Novel Dimeric Metabolites from *Alternaria tagetica*

M. Marcela Gamboa-Angulo,[†] Fátima Alejos-González,[†] Fabiola Escalante-Erosa,[†] Karlina García-Sosa,[†] Guillermo Delgado-Lamas,[‡] and Luis M. Peña-Rodríguez*[,†]

Grupo de Química Orgánica, Unidad de Biotecnología, Centro de Investigación Científica de Yucatán A.C., Apdo. Postal 87 Cordemex 97310, Mérida, Yucatán, México, and Instituto de Química, Universidad Nacional Autónoma de Mèxico, Circuito Exterior, Ciudad Universitaria, México D.F.

Received August 27, 1999

Two novel polyketides, *bis*-7-*O*-8″.8-*O*-7″- and *bis*-7-*O*-7″.8-*O*-8″-zinniol (**2** and **3**, respectively) were isolated from the organic crude extract of culture filtrates from *Alternaria tagetica*. Both structures were determined on the basis of their spectroscopic data (IR, MS, ^{1}H NMR, ^{13}C NMR, and 2D NMR experiments) and confirmed by chemical synthesis. Zinniol (**1**) was isolated as a major component, and its ^{13}C NMR data was correctly assigned after careful analysis of data from its 2D NMR experiments (HMQC and HMBC).

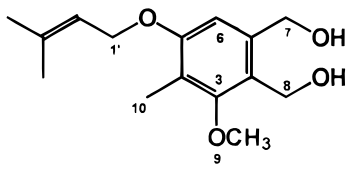
Among fungi, the *Alternaria* genus is known for the high pathogenicity of some of its members, including *A. mali*, *A. kikuchiana*, and *A. solani*, which infect apple, pear, and potato plants, respectively.[1] It has long been recognized that these and other phytopathogenic fungi produce phytotoxic metabolites that play a significant role during the infection process; these phytotoxins have been classified as host specific and non-host specific toxins (HSTs and non-HSTs, respectively), depending on whether they are considered to be essential or not for pathogenicity.[2] One of the most important applications of phytotoxins, both HSTs and non-HSTs, is their use as selection agents for the development of disease-resistant plant lines.[1−3]

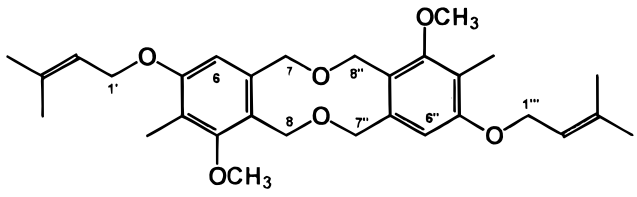
One *Alternaria* species that has not received much attention for its production of phytotoxic metabolites is *A. tagetica*, the causal agent of early blight in marigold (*Tagetes erecta*), an important economic crop in Mexico for the industrial production of xantophyll pigments that are extracted from its flowers.[4] It has been reported that, under favorable conditions, *A. tagetica* can severely infect marigold plants causing losses of up to 100% in flower yields.[4−5] Previous studies carried out on *A. tagetica* reported zinniol, a non-HST frequently isolated from *Alternaria* species,[6] as one of the phytotoxic components present in the organic crude extract.[7]

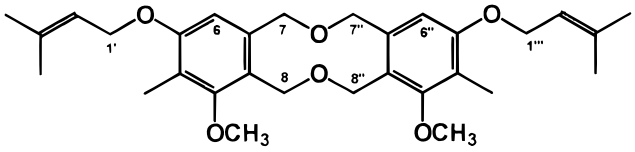
As part of a study directed toward the detection, isolation, and identification of the phytotoxic metabolites produced by *A. tagetica* cultured in liquid medium, we report herein the purification and identification of two novel dimeric metabolites, *bis*-7-*O*-8″.8-*O*-7″- and *bis*-7-*O*-7″.8-*O*-8″-zinniol (**2** and **3**, respectively) and the correct assignment of the ^{13}C NMR data for zinniol (**1**).

Results and Discussion

An initial solvent partitioning of the phytotoxic organic crude extract (necrotic area: 0.3 mm^2) from cultures of *A. tagetica* yielded three crude fractions of increasing polarity. Testing of the fractions in the leaf-spot assay[8] showed that the low polarity (hexane) fraction possessed the strongest phytotoxic activity (necrotic area: 0.41 mm^2). A bioassay-guided purification of the low polarity fraction[9] resulted in the isolation of a single phytotoxic (necrotic area: 0.7 mm^2) metabolite identified as zinniol (**1**) by comparing its spectroscopic data with those reported in the literature.[10−12] A careful analysis of both ^{1}H and ^{13}C NMR data of **1**, together with those from DEPT, HMQC, and HMBC experiments, allowed for the correct assignment of all carbons and protons of zinniol (Table 1). The three oxomethylene groups in **1** were easily detected from DEPT and HMQC analyses; a doublet at δ 4.52 showed a direct correlation with the carbon at 65.3 ppm, while two singlets at δ 4.68 and 4.76 were shown to be connected with carbons at 64.7 and 56.8 ppm, respectively. In the HMBC of **1**, the long-range correlation (^3J) observed between the aromatic proton (δ 6.68) and the carbons resonating at 124.7, 120.0, and 64.7 ppm, together with the ^3J interactions observed between the methylene protons at δ 4.76 and the carbons at 158.2, 138.9, and 124.7 ppm, allowed the unequivocal assignment of the signal at 64.7 ppm to C-7 and that at 56.8 ppm to C-8. In view of these results, the remaining oxygen-bearing carbon at 65.3 ppm must correspond to the C-1′ methylene. Similarly a ^3J interaction between the C-7 oxomethylene protons (δ 4.68) and the carbon at 124.7 ppm,







* To whom correspondence should be addressed. Tel.: +52-9-981-3923. Fax: +52-9-981-39-00. E-mail: lmanuel@cicy.mx.
[†] Grupo de Química Orgánica.
[‡] Instituto de Química.

10.1021/np990422q CCC: $19.00
Published on Web 07/22/2000

Table 1. ^{13}C and ^{1}H NMR ($CDCl_3$) Data for Compounds **1**−**3**[a,b]

	1		**2**		**3**	
position	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H
1	138.9 s		135.1 s		140.1 s	
2	124.7 s		125.8 s		120.7 s	
3	158.2 s		158.5 s		158.4 s	
4	120.0 s		120.3 s		119.4 s	
5	157.7 s		157.5 s		158.2 s	
6	109.0 d	6.68 br s	109.6 d	6.67 br s	109.0 d	6.74 br s
7	64.7 t	4.68 s	71.6 t	4.66 s	63.6 t	4.59 s
8	56.8 t	4.76 s	56.8 t	4.67 s	64.1 t	4.73 s
9	61.9 q	3.78 s	61.8 q	3.79 s	61.7 q	3.71 s
10	9.2 q	2.16 s	9.2 q	2.16 s	9.2 q	2.15 s
1′	65.3 t	4.52 br d (6.5)	65.4 t	4.52 br d (6.6)	65.3 t	4.52 br d (6.6)
2′	119.9 d	5.49 m (6.5, 1.5)	119.9 d	5.48 m (6.6, 1.5)	119.9 d	5.48 m (6.6,1.5)
3′	137.6 s		137.5 s		137.6 s	
4′	25.8 q	1.8 s	25.7 q	1.79 s	25.8 q	1.78 s
5′	18.2 q	1.74 s	18.2 q	1.75 s	18.2 q	1.73 s
1″			140.2 s		140.1 s	
2″			120.6 s		120.7 s	
3″			158.5 s		158.4 s	
4″			119.4 s		119.4 s	
5″			158.3 s		158.2 s	
6″			108.9 d	6.76 br s	109.0 d	6.74 br s
7″			63.4 t	4.61 s	63.6 t	4.59 s
8″			63.9 t	4.71 s	64.1 t	4.73 s
9″			61.8 q	3.7 s	61.7 q	3.71 s
10″			9.2 q	2.15 s	9.2 q	2.15 s
1‴			65.4 t	4.52 br d (6.6)	65.3 t	4.52 br d (6.6)
2‴			119.9 d	5.48 m (6.6, 1.5)	119.9 d	5.48 m (6.6,1.5)
3‴			137.5 s		137.6 s	
4‴			25.7 q	1.79 s	25.8 q	1.78 s
5‴			18.2 q	1.75 s	18.2 q	1.73 s

[a] Assignments were established by DEPT, HMQC, and HMBC spectra. [b] *J* values (in Hz) in parentheses.

together with a ^2J interaction between the methyl group protons at 2.16 ppm and the carbon at 120.0 ppm, allowed for the correct assignment of the latter signal to C-4 and that at 124.7 ppm to C-2. These assignments, particularly that of the C-8 methylene in the ^{13}C NMR spectrum, differ from those previously reported for zinniol. The chemical shift values for C-8 and C-1′ were originally reported at 65.3 and 56.6 ppm,[11] an assignment that can now be reversed on the basis of the results shown here. In a second report on the isolation of zinniol, chemical shift values were assigned by comparison with a model compound and shift calculations.[12] In this report, C-8 is assigned to the signal at 63.9 ppm; however, there was no signal reported with a chemical shift of 56−57 ppm. Because the identity of zinniol was reportedly confirmed by direct comparison with an authentic sample, it is difficult to explain the significant difference observed for the C-8 assignment and the absence of the signal at 56−57 ppm in the work described by these authors.

During the isolation of **1**, a second, less polar and non-phytotoxic, metabolite was obtained in pure form. Both the IR and ^{1}H NMR spectra of the new metabolite showed significant similarities with those of **1**, suggesting a related structure. The ^{1}H NMR of the new metabolite showed signals having chemical shifts almost identical to those of **1**; however, while individual signals for the protons in the dimethylallyloxy chain were observed [δ 5.48 (H-2′), 4.52 (H-1′), 1.79 (Me-C-3′) and 1.75 (Me-C-3′)], the rest of them appeared duplicated (Table 1). The spectrum showed the presence of two aromatic protons at δ 6.67 and 6.76, two methoxy groups at δ 3.79 and 3.70, and two methyl groups at δ 2.16 and 2.15, in addition to four singlets at δ 4.71, 4.67, 4.66, and 4.61 corresponding to the same number of oxygen-bearing methylene groups. The presence of two

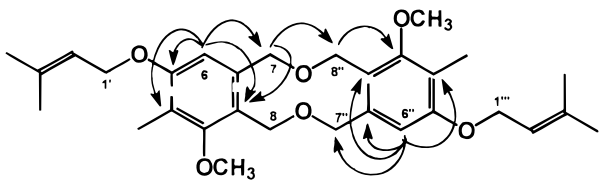


Figure 1. Selected HMBC correlations (H−C) observed for **2**.

aromatic rings in the structure of the new metabolite was confirmed by the 12 sp^2 carbon signals (10 quaternary and two tertiary) observed in the aromatic region of its ^{13}C NMR spectrum. These data, together with the molecular ion peak at *m*/*z* 496 ($C_{30}H_{40}O_6$) observed in the EIMS of the new metabolite and the absence of hydroxyl group bands in its IR spectrum, suggested a dimeric structure in which two zinniol units are connected via ether linkages. A detailed analysis of the HMQC and HMBC experiments performed on the new metabolite allowed for the assigment of all the signals observed in the ^{1}H and ^{13}C NMR spectra and permitted the establishment of the linkage positions between the two units of zinniol. HMQC data showed that the methylene protons at δ 4.61, 4.66, 4.67, and 4.71 correlated with carbons at δ 63.4, 71.6, 56.8, and 63.9, respectively. Similarly, the aromatic protons at δ 6.67 and 6.76 were shown to be bonded to their corresponding sp^2 carbons at δ 109.6 and 108.9. In the HMBC of the new metabolite (Figure 1), the aromatic proton at δ 6.67 (H-6) showed long-range couplings with C-5 (157.5 ppm), C-4 (120.3 ppm), and C-2 (125.8 ppm), while the one at 6.76 (H-6″) showed similar correlations with C-7″ (63.4 ppm), C-5″ (158.3 ppm), C-4″ (119.4 ppm), and C-2″ (120.6 ppm). Finally, the long-range interaction through oxygen between the methylene protons at δ 4.66 (H-7) and the C-8″ signal at δ 63.9 ppm provided the evidence required to assign a C-7-*O*-C-8″/C-8-*O*-C-7″ ether linkage between the two zinniol units and to identify the new metabolite as *bis*-7-*O*-8″.8-*O*-7″-zinniol (**2**).

The EIMS of a third, non-phytotoxic, purified metabolite showed a parent ion peak (*m*/*z* 496) identical with that of **2**, suggesting an isomeric structure for the two metabolites. Although a molecular weight of 496 corresponds to a molecular formula of $C_{30}H_{40}O_6$, both the ^{1}H and ^{13}C NMR spectra of the third metabolite showed signals corresponding to only 20 protons and 15 carbons, all with chemical shift values very similar to those of **1** (Table 1). These data suggested that the third metabolite was also a dimer, having two zinniol units bonded through two ether linkages, where the presence of one set of signals in both the ^{1}H and ^{13}C NMR spectra can be explained by a high symmetry in the molecule. The third metabolite was thus identified as *bis*-7-*O*-7″.8-*O*-8″-zinniol (**3**).

To confirm the structure of both metabolites, **1** was treated under Lewis acid ($ZnCl_2$)[13] conditions. Both dimers (**2** and **3**) were formed, and, after purification, their spectroscopic data and their TLC and HPLC behavior proved to be identical to those observed for the natural products.

To rule out the possibility of **2** and **3** being artifacts formed as a result of the extraction and purification process, a careful HPLC analysis of all extracts and purified fractions was carried out. Both dimers were detected in both the original organic crude extract and the low polarity fraction obtained after the solvent partition procedure. Although **2** and **3** could not be detected in the lyophylized culture filtrate due to interference by other components, their presence was clearly observed in the organic extracts obtained after treatment of the filtrate with DIAION HP-

20 resin. These experiments provided enough evidence to establish the natural origin of the two novel metabolites.

Several metabolites structurally related to zinniol have been reported from *A. porri*,[11,14−17] *A. solani*,[18,19] and *A. cichorii*.[12] These include structures in which the primary alcohol groups have been oxidized and condensed into a lactone (e.g., 6-[3′,3′-dimethylallyloxy]-4-methoxy-5-methylphthalide and zinnolide) or a lactam ring (e.g., zinnimidine and porritoxine), and others in which the isoprene side chain has been modified (e.g., homozinniol and zinndiol) or lost (e.g., zinnol). Reports on the production of dimeric metabolites by *Alternaria* species are limited, with the best known example being the C−C linked alterporriolides.[20] However, **2** and **3** represent the first examples of zinniol dimers linked through an ether bond, similar to that reported in the literature for lignans from *Illicium verum*.[21] Finally, the non-phytotoxic activity of **2** and **3** is in agreement with the existing reports that the hydroxymethyl groups are essential for the expression of biological activity.[19]

Experimental Section

General Experimental Procedures. IR spectra were measured on a FT-IR Nicolet Magna 750 or on a FT-IR Protege 460. EIMS were recorded at 70 eV on a JEOL-JMS-AX505HA mass spectrometer. GC analyses were performed on a Hewlett-Packard 5890 gas chromatograph under the following conditions: 0.5 μL of sample; Ultra 1 column (Hewlett-Packard, cross-linked methyl silicone gum, 25 m long, 0.32 mm i.d., 0.52 μm fill thickness); flow rate = 1 mL/min; temperature programs: (a) T_1 = 180 °C, T_2 = 290 °C; (b) T_1 = 70 °C, T_2 = 290 °C, gradient = 10 °C/min. HPLC analyses were carried out using a Milton Roy CM-4000 series chromatograph equipped with a Milton Roy SM-4000 UV detector and a Milton Roy CS-4100 integrator. Conditions for HPLC analyses were as follows: Hypersil ODS C_{18} column (Alltech, 5 micron, i.d. = 4.6 mm, length = 250 mm); gradient elution using MeCN (A) and H_2O (B) [Method a: A/B 10:90 to 90:10 (6 min.), flow rate = 1 mL/min, UV detector set at 254 nm; and Method b: A/B 10:90 to 80:20 (12 min), flow rate = 1.5 mL/min, UV set at 220 nm]. NMR spectra were recorded on Varian Unity-300 and -500 spectrometers, at 300 and/or 500 MHz for ^{1}H and 75 or 125 MHz for ^{13}C, using $CDCl_3$ as solvent and tetramethylsilane (TMS) as internal standard. Two-dimensional NMR experiments (DEPT, HMQC, and HMBC) were obtained on a Varian Unity-500 instrument. Industrial-grade solvents were glass-distilled in the laboratory prior to use. Flash column and vacuum liquid chromatography (VLC) purifications were carried out using Si gel (200−400 mesh and TLC grade, respectively) from Aldrich Chemical Co. Preparative TLC purifications were performed on E. M. Merck glass-coated Si gel plates (GF_{254}, 20 × 20 mm, 0.25 mm thickness).

Fungal Material. The strain of *A. tagetica* Shome and Mustafee (ATCC 58771) was maintained on V-8 juice agar (V8A) slant tubes, kept in the dark at 4 °C. The tubes were used to inoculate V8A-containing Petri dishes, on which the fungus was allowed to grow for 15 days at 26 °C, under 16/8 h of light/darkness conditions. A spore suspension (1 mL), prepared by adding 5 mL of sterile distilled water to a Petri dish, was used to inoculate 1 L of a liquid culture medium consisting of casamino acid enriched with marigold infusion (20 g of leaves/L of medium), and the fungus was cultured under stationary conditions for 28 days at 26 °C, under natural light (diurnal cycle).

Extraction and Isolation. The mycelial mat was separated from the culture filtrate by filtration through two layers of cheesecloth. The aqueous filtrate was extracted with EtOAc (× 3, 1:1, v:v) and the resulting organic layer concentrated under reduced pressure to yield the organic crude extract (ca. 0.29 g/L). A suspension of the organic crude extract (ca. 29 g) in H_2O/MeOH 9:1 (ca.100 mL of solution/300 mg of extract) was successively partitioned between hexane (× 3, 1:1, v:v) and EtOAc (× 3, 1:1, v:v), yielding the corresponding low (5.2 g) and medium (16.0 g) polarity fractions, respectively. VLC purification of the low polarity fraction, using an stepwise gradient elution (hexane/acetone, 98:2−100%, 13 fractions) yielded **1** (fractions 11−12, 500 mg) as yellow oil. Fraction 10 (662 mg) was further purified by flash column chromatography (CH_2Cl_2/acetone/MeOH, 95:3:2) to produce several fractions, one of which contained **2** (15.7 mg) in pure form. Pure **3** (20 mg) was obtained by preparative TLC (CH_2Cl_2/acetone, 95:5, × 3) purification.

Phytotoxicity Evaluation. Aqueous filtrates, before and after extraction, together with the corresponding organic and mycelium extracts, as well as crude and purified fractions, were tested for phytotoxic activity using the leaf-spot assay[8] on marigold leaves (0.2 mg/application).

Zinniol (1): yellow oil; R_f 0.22 (CH_2Cl_2/acetone/MeOH, 95:3:2); t_R (HPLC, Method a) 11.02 min; t_R (GC, method a) 10.09 min; t_R (GC, method b) 23.41 min; identified by comparison of spectroscopic data with those reported in the literature.[8,9] For the corrected assignments of the ^{13}C NMR data, see Table 1.

***bis*-7-*O*-8″.8-*O*-7″ Zinniol (2):** white solid; R_f 0.33 (CH_2Cl_2/acetone/MeOH, 95:3:2); t_R (HPLC, method a) 14.63 min; t_R (GC, method a) 9.69 min; t_R (GC, method b) 23.38 min; IR (film) ν_{max} 3000, 2940, 1610, and 1120 cm^{-1}; HREIMS *m/z* 496.2790 (calcd for $C_{30}H_{40}O_6$, 496.2825); LREIMS (70 eV) *m/z* 496 (M^+, 1.0), 428 ($M^+ - 68$, 3), 410 (5), 360 (5), 342 (12), 249 (10), 181 (100), 164 (12), 69 (22); ^{1}H and ^{13}C NMR data, see Table 1.

***bis*-7-*O*-7″.8-*O*-8″ Zinniol (3):** white solid; R_f 0.44 (CH_2Cl_2/acetone/MeOH, 95:3:2); t_R (HPLC, method a) 14.24 min; t_R (GC, method a) 9.69 min; t_R (GC, method b) 23.38 min; IR ($CHCl_3$) ν_{max} 3000, 2940, 1610 and 1120 cm^{-1}; EIMS (70 eV) *m/z* 496 (M^+, 14), 428 ($M^+ - 68$, 10), 360 (6), 342 (24), 248 (14), 181 (100), 164 (55), 69 (52); ^{1}H and ^{13}C NMR data, see Table 1.

Preparation of 2 and 3. Hydrated $ZnCl_2$ (42 mg, Aldrich Chemical Co.) was added to a solution of **1** (23 mg, 0.086 mmol) in CH_2Cl_2 (10 mL). The reaction mixture was stirred at room temperature for 66 h, quenched with water, and extracted with CH_2Cl_2. The organic solvent was evaporated and the resulting crude reaction product purified by flash column chromatography, using stepwise elution with CH_2Cl_2/acetone (97.5:2.5 and 95:5), to produce **2** (2.5 mg) and **3** (0.5 mg) in pure form.

Acknowledgment. We thank Isabel Chávez, Hector Ríos, Beatriz Quiroz, Rocío Patiño, Luis Velasco-Ibarra, and Javier Pérez-Flores (Instituto de Química, UNAM) for recording the NMR, IR, and MS spectra and Ileana Borges-Argáez (CICY) for her assistance in the initial microbiological work. Financial support for the project, through grants from PADEP-UNAM (Nos. 5356 and 5370) and CONACyT (4871-E9406), is also greatly appreciated. M.M.G.A. wishes to thank CONACyT for a graduate student fellowship.

References and Notes

(1) Templeton, G. E. In *Microbial Toxins VIII. Fungal Toxins*; Kadis, S., Ciegler, A., Ajl, S. J., Eds.; Academic: New York, 1972; Chapter 7, pp 169−192.
(2) Scheffer, R. P.; Briggs, S. P. In *Toxins in Plant Disease*; Durbin, R. D., Ed.; Academic: New York, 1981; Chapter 2, pp 1−17.

(3) Macko, V. In *Toxins and Plant Pathogenesis*; Daly, J. M., Deverall, B. J., Eds.; Academic: New York, 1983; p 41−74.
(4) García Aguirre, A.; Oviedo Zárate, A.; Ramírez Ramírez, R.; Vázquez Gómez, A. *Cultivo de la flor de Cempazúchitl*; Laboratorios Bioquímex S. A. de C. V.; México, 1992; p 7.
(5) Hotchkiss, E. S.; Baxter, L. W. *Plant Dis.* **1983**, *67*, 1288−1290.
(6) Cotty, P. J.; Mishagi, I. J. *Phytopathology* **1984**, *74*, 785−788.
(7) Cotty, P. J.; Mishagi, I. J.; Hine, R. B. *Phytopathology* **1983**, *73*, 1326−1328.
(8) Pena-Rodríguez, L. M.; Armingeon, N. A.; Chilton, W. S. *J. Nat. Prod.* **1988**, *51*, 821−828.
(9) Weaker activity was detected in other purified fractions, as well as in the medium polarity fraction (ethyl acetate). Isolation of the metabolites responsible for the activity observed is currently in progress; their structures will be published elsewhere.
(10) Starratt, A. N. *Can. J. Chem.* **1968**, *46*, 767−770.
(11) Suemitsu, R.; Ohnishi, K.; Horiuchi, M.; Kitaguchi A.; Odamura, K. *Phytochemistry* **1992**, *31*, 2325−2326.
(12) Stierle, A.; Hershenhorn, J.; Strobel, G. *Phytochemistry* **1993**, *32*, 1145−1149.
(13) Kim, S.; Chung, K. N.; Yang, S. *J. Org. Chem.* **1987**, *52*, 3917−3919.
(14) Suemitsu, R.; Ohnishi, K.; Horiuchi, M.; Morikawa Y. *Biosci. Biotech. Biochem.* **1992**, *56*, 986.
(15) Suemitsu, R.; Ohnishi, K.; Horiuchi, M.; Morikawa, Y.; Sakaki, Y.; Matsumoto, Y. *Biosci. Biotech. Biochem.* **1993**, *57*, 334−335.
(16) Suemitsu, R.; Ohnishi, K.; Morikawa, Y.; Ideguchi, I.; Uno, H. *Phytochemistry* **1994**, *35*, 603−605.
(17) Suemitsu, R.; Ohnishi, K.; Morikawa, Y.; Nagamoto, S. *Phytochemistry* **1995**, *38*, 495−497.
(18) Ichihara, A.; Tazaki, H.; Sakamura, S. *Agric. Bio. Chem.* **1985**, *49*, 2811−2812.
(19) Gamboa-Angulo, M. M.; Alejos-González, F.; Peña-Rodríguez, L. M. *J. Agric. Food Chem.* **1997**, *45*, 282−285.
(20) Suemitsu, R.; Horiuchi, K.; Kubota, M.; Okamatsu, T. *Phytochemistry* **1990**, *29*, 1509−1511.
(21) Sy, L.; Brown, G. D. *J. Nat. Prod.* **1998**, *61*, 987−992.

NP990422Q