10):** white solid, mp 100.6−101.7 °C; ^{1}H NMR ($CDCl_3$) δ (ppm) 7.70 (d, J = 15.5 Hz, 1H), 7.53 (d, J = 7.0 Hz, 2H), 7.36 (m, 3H), 6.74 (d, J = 15.5, 1H), 3.63 (t, J = 7.0, 2H), 3.60 (t, J = 7.0, 2H), 2.01 (quint, J = 7.0, 2H), 1.91 (quint, J = 7.0, 2H); ESI$^+$ m/z = $[M + H]^+$ = 202.

***N*-(Decanoyl)piperidine (11):** ^{1}H NMR ($CDCl_3$) δ (ppm) 3.55 (t, J= 5.20, 2H), 3.39 (t, J = 5.20, 2H), 2.31 (t, J = 7.60, 2H), 1.58−1.65 (m, 4H), 1.52−1.57 (m, 4H), 1.20−1.30 (m, 12H), 0.87 (t, J=7.20, 3H); ESI$^+$ m/z= $[M + H]^+$ = 240.

***N*-(2-Decenoyl)piperidine (12):** ^{1}H NMR ($CDCl_3$) δ (ppm) 6.82 (dt, J_1 = 15.20, J_2 = 7.00, 1H), 6.23 (d, J = 15.20, 1H), 3.59 (t, J = 6.30, 2H), 3.47 (t, J = 6.30, 2H), 2.17 (m, 2H), 1.64 (quint, J = 5.60, 2H), 1.56 (quint, J = 5.60, 4H), 1.44 (quint, J = 7.00, 2H), 1.28 (m, 8H), 0.88 (t, J = 7.00, 3H); ESI$^+$ m/z = $[M + H]^+$ = 238.

(2*E*,4*Z*-Decadienoyl)piperidine (13): ^{1}H NMR ($CDCl_3$) δ (ppm) 7.59 (dd, J_1 = 14.60, J_2 = 11.70, 1H), 6.34 (d, J = 14.60, 1H), 6.13 (t, J = 11.70, 1H), 5.78 (m, 1H), 3.62 (t, J = 7.00, 2H), 3.45 (t, J = 7.00, 2H), 2.31 (q, J = 7.40, 2H), 1.67 (quint, J = 7.40, 4H), 1.57 (quint, J = 7.40, 2H), 1.40 (m, 2H), 1.27 (m, 4H), 0.88 (t, J = 7.00, 3H); ESI$^+$ m/z = $[M + H]^+$ = 236.

***N*-(*trans*-Cinnamoyl)piperidine (14):** white solid, mp 118.9−119.9 °C; ^{1}H NMR ($CDCl_3$) δ (ppm) 7.64 (d, J=15.5 Hz, 1H), 7.52 (d, J=7.2 Hz, 2H),

7.36 (m, 3H), 6.90 (d, J = 15.5, 1H), 3.67 (s, 2H), 3.59 (s, 2H), 1.68 (m, 2H), 1.62 (m, 4H); ESI^+ m/z = $[M + H]^+$ = 216.

***N*-(Decanoyl)hexamethyleneimine (15):** ^1H NMR ($CDCl_3$) δ (ppm) 3.52 (t, J = 6.00, 2H), 3.42 (t, J = 6.00, 2H), 2.30 (t, J = 7.80, 2H), 1.66−1.74 (m, 4H), 1.60−1.66 (m, 2H), 1.50−1.6.0 (m, 4H), 1.20−1.30 (m, 12H), 0.87 (t, J = 7.20, 3H); ESI^+ m/z = $[M + H]^+$ = 254.

***N*-(2-Decenoyl)hexamethleneimine (16):** ^1H NMR ($CDCl_3$) δ (ppm) 6.91 (dt, J_1 = 15.20, J_2 = 7.00, 1H), 6.21 (d, J = 15.20, 1H), 3.57 (t, J = 6.00, 2H), 3.49 (t, J = 6.00, 2H), 2.17 (m, 2H), 1.73 (m, 4H), 1.56 (m, 4H), 1.45 (m, 2H), 1.28 (m, 8H), 0.88 (t, J = 7.00, 3H); ESI^+ m/z = $[M + H]^+$ = 252.

(2*E*,4*Z*-Decadienoyl)hexamethleneimine (17): ^1H NMR ($CDCl_3$) δ (ppm) 7.64 (dd, J_1 = 14.60, J_2 = 11.70, 1H), 6.30 (d, J = 14.60, 1H), 6.16 (t, J = 11.70, 1H), 5.78 (m, 1H), 3.60 (t, J = 7.00, 2H), 3.51 (t, J = 7.00, 2H), 2.31 (q, J = 7.40, 2H), 1.76 (m, 4H), 1.57 (m, 4H), 1.40 (m, 2H), 1.30 (m, 4H), 0.88 (t, J = 7.00, 3H); ESI^+ m/z = $[M + H]^+$ = 250.

***N*-(*trans*-Cinnamoyl) hexamethleneimine (18):** white solid, mp 122.6−123.3 °C; ^1H NMR ($CDCl_3$) δ (ppm) 7.70 (d, J = 15.4 Hz, 1H), 7.52 (d, J = 7.6 Hz, 2H), 7.36 (m, 3H), 6.88 (d, J = 15.4, 1H), 3.63 (t, J = 6.0, 2H), 3.61 (t, J = 6.0, 2H), 1.76 (m, 4H), 1.59 (m, 4H); ESI^+ m/z = $[M + H]^+$ = 230.

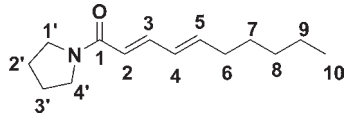
Comparison of the Percent Content of the Sarmentine (1) from Different Fruit Samples. Freshly ground fruit powder (10 g) of different samples was soaked in ethyl acetate (50 mL) for 20 h at room temperature. The solution was filtered by a Whatman qualitative filter paper (No. 1, ∅ 155 mm). The residue and filter paper were washed with ethyl acetate (25 mL). The combined organic phase was dried under vacuum. The weight of each extract was recorded. The active compound in the extracts was quantified by LC/MS.

Quantification of Sarmentine (1) in the Ethyl Acetate Extract of Dry Fruits by (LC/MS). Conditions of liquid chromatography and mass spectrometry are specifically described as follows. Chromatographic separation was performed at 25 °C on a Thermo high performance liquid chromatography (HPLC) instrument equipped with Finnigan Surveyor PDA plus detector, autosampler plus, MS pump and a 4.6 mm × 100 mm Luna C18 5 μm column (Phenomene, Torrance, CA). The solvent system consisted of water (solvent A) and acetonitrile (solvent B). The mobile phase began at 10% solvent B and was linearly increased to 100% solvent B over 20 min and then held for 4 min, and finally returned to 10% solvent B over 3 min and kept for 3 min. The flow rate was 0.5 mL/min. The injection volume was 10 μL, and the samples were kept at room temperature in an auto sampler. The active compound was detected by a positive electrospray ionization mode in a full scan mode (m/z 100−1500 Da) on a LCQ DECA XP^{plus} mass spectrometer (Thermo Electron Corp., San Jose, CA). The flow rate of nitrogen gas was fixed at 30 and 15 arb for the sheath and aux/sweep gas flow rate, respectively. Electrospray ionization (ESI) was performed with a spray voltage set at 5000 V and a capillary voltage at 35.0 V. The capillary temperature was set at 400 °C.

The active compound standard was obtained by repeated crystallization in laboratory until only one peak at 1 mg injection level was shown under a wavelength of 210 nm with two different mobile phases. A series of standard concentrations (125, 62.5, 31.25, 15.6, 7.8, 3.9, 1.95, and 0.976 ng/mL) was made in ethanol. Three independent samples (2.5 μg/mL in ethanol) for each extract were made. Mass spectra were run in a SIM mode with a mass range of 221.0−224.0 and retention time of 16.94 min. The limits of detection (LOD) of the active compound were determined by running decreasing amounts of standard solution until the ratio of the signal of the active compound over the background was greater than or equal to 3. The concentration of sarmentine in fruit samples was presented by an average of three independent samples with a standard deviation.

Evaluation of Herbicidal Activity. Herbicidal activity of the active compound and synthesized compounds was evaluated by foliar spraying. Carrier solution contained 2% ethanol, 0.2% glycosperse O-20 and 0.1% sodium lauryl sulfate. Freshly prepared solution with the evaluated compound at a concentration of 5 mg/mL was used. In the spectrum study, spraying volume was dependent on the foliar surface area, ranging from approximately 1 to 3 mL/pot. One or two pots of plants were treated for each. Number of pots treated was dependent on both foliar area and availability. In the structure−activity relationship study, one pot of barnyard grass was used per treatment and the spraying volume of each compound was 3 mL of 5 mg/mL. Phytotoxicity was evaluated 3 days after spraying. Efficacy of phytotoxicity was graded as I (no effect), II (< 20% mortality), III (20−40% mortality), IV (40−60% mortality), V (60−80% mortality) and VI (80−100% mortality).

Table 1. ^1H and ^{13}C NMR ($CDCl_3$) Data of Sarmentine (**1**)



position	^{13}C	^1H (J = Hz)
1	165.4	
2	120.0	6.08 1H d (14.4)
3	142.4	7.26 1H dd (14.4, 10.8)
4	128.8	6.16 1H dd (10.8, 9.6)
5	143.4	6.07 1H dt (14.4, 7.2)
6	33.1	2.13 2H quartet (7.2)
7	26.3	1.39 2H quintet (7.2)
8	24.5	1.27 2H m
9	22.7	1.27 2H m
10	14.2	0.87 3H t (7.2)
1′	46.6	3.50 2H t (7.2)
2′	31.5	1.95 2H quintet (7.2)
3′	28.6	1.85 2H quintet (7.2)
4′	46.0	3.52 2H t (7.2)

RESULTS AND DISCUSSION

Structure Elucidation. The active compound was identified on the basis of the following evidence: High resolution mass data (TOF MS ESI^+) is 222.5386, indicating that the molecular formula of the active compound is most likely $C_{14}H_{23}NO$. The data from ^1H and ^{13}C NMR listed in **Table 1** further support this molecular formula. The ^1H NMR spectrum indicated the presence of 23 protons including four olefinic (−CH═), one methyl ($-CH_3$) and eight methylene ($-CH_2-$) protons. The ^{13}C NMR and DEPT-135 spectrum (not shown) confirmed the presence of 14 carbons accounting for one amide carbonyl (N−CO−), four olefinic carbons (−CH═), eight methylene carbons ($-CH_2-$) and one methyl carbon ($-CH_3$). From the $^1H-^1H$ COSY spectrum (not shown), two spin systems were constructed. One contained four consecutive methylenes ($-CH_2CH_2CH_2CH_2-$), and the other contained four conjugated olefin protons further connected to $CH_2CH_2CH_2CH_3$. Moreover, data from HMBC (not shown) indicated that the above-mentioned two spin systems were connected through an amide to give the planar structure of sarmentine (**1**). Finally, the stereochemistry of the two double bonds were assigned as *trans* based on the coupling constants (J = 14.4 Hz). The search in the literature (SciFinder) indicated that the compound **1** is a known compound called sarmentine, which was first isolated from dry *P. sarmentosum* fruit powder (*17*) and also from *P. nigrum* (*18*). However, this is the first report of isolation from *P. longum* and of exhibiting phytotoxicity.

Herbicidal Spectrum of Sarmentine (1). The phytotoxicity of sarmentine (**1**) depended on its concentration and plant species. The optimal concentration of sarmentine (5 mg/mL) for excellent control of barnyard grass (**Figure 1**) was chosen for herbicidal spectrum study. Due to the hydrophobic property of sarmentine (**1**), a carrier solution containing 0.2% glycosperse O-20, 2% ethanol and 0.1% sodium lauryl sulfate was used. This carrier solution contains a high concentration of surfactants, and sarmentine (**1**) can be suspended in this solution for 15 min. To mitigate the poor suspension, the sarmentine (**1**) solution was prepared immediately prior to foliar application. The carrier solution alone did not show any phytotoxicity toward tested plants when results were recorded.

Sarmentine (**1**) displayed phytotoxicity against a variety of plants including crop plants and weeds (**Table 2**). Phytotoxicity of

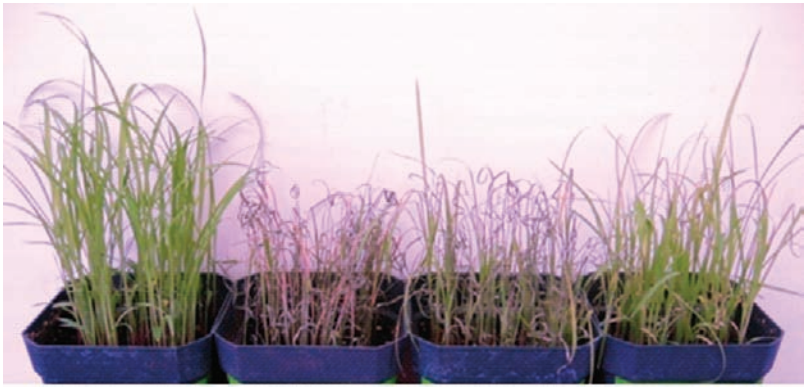


Figure 1. Concentration dependence of sarmentine phytotoxicity on barnyard grass. Pots from left to right were treated with the carrier solution, 5.0, 2.5, and 1.25 mg/mL of sarmentine, respectively. The carrier solution consisted of 2% ethanol and 0.2% glycosperse O-20 KFG. Each treatment was 3 mL/pot.

Table 2. Phytotoxicity Spectrum of Sarmentine (**1**)

no.	plant name	efficacy[a]	no.	plant name	efficacy[a]
1	pigweed (*Amaranthus retroflexus* L.)	VI	9	lambsquarter (*Chenopodium album* L.)	VI
2	barnyard grass (*Echinochloa crus-galli* L.)	VI	10	annual bluegrass (*Poa annua* L.)	VI
3	bindweed (*Convolvulus arvensis* L.)	VI	11	wild mustard (*Brassica kaber* L.)	VI
4	crabgrass (*Digitaria sanguinalis* L.)	VI	12	black nightshade (*Solanum nigrum* L.)	VI
5	horseweed (*Conyza canadensis* L.)	II	13	curly dock (*Rumex crispus* L.)	VI
6	sedge (*Cyperus difformis* L.)	III	14	sweet corn (*Zea mays* L.)	VI
7	sprangletop (*Leptochloa fascicularis* Lam.)	VI	15	wheat (PR 1404) (*Triticum aestivum* L.)	VI
8	dandelion (*Taraxacum officinale* F.)	VI	16	rice (M-104) (*Oryza sativa* L)	I

[a] Efficacy of phytotoxicity was graded as I (no effect), II (<20% mortality), III (20−40% mortality), IV (40−60% mortality), V (60−80% mortality) and VI (80−100% mortality).

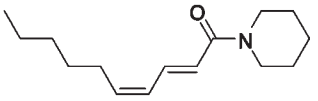
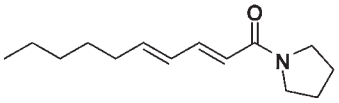
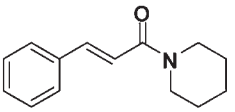
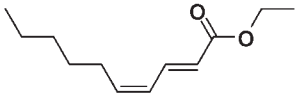
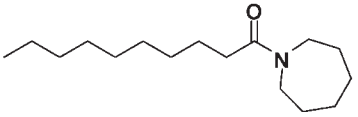
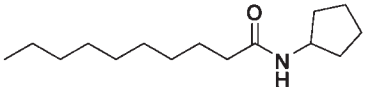
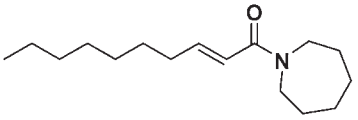
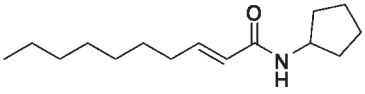
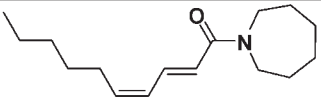
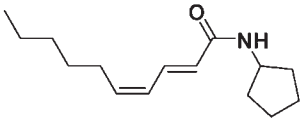
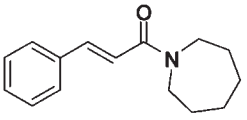
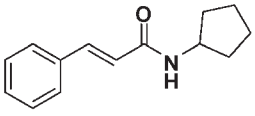
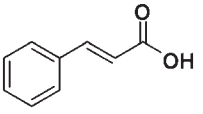
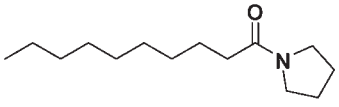
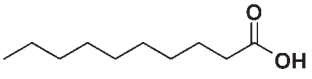
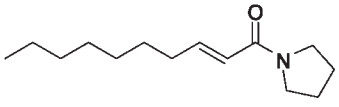
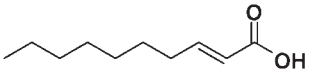
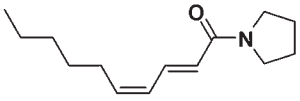
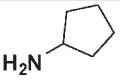
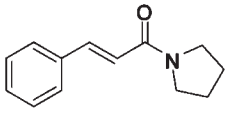
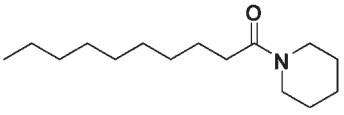
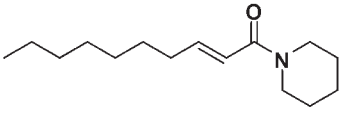
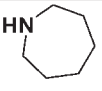
sarmentine (**1**) was dependent on the plant species, ranging from zero to 100% control. No visible phytotoxicity (after 10 days) was shown on rice plants toward sarmentine (**1**). Slight phytotoxicity of sarmentine (**1**) on sedge and horseweed was shown. However, high phytotoxicity of sarmentine (**1**) was observed on pigweed, barnyard grass, bindweed, crabgrass, sprangletop, dandelion, lambsquarter, annual bluegrass, wild mustard, black nightshade, curly dock, sweet corn and wheat. Phytotoxicity may be related to age of the plants. For example, horseweed (70 days old) was much older than other weeds, and less phytotoxicity was shown.

Structure−Activity Relationship (SAR). To quickly understand which part of the sarmentine (**1**) molecule plays a crucial role on the phytotoxicity, a series of sarmentine analogues were synthesized and evaluated on barnyard grass. The SAR study (**Table 3**) suggested that neither the long unsaturated fatty acid nor pyrrolidine of sarmentine (**1**) alone is crucial for phytotoxicity, but the amide bond with a secondary amine seemed to be necessary. This conclusion was supported by the following experimental results. Phytotoxicity remained the same when the acid moiety of sarmentine (**1**) was replaced by structurally similar fatty acids such as 2*E*,4*Z*-decadienoic acid (i.e., geometric isomer of 2*E*,4*E*-decadienoic acid) with two double bonds (**9**), 2*E*-decenoic acid with one double bond (**8**) and decanoic acid without any double bonds (**7**), and even structurally different acid such as *trans*-cinnamic acid (**10**). This suggested that the acid moiety of sarmentine (**1**) can vary when the amine is pyrrolidine. Similarly, when the acid moiety remained the same, the amine could vary. For example, phytotoxicity remained the same with decanoic acid when the amine was changed from a five-membered ring (**7**) to a six- or seven-membered ring (**11** and **15**, respectively). However, phytotoxicity dropped dramatically when the amide bond with a secondary amine was changed into an ester bond (e.g., **2**) and an amide bond with a primary amine (e.g., **3**−**6**). In addition, results from the SAR study (**Table 3**) also indicate that the amine moiety of sarmentine, pyrrolidine (**23**), and its analogues such as cyclopentylamine (**22**), hexamethyleneimine (**25**) and piperidine (**24**) were nontoxic to barnyard grass; but the analogues of the acid moiety of sarmentine such as decanoic acid (**20**), 2*E*-decenoic acid (**21**) and *trans*-cinnamic acid (**19**) were very active. To obtain a better SAR, the length of the carbon chain in the acid moiety, disubstituted amines (nonring system) and changing carbonyl group into other groups such as phosphate or sulfone should be further investigated.

Symptoms of Phytotoxicity. Sarmentine (**1**) is phytotoxic by contact. When plants were exposed to sarmentine, phytotoxic symptoms included bent stems, closed-up leaves, or slight black tiny spots (scorching) on the leaves which then became bigger and bigger until they covered the whole leaves. These symptoms could be clearly observed within half an hour to 2 h after spraying. Most phytotoxicity occurred within 7 h after spraying. These observed symptoms were very similar to that of middle-chain fatty acids such as decanoic acid (**20**) (**Figure 2**).

Our results suggest that sarmentine (**1**) and its analogues most possibly possess the same mode of action as the middle-chain fatty acids such as decanoic acid (**20**) and 2*E*-decenoic acid (**21**), which disrupt the cell membrane and then initiate peroxidation driven by radicals (*19*, *20*). Phytotoxicity of sarmentine (**1**) and its analogues can be a direct and indirect (or hydrolytic) action. The indirect mechanism involves the acid moiety of sarmentine and its analogues acting after the active ingredients were hydrolyzed plant amide hydrolases (*21*). For example, 2*E*,4*E*-decadieneoic acid, one of the hydrolyzed metabolites of sarmentine (**1**), is structurally similar to decanoic acid (**20**) and 2*E*-decenoic acid (**21**). It seems reasonable to assume that phytotoxicity of sarmentine (**1**) results from the formation of 2*E*,4*E*-decadieneoic acid. However, this possible hydrolytic mechanism may be totally excluded. First of all, esters are typically hydrolyzed by serine proteases with similar or much higher rates when compared with structurally similar amides (*22*−*24*). Therefore, *trans*-2-*cis*-4-decadienoate (**2**) should be at least as active as its amide analogues

Table 3. Phytotoxicity of Sarmentine (**1**) and Its Analogues on Barnyard Grass

No	Structures	Efficacy *	No	Structures	Efficacy*
	vector	I	13		VI
1	 (sarmentine)	VI	14		IV
2		II	15		VI
3		II	16		VI
4		II	17		VI
5		II	18		II
6		II	19		V
7		VI	20		VI
8		VI	21		VI
9		VI	22		I
10		VI	23		I
11		VI	24		I
12		VI	25		I

* Efficacy of phytotoxicity was graded as I (no effect), II (<20% mortality), III (20−40% mortality), IV (40−60% mortality), V (60−80% mortality) and VI (80−100% mortality).

(e.g., **9**, **13** and **17**), which is not supported by our experimental results (**Table 3**). In addition, when LY2183240 [a nonselective and highly potent inhibitor for human serine hydrolases such as fatty acid amide hydrolase (IC_{50} = 13 nM) and MAG-lipase (IC_{50} = 5.3 nM) (*25*)] at 32.5 and 325 μM was separately coapplied with *N*-(decanoyl) pyrrolidine (**7**), the phytotoxicity of **7** was not affected at all (data are not shown here). Of course, the fact that this inhibitor may not inhibit serine hydrolases (e.g., the

Figure 2. Comparison of phytotoxic symptoms and time course of phytotoxicity of sarmentine and decanoic acid (*21*). Mustard and barnyard grass are displayed in the back and front row, respectively. Plants in the left, middle and right columns were treated with carrier solution, sarmentine and decanoic acid, respectively. The carrier solution consisted of 2% ethanol, 0.2% glycosperse O-20 KFG and 0.1% sodium lauryl sulfate.

Table 4. The Concentration of Sarmentine (**1**) in Different Samples of Dry *P. longum* Fruits

sample	resources	purchase time	% of sarmentine in ethyl acetate extract	extract wt (g) from 10 g of ground dry fruit powder	% content of sarmentine in dry fruit
1	WAN FUNG	May 25, 2008	12.66 ± 0.45	0.45 ± 0.03	0.5697 ± 0.0380
2	WAN FUNG	March, 23, 2009	1.83 ± 0.10	0.51 ± 0.03	0.0933 ± 0.0055
3	WAH TSUN	June 2, 2008	0.01 ± 0.001	0.55 ± 0.02	0.00055 ± 0.00002
4	WAH TSUN	March 4, 2009	0.008 ± 0.001	0.66 ± 0.03	0.00053 ± 0.00002

amide hydrolases, lipases) in barnyard grass may explain the negative results of this experiment. Finally, it is impossible for serine hydrolases to metabolize sarmentine (**1**) and its analogues so quickly that the time course of phytotoxic symptoms of sarmentine and its analogues matches very well with those of middle chain fatty acids (**Figure 2**). The direct action mechanism with sarmentine (**1**) and its analogues directly disrupting cell membrane and then initiating the peroxidation reactions is supported by the following observed experimental phenomenon (**Figure 2**): the appearance and progression of phytotoxic symptoms of sarmentine (**1**) and its analogues was similar to that of decanoic acid (**20**). Using this mode of action mechanism it is, however, difficult to explain why esters (e.g., **2**) and amides with a primary amine (e.g., **3**−**6**) did not display the same phytotoxicity. Therefore, more biochemical and molecular biology data are needed to elucidate the mode of action of sarmentine and its analogues.

Quantification of Sarmentine in Different *P. longum* Fruit Samples. In order to successfully use long pepper fruit as raw material for a commercial bioherbicide, the variability of sarmentine content in dry long pepper fruits obtained from different sources was investigated. Due to the fact that freshly dried fruits are not readily available, dry fruits from herb stores were investigated instead. For quantitative analysis, the LC/MS method was developed and the standard curve of sarmentine ($Y = 83324X - 30784$, $R^2 = 0.9998$; X and Y stand for the concentration of sarmentine and the peak area, respectively) was obtained. For the method, the limit of detection for sarmentine was 12 pg per injection (or 1.2 ng/mL). Based on this external standard curve, the concentration of sarmentine in the extracts of four different *P. longum* fruit samples varied dramatically, ranging from 0.0005% to 0.57% (**Table 4**). This high variability may result from the age and/or origin of the dry fruits, which parameters were not available for the test samples. The first sample we obtained in this study contained the highest content of sarmentine. However, this may give us an indication that freshly dried material should be used to study phytochemicals. In addition, from this study, it seems that the quantity of sarmentine in the ground powder of dry *P. longum* fruits can be evaluated by physical appearance because the higher the concentration of sarmentine, the oilier the ground powder is. For example, no oil was visible for the ground powder of the two samples from WAH TSUN, but oily powder was seen for the ground powder of those from WAN FUNG, especially the sample obtained on May 25, 2008.

Potential Use of Sarmentine and Its Analogues. Biological effects of sarmentine have been investigated in the past. Sarmentine has been found to be an *in vivo* skin antioxidant protecting photoaged skin (*26*) and to display antiplatelet aggregation activity (*27*), antiplasmodial and antimycobacterial activities (*28*) and antituberculosis activity (*29*). In addition, sarmentine is used to solubilize hydrophobic compounds in cosmetics and pharmaceuticals (PCT Publication No: WO/2008/065451 (*30*)). In this study, sarmentine has shown potential for use as a bioherbicide and/or a lead molecule for synthetic herbicide. However, chemical instability of sarmentine under environmental (e.g., light, oxygen) and biological conditions (e.g., P450s, hydrolases) is currently limiting the commercial use of sarmentine. Structural modification of sarmentine as demonstrated in this study will probably help solve instability problem,

and lead to more commercial application and use of this known natural compound.

ACKNOWLEDGMENT

We thank Drs. Jun Yang, Junyan Liu and Ms. Shirley Gee of the University of California at Davis for assistance in measuring the high resolution mass of sarmentine and providing some reagents.

LITERATURE CITED

(1) Gianessi, L. P.; Reigner, N. P. The value of herbicides in U.S. crop production. *Weed Technol*. **2007**, *21*, 559–566.
(2) Solomon, G. M.; Schettler, T. Environment and health: 6. Endocrine disruption and potential human health implications. *Can. Med. Assoc. J*. **2000**, *163*, 1471–1476.
(3) Stillerman, K. P.; Mattison, D. R.; Giudice, L. C.; Woodruff, T. J. Environmental exposures and adverse pregnancy outcomes: A review of the science. *Reprod. Sci*. **2008**, *15*, 631–650.
(4) Whaley, C. M.; Wilson, H. P.; Westwood, J. H. A new mutation in plant ALS confers resistance to five classes of ALS-inhibiting herbicides. *Weed Sci*. **2007**, *55*, 83–90.
(5) Duke, S. O.; Dayan, F. E.; Rimando, A. M.; Schrader, K. K.; Aliotta, G.; Oliva, A.; Romagni, J. G. Chemicals from nature for weed management. *Weed Sci*. **2002**, *50*, 138–151.
(6) Batish, D. R.; Setia, N.; Singh, H. P.; Kohli, R. K. Phytotoxicity of lemon-scented eucalypt oil and its potential use as a bioherbicide. *Crop Prot*. **2004**, *23*, 1209–1214.
(7) Macias, F. A.; Molinillo, J. M. G.; Varela, R. M.; Galindo, J. C. J. Allelopathy—a natural alternative for weed control. *Pest Manage. Sci*. **2007**, *63*, 327–348.
(8) Bertin, C.; Weston, L. A.; Huang, T.; Jander, G.; Owens, T.; Meinwald, J.; Schroeder, F. C. Grass roots chemistry: meta-Tyrosine, an herbicidal nonprotein amino acid. *Proc. Natl. Acad. Sci. U.S.A*. **2007**, *104*, 16964–16969.
(9) Dayan, F. E.; Cantrell, C. L.; Duke, S. O. Natural products in crop protection. *Bioorg. Med. Chem*. **2009**, *17*, 4022–4034.
(10) Krishnamurthi, A. *The Wealth of India Raw Materials*; CSIR: New Delhi, India, 1969; Vol. 8, p 96.
(11) Ghoshal, S.; Prasad, B. N. K.; Lakshmi, V. Antiamoebic activity of *Piper longum* fruits against *Entamoeba histolytica* in vitro and in vivo. *J. Ethnopharmacol*. **1996**, *50*, 167–170.
(12) Choi, E. M.; Hwang, J. K. Investigations of anti-inflammatory and antinociceptive activities of *Piper cubeba, Physalis angulata* and Rosa hybrid. *J. Ethnopharmacol*. **2003**, *89*, 171–175.
(13) Mata, R.; Morales, I.; Perez, O.; Rivero-Cruz, I.; Acevedo, L.; Enriquez-Mendoza, I.; Bye, R.; Franzblau, S.; Timmermann, B. Antimycobacterial compounds from *Piper sanctum*. *J. Nat. Prod*. **2004**, *67*, 1961–1968.
(14) Vedhanayaki, G.; Shastri, G. V.; Kuruvilla, A. Analgesic activity of *Piper longum* Linn. Root. *Indian J. Exp. Biol*. **2003**, *41*, 649–651.
(15) Biswanath, D.; Kashinatham, A.; Srinivas, K. V. N. S. Alkamides and other constituents of *Piper longum*. *Planta Med*. **1996**, *62*, 582.
(16) Yang, Y. C.; Lee, S. G.; Lee, H. K.; Kim, M. K.; Lee, S. H.; Lee, H. S. A piperidine amide extracted from *Piper longum* L. fruit shows activity against *Aedes aegypti* Mosquito Larvae. *J. Agric. Food Chem*. **2002**, *50*, 3765–3767.
(17) Likhitwitayawuid, K.; Ruangrungsi, N.; Lange, G. L.; Decicco, C. P. Structural elucidation and synthesis of new components isolated from *Piper sarmentosum* (Piperaceae). *Tetrahedron* **1987**, *43*, 3689–3694.
(18) Kiuchi, F.; Nakamura, N.; Tsuda, Y.; Kondo, K.; Yoshimura, H. Studies on crude drugs effective on visceral larva migrans, IV isolation and identification of larvicidal principles in Pepper. *Chem. Pharm. Bull*. **1988**, *36*, 2452–2465.
(19) Lederer, B.; Fujimori, T.; Tsujino, Y.; Wakabayashi, K.; Böger, P. Phytotoxic activity of middle-chain fatty acids II: peroxidation and membrane effects. *Pest. Biochem. Physiol*. **2004**, *80* (3), 151–156.
(20) Fukuda, M.; Tsujino, Y.; Fujimori, T.; Wakabayashi, K.; Böger, P. Phytotoxic activity of middle-chain fatty acids I: effects on cell constituents. *Pest. Biochem. Physiol*. **2004**, *80* (3), 143–150.
(21) Shrestha, R; Dixon, R. A.; Chapman, K. D. Molecular identification of a functional homologue of the mammalian fatty acid amide hydrolase in *Arabidopsis thaliana*. *J. Biol. Chem*. **2003**, *278* (37), 34990–34997.
(22) Carter, P.; Abrahmsen, L.; Wells, J. A. Probing the mechanism and improving the rate of substrate-assisted catalysis in subtilisin BPN. *Biochemistry* **1991**, *30*, 6142–6148.
(23) Corey, D. R.; Craik, C. S. An investigation into the minimum requirements for peptide hydrolysis by mutation of the catalytic triad of trypsin. *J. Am. Chem. Soc*. **1992**, *114*, 1784–1790.
(24) Patricelli, M. P.; Cravatt, B. F. Fatty acid amide hydrolase competitively degrades bioactive amides and esters through a nonconventional catalytic mechanism. *Biochemistry* **1999**, *38*, 14125–14130.
(25) Alexander, J. P.; Cravatt, B. F. The putative endocannabinoid transport blocker LY2183240 is a potent inhibitor of FAAH and several other brain serine hydrolases. *J. Am. Chem. Soc*. **2006**, *128*, 9699–9704.
(26) Cornacchione, S.; Sadick, N. S.; Neveu, M.; Talbourdet, S.; Lazou, K.; Viron, C.; Renimel, I.; de Quéral, D.; Kurfurst, R.; Schnebert, S.; Heusèle, C.; André, P.; Perrier, E. In vivo skin antioxidant effect of a new combination based on a specific *Vitis vinifera* shoots extract and a biotechnological extract. *J. Drugs Dermatol*. **2007**, *6S*, 8–13.
(27) Li, C. Y.; Tsai, W.; Damu, A. G.; Lee, E. J.; Wu, T. S.; Dung., N. X.; Thang, T. D.; Thanh, L. Isolation and identification of antiplatelet aggregatory principles from the leaves of *Piper lolot*. *J. Agric. Food Chem*. **2007**, *55*, 9436–9442.
(28) Tuntiwachwuttikul, P.; Phansa, P.; Pootaeng-on, Y.; Taylor, W. C. Chemical constituents of the roots of *Piper Sarmentosum*. *Chem. Pharm. Bull*. **2006**, *54*, 149–151.
(29) Rukachaisirikul, T.; Siriwattanakit, P.; Sukcharoenphol, K.; Wongvein, C.; Ruttanaweang, P.; Wongwattanavuch, P.; Suksamrarn, A. Chemical constituents and bioactivity of *Piper sarmentosum*. *J. Ethnopharmacol*. **2004**, *93*, 173–176.
(30) Stephen, T.; Andrew, H. Compositions comprising macromolecular assembles of lipid surfactant, WIPO Patent Application WO/2008/065451.

Received for review June 1, 2010. Revised manuscript received August 2, 2010. Accepted August 3, 2010.