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New Potato Micro-Tuber-Inducing Cyclohexene Compounds Related to Theobroxide from *Lasiodiplodia theobromae*

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Two new cyclohexene compounds related to theobroxide (3) were isolated from the mycelia of *Lasiodiplodia theobromae* OCS71. The structures of these compounds were determined to be (4*S*,5*S*)-4,5-dihydroxy-2-methylcyclohex-2-enone (1) and (3a*S*,4*R*,5*S*,7a*R*)-4,5-dihydroxy-7-methyl-3a,4,5,7a-tetrahydrobenzo[1,3]dioxol-2-one (2) by means of spectroscopic analyses and chemical correlation to 3. Compound 2 was shown to take up the carbonate ion to form a carbonic acid ester non-enzymatically. The compounds also showed potato micro-tuber-inducing activities at a concentration of 10^{-3} M, using a culture of single-node segments of potato stems *in vitro*.

Key words: theobroxide related compounds; potato micro-tuber-inducing activity; *Lasiodiplodia theobromae*

Lasiodiplodia theobromae (synonym *Botryodiplodia theobromae*) is a common pathogenic fungus found in the tropics and subtropics on a great variety of host plants.[1] This fungus causes various diseases in fruit and root crops during storage. The fungus is also known to produce jasmonic acid related compounds and some other metabolites that exhibit interesting biological activities. Theobroxide (**3**) and its related compound (**4**) produced by *L. theobromae* have induced potato micro-tuber formation under non-inductive conditions.[2–4] A biosynthetic study of theobroxide has recently shown that four acetate units were incorporated into it by a polyketide biosynthetic pathway.[5] *L. theobromae* is believed to produce other theobroxide-related compounds that possess biological activity. In our screening of *L. theobromae* OCS71, new cyclohexene compounds **1** and **2**, which are structurally related to theobroxide, were found in the mycelia of the fungus. We report here the isolation, structural elucidation, and biological activities of **1** and **2**. Non-enzymatic formation of compounds **2** and **4** from **3** in the presence of CO_3^{2-} is also reported.

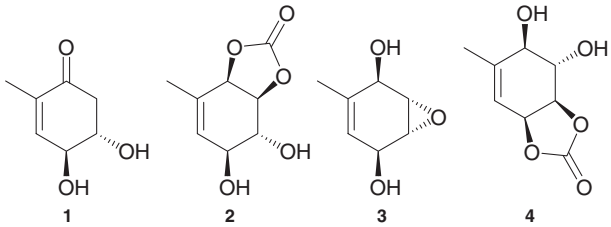

Fig. 1. New Compounds **1** and **2** and Their Related Compounds **3** and **4**.

Results and Discussion

The *L. theobromae* OCS71 fungus was statically grown in 500-ml flasks containing 200 ml of a 2% potato-dextrose medium at 25 °C in the dark for 21 d. Compounds **1** (colorless oil, 1.5 mg) and **2** (colorless oil, 6.1 mg) were isolated from an acetone extract of mycelia (DW = 139 g) of *L. theobromae* OCS71 by silica gel column chromatography and HPLC.

The molecular formula of **1** was established as $C_7H_{10}O_3$ on the basis of HREI-MS data. The value for the specific rotation was +128° (*c* 0.2, MeOH). The IR absorption peaks at 3452 and 1674 cm^{-1} respectively indicated the presence of hydroxyl groups and a carbonyl group. The UV absorption maximum at 231 nm suggested the presence of a conjugated enone group in the structure. The NMR spectral data for **1** resembled those of theobroxide. The ^{1}H- and ^{13}C-NMR spectra showed the presence of a methyl, sp^3 methylene, two sp^3 oxymethines, sp^2 methine, and two sp^2 quaternary carbons, including a ketone. Three degrees of unsaturation were inferred from the molecular formula of **1**. HMBC correlations between CH_3/C-1 and C-2, and H-3/C-2 suggested a double bond connecting a methyl group at C-2. The ^{1}H-^{1}H spin coupling system of H-4, H-5, and H_2-6 was deduced by COSY correlations between H-4/H-5 and H-5/H_2-6. Considering the molecular formula, IR spectral data, and the carbon

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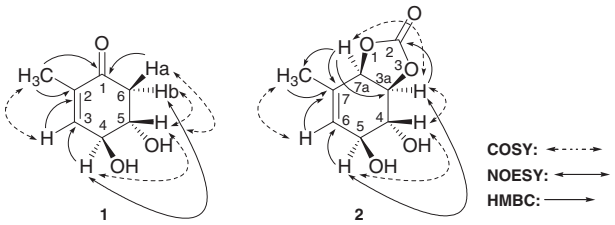

Fig. 2. Important 2D-NMR Correlations of **1** and **2**.

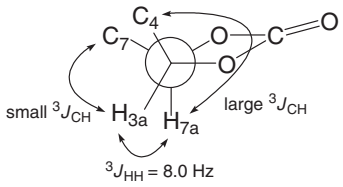

Fig. 3. Newman Projection of the Carbonic Acid Ester Moiety of **2**.

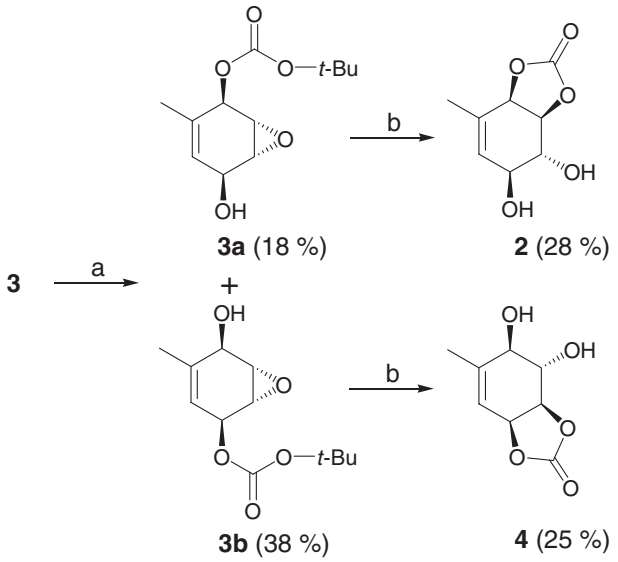

Fig. 4. Preparation of **2** and **4** from **3**.
a, $(Boc)_2O$, DMAP, CH_3CN, room temperature, 24 h. b, BF_3-OEt_2, CH_2Cl_2, $-40\,^\circ C$, 30 min.

chemical shift values at C-4 (δ 73.4) and C-5 (δ 73.6), each carbon was attached to a hydroxyl group. The linkage of the two partial structures just described was confirmed by HMBC correlations of H_2-6/C-1 and H-4/C-3, suggesting that the planar structure of **1** was 4,5-dihydroxy-2-methyl-cyclohex-2-enone. The coupling constant values between H-4/H-5 and H-5/H-6b were 7.7 and 11.4 Hz, respectively. NOESY correlations observed between H-4/H-6b suggested that H-4 and H-6b resided on the same face of **1**, while H-5 and H-6a resided on the other side of the molecule. To determine the absolute stereochemistry at C-4 and C-5 of **1**, compound **1** was converted to its dibenzoyl derivative (**1a**). The CD spectra of **1a** showed positive and negative Cotton effects at 239.0 and 221.5 nm, respectively. The absolute structure of **1** was therefore deduced to be (4*S*,5*S*)-4,5-dihydroxy-2-methyl-cyclohex-2-enone (Fig. 2).

The molecular formula of **2** [-12.1° (*c* 0.5, MeOH)] was determined to be $C_8H_{10}O_5$ by HREI-MS data, which is the same as that of theobroxide-related compound **4**. The IR absorption peaks at 3406 and 1784 cm^{-1} respectively indicated the presence of hydroxyl groups and an ester group. Four degrees of unsaturation were inferred from the molecular formula of **2**. The NMR spectral data for **2** were similar to those of **4**.[4] The ^{1}H- and ^{13}C-NMR spectra showed the presence of a methyl, four sp^3 oxymethines, sp^2 methine, and two sp^2 quaternary carbons, including a carbonyl carbon. The most significant difference in the ^{1}H-NMR spectra between these compounds was observed at around 5.0 ppm, *i.e.*, a broad doublet signal at δ_H 5.06 in **2** changed to a multiplet signal at δ_H 5.13 in **4**. HMBC correlations between H-6 and CH_3/C-7, and H-7a/C-7 and CH_3 suggested a double bond connecting a methyl group at C-7. The ^{1}H-^{1}H spin coupling sequence of H-3a, H-4, H-5, and H-7a was deduced by COSY correlations between H-3a/H-4 and H-7a, and H-4/H-5. HMBC correlations of H-5/C-6 suggested the presence of a cyclohexene ring in the structure of **2**. The proton chemical shift values for the oxymethine protons, H-7a (δ 5.06) and H-3a (δ 4.64), suggested that these protons were deshielded by an ester group. Considering the HMBC relationship of H-3a/C-2 and the unsaturation degree, a carbonic acid ester had been formed at C-3a and C-7a as a five-membered ring, enabling the planar structure of **2** to be determined as 4,5-dihydroxy-7-methyl-3a,4,5,7a-tetrahydrobenzo[1,3]-dioxol-2-one. To determine the absolute configurations at C-4 and C-5, compound **2** was converted to its dimethylaminobenzoate derivative (**2a**). The CD spectra for **2a** showed positive and negative Cotton effects at 324.5 nm and 301.0 nm, respectively, allowing the absolute configurations at C-4 and C-5 to be respectively deduced as *R* and *S*. To narrow down the possible structure of the carbonic acid ester moiety, a *J*-based configuration analysis[6] was conducted by the NMR spectral data. HMBC data revealed that ^3J (H-3a/C-7) and ^3J (H-7a/C-4) were small and large, respectively (Fig. 3). The ^3J (H-3a/H-7a) value of 8.0 Hz indicated that the relative configuration of H-3a and H-7a was *cis*. The NOESY correlation of H-5/H-3a demonstrated that H-5 and H-3a resided on the same face of the molecule. Accordingly, the absolute structure of **2** was determined to be (3a*S*,4*R*,5*S*,7a*R*)-4,5-dihydroxy-7-methyl-3a,4,5,7a-tetrahydrobenzo[1,3]dioxol-2-one (Fig. 2). To confirm this structure of **2**, chemical conversion of **3** into **2** was conducted (Fig. 4). Compound **3** was reacted with di-*t*-butyl dicarbonate to introduce a carbonic acid ester into the structure,[7,8] yielding **3a** and **3b** as a mixture. After HPLC separation, compounds **3a** and **3b** were cyclized to afford expected

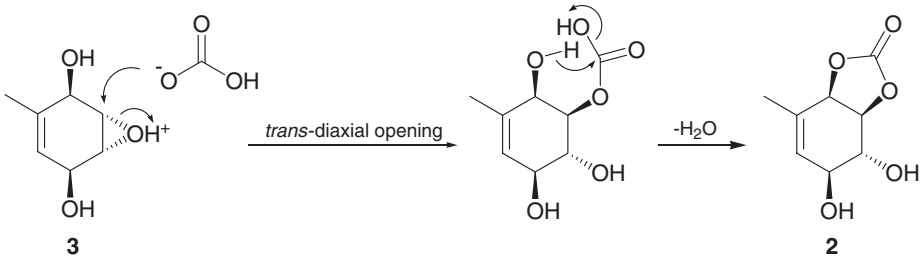

Fig. 5. Proposed Biosynthetic Reaction from **3** to **2**.

bicyclic carbonates **2** and **4**, respectively. The EI-MS and ^{1}H-NMR data for chemically prepared **2** were consistent with those of **2** isolated from *L. theobromae*, supporting the absolute structure of **2** as shown in Fig. 2.

Our previous study showed that a theobroxide (**3**) was formed by a polyketide biosynthetic pathway,[5] and that the carbon of the carbonic acid ester in the carbonyldioxy derivative (**4**) originated from the carbonyl carbon of an acetate. We examined whether the conversion of **3** into **2** and **4** occurred enzymatically in the reaction mixture containing Na_2CO_3. It was found that **2** and **4** were produced with or without a cell-free extract prepared from *L. theobromae*. Accordingly, **2** and **4** were postulated to be non-enzymatically generated by CO_3^{2-}, which was enzymatically formed from carboxylic acids such as an acetate within the culture of *L. theobromae*. Taking into consideration the stereochemistry of **2**, **3**, and **4**, one of the epoxycarbons was probably attacked by the HCO_3^- anion *via trans*-diaxial opening, and the resulting monocarbonate intermediate was further converted to the carbonyl dioxy compound with the elimination of H_2O (Fig. 5).[9,10]

The potato micro-tuber-inducing activity of **1** and **2** was evaluated by the method of Koda *et al.*[11] While the activity of the theobroxide (**3**) was observed at a concentration of 10^{-5} M, the activity of **1** and **2** appeared at a concentration of 10^{-3} M, showing that both compounds had weaker activity than **3**. No obvious difference between potato micro-tubers treated with **1** and **2** was apparent in the experiment.

Materials and Methods

General. Data were obtained with the following instruments: IR, Hitachi 270-30 Infrared spectrophotometer; optical rotation, Jasco DPI-370 polarimeter; NMR, Bruker AMX-500 FT-NMR and Jeol JNM-EX 270 FT-NMR spectrometers; FD-, EI- and HREI-MS, Jeol JMS SX-102A mass spectrometer; UV, Hitachi U-2800 spectrophotometer; CD, Jasco J-720W spectropolarimeter.

Isolation. Lasiodiplodia theobromae OCS71 was grown statically in fifty 500-ml flasks each containing 200 ml of a 2% potato-dextrose medium at 25 °C in the dark for 21 d. The mycelia were washed with distilled water, filtered, and dried at room temperature in the dark (DW = 139 g). The dried mycelia were extracted with acetone (21 × 2), and concentrated *in vacuo* to give an aqueous residue. This was extracted with EtOAc (350 ml × 5). The extract was dried over Na_2SO_4 and evaporated to give a crude extract (1.86 g). This was subjected to silica gel column chromatography (Kanto Chemical, silica gel 60N, 200 g; MeOH:$CHCl_3$ = 0:100, 3:97, 10:90, 20:80, v/v). The fraction from MeOH:$CHCl_3$ = 10:90 (v/v) was applied to silica gel column chromatography (Kanto Chemical, silica sel 60N, 30 g; MeOH:$CHCl_3$ = 10:90), and then purified by HPLC (Inertsil ODS, 20 × 250 mm; MeOH:H_2O = 10:90, v/v, 5 ml/min, $A_{210\,nm}$) to afford **1** (1.5 mg) and **2** (6.1 mg).

Compound **1**: $[\alpha]_D^{22}$ +128° (*c* 0.21, MeOH); EI-MS *m/z* (rel. int., %): 142[M]$^+$ (8), 124 (5), 98 (100), 70 (38), 69 (35), 43 (17), 41 (15); HREI-MS *m/z*: 142.0616 [M]$^+$ (calcd. for $C_7H_{10}O_3$: 142.0629); ^{1}H-NMR (500 MHz, CD_3OD) δ (ppm): 6.64 (1H, m, H-3), 4.21 (1H, ddq, 7.7, 2.1, 2.1 Hz, H-4), 3.81 (1H, ddd, *J* = 11.4, 7.7, 4.6 Hz, H-5), 2.71 (1H, dd, *J* = 15.9, 4.6 Hz, H-6a), 2.40 (1H, dd, *J* = 15.9, 11.4 Hz, H-6b), 1.72 (3H, m, 2-CH_3); ^{13}C-NMR (125 MHz, CD_3OD) δ (ppm): 199.7 (C-1), 148.0 (C-3), 136.5 (C-2), 73.6 (C-5), 73.4 (C-4), 45.5 (C-6), 15.3 (2-CH_3); IR ν_{max} cm^{-1}: 3452, 2924, 1674, 1438, 1368, 1078, 982, 898; UV (MeOH) λ_{max} nm (ε): 231 (5980).

Compound **2**: $[\alpha]_D^{22}$ −12.1° (*c* 0.49, MeOH); EI-MS *m/z* (rel. int., %): 186[M]$^+$ (8), 124 (11), 113 (9), 100 (100), 95 (33), 71 (54), 55 (33), 41 (32); HREI-MS *m/z*: 186.0525 [M]$^+$ (calcd. for $C_8H_{10}O_5$: 186.0528); ^{1}H-NMR (500 MHz, CD_3OD) δ (ppm): 5.69 (1H, m, H-6), 5.06 (1H, br d, *J* = 8.0 Hz, H-7a), 4.64 (1H, dd, *J* = 8.0, 9.0 Hz, H-3a), 3.95 (1H, m, H-5), 3.44 (1H, dd, *J* = 9.0, 9.0 Hz, H-4), 1.83 (3H, m, 7-CH_3); ^{13}C-NMR (125 MHz, CD_3OD) δ (ppm): 155.9 (C-2), 132.7 (C-6), 129.7 (C-7), 80.3 (C-3a), 78.5 (C-7a), 74.8 (C-4), 69.7 (C-5), 19.5 (7-CH_3); IR ν_{max} cm^{-1}: 3406, 2924, 1784, 1440, 1365, 1174, 1062.

*Preparation of the dibenzoyl derivative (**1a**).* Compound **1** (1.5 mg, 0.01 mmol) and benzoyl chloride (25 µl, 0.2 mmol) were dissolved in pyridine (1 ml). The reaction mixture was stirred at room temperature for

24 h and then treated with MeOH (1 ml). After removing the solvent *in vacuo*, the residue was purified by HPLC (Inertsil ODS, 20 × 250 mm; MeOH:H_2O = 80:20, v/v, 5 ml/min, $A_{280\,nm}$) to give the dibenzoate of **1** (**1a**; 3.1 mg, 89%). **1a**: EI-MS *m/z* (rel. int., %): 350$[M]^+$ (2), 229 (6), 228 (20), 105 (100), 77 (22); UV (MeOH) λ_{max} nm (ε): 232 (21700); ^{1}H-NMR (270 MHz, $CDCl_3$) δ (ppm): 7.98 (4H, m), 7.52 (2H, m), 7.40 (4H, m), 6.70 (1H, m), 6.04 (1H, m), 5.71 (1H, ddd, J = 10.4, 7.8, 4.8 Hz), 3.17 (1H, dd, J = 16.5, 4.8 Hz), 2.75 (1H, dd, J = 16.5, 10.4 Hz), 1.88 (3H, m).

*Preparation of the dimethylaminobenzoyl derivative (**2a**).* Compound **2** (1 mg, 0.005 mmol) and 4-(dimethylamino)benzoyl chloride (19.9 mg, 0.11 mmol) were dissolved in pyridine (1 ml). The reaction mixture was stirred at room temperature for 24 h and then treated with MeOH (1 ml). After removing the solvent *in vacuo*, the residue was purified by HPLC (Inertsil ODS, 20 × 250 mm; CH_3CN:H_2O = 70:30, v/v, 5 ml/min, $A_{280\,nm}$) to give the dibenzoate of **2** (**2a**; 0.6 mg, 25%). **2a**: EI-MS *m/z* (rel. int., %): 480$[M]^+$ (8), 316 (29), 164 (51), 148 (100), 120 (11); UV (MeOH) λ_{max} nm (ε): 316 (37200); ^{1}H-NMR (270 MHz, $CDCl_3$) δ (ppm): 7.83 (4H, m), 6.61 (4H, m), 5.91 (1H, m), 5.68 (1H, dd, J = 7.3, 7.3 Hz), 5.62 (1H, m), 5.07 (1H, m), 4.98 (1H, dd, J = 7.3, 7.3 Hz), 3.00 (6H, m), 2.99 (6H, m), 1.97 (3H, m).

*Chemical conversion of theobroxide (**3**) into **2** and **4**.* Theobroxide **3** (100 mg, 0.7 mmol) and $(Boc)_2O$ (152.6 mg, 0.7 mmol) were dissolved in CH_3CN (5 ml).[7] DMAP (7 mg) was added, and the reaction mixture was stirred at room temperature for 24 h. After removing the solvent *in vacuo*, the residue was purified by silica gel column chromatography (Kanto Chemical, silica gel 60N, 20 g; MeOH:$CHCl_3$ = 3:97, v/v) and HPLC (Inertsil ODS, 20 × 250 mm; CH_3CN:H_2O = 30:70, v/v, 8 ml/min, $A_{210\,nm}$) to give **3a** (30.0 mg, 0.12 mmol, 18%; retention time, 63.4 min) and **3b** (64.6 mg, 0.27 mmol, 38%; retention time, 65.7 min). Compound **3a** (25.2 mg, 0.10 mmol) and BF_3-OEt_2/CH_2Cl_2 (0.2 mM, 0.5 ml, 0.10 mmol) were dissolved in CH_2Cl_2 (3 ml).[8] The reaction mixture was stirred at −40 °C for 30 min. The reaction solution was poured into saturated $NaHCO_3$ aq. (6 ml) and successfully extracted with CH_2Cl_2 and EtOAc (6 ml × 3). Each extract was washed with saturated brine (5 ml × 1) and dried over Na_2SO_4. The EtOAc extract was evaporated and the resulting residue (5.7 mg) was purified by HPLC (Inertsil ODS, 20 × 250 mm; MeOH:H_2O = 10:90, v/v, 5 ml/min, $A_{210\,nm}$) to give compound **2** (5.3 mg, 0.03 mmol, 28%). Compound **4** was converted from **3** in the same manner above (yield, 25%). The specific rotation values, EI-MS and ^{1}H-NMR spectral data for **2** and **4** were identical with those of both compounds isolated from *L. theobromae*.[4] Compound **3a**: FD-MS *m/z*, 242 $[M]^+$; ^{1}H-NMR (500 MHz, $CDCl_3$) δ (ppm): 5.41 (1H, m, H-2), 5.15 (1H, m, H-3), 4.16 (1H, br d, J = 8.9 Hz, H-6), 3.38 (1H, m, H-5), 3.30 (1H, m, H-4), 1.81 (3H, m, H-7), 1.45 [9H, s, $C(CH_3)_3$]. Compound **3b**: FD-MS *m/z*, 242 $[M]^+$; ^{1}H-NMR (500 MHz, $CDCl_3$) δ (ppm): 5.64 (1H, m, H-2), 5.05 (1H, m, H-6), 4.35 (1H, m, H-3), 3.33 (2H, m, H-4, 5), 1.72 (3H, m, H-7), 1.46 [9H, s, $C(CH_3)_3$].

In vitro *conversion of **3** into **2** and **4**.* Frozen mycelia of *L. theobrome* (1.83 g) were ground in liquid nitrogen and suspended in 20 ml of a 100 mM phosphate buffer (pH 6.4, 1 mM DTT). After centrifuging the suspension (13,000 × *g*), the resulting supernatant was used for a cell-free experiment. *In vitro* conversion was carried out in a final volume of 6 ml containing the 100 mM phosphate buffer (pH 6.4), 1 mM theobroxide, and 1 mM Na_2CO_3 with or without 1 ml of the cell-free extract. The reaction mixture was incubated at 25 °C. Seven days after starting the reaction, the reaction mixture (6 ml) was extracted with the same volume of EtOAc and then evaporated. The residue was dissolved in a small amount of MeOH and subjected to HPLC (Inertsil ODS, 20 × 250 mm, MeOH:H_2O = 10:90, 5 ml/min, $A_{210\,nm}$). The retention times of the products in this experiment and of authentic samples were same (**2**, retention time, 7.5 min: **4**, retention time, 7.0 min). The molecular ion peaks of both products (*m/z* 186) were observed by EI-MS.

Bioassay. The potato micro-tuber-inducing activity was assayed by cultures of single–node segments of the potato stem *in vitro* as previously described.[9]

Acknowledgments

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References

1) Aldridge, D. C., Galt, S., Giles, D., and Turner, W. B., Metabolites of *Lasiodiplodia theobromae*. *J. Chem. Soc. (C)*, 1623–1627 (1971).
2) Nakamori, K., Matsuura, H., Yoshihara, T., Ichihara, A., and Koda, Y., Potato micro-tuber inducing substances from *Lasiodiplodia theobromae*. *Phytochemistry*, **45**, 835–839 (1994).
3) Yoshihara, T., Ohmori, F., Nakamori, K., Amanuma, M., Tsutsumi, T., Ichihara, A., and Matsuura, H., Induction of plant tubers and flower buds under noninducing photoperiod condition by a natural product, theobroxide. *J. Plant Growth Regul.*, **19**, 457–461 (2000).
4) Matsuura, H., Obara, N., Chisaka, N., Ichihara, A., and Yoshihara, T., Novel cyclohexene compound from *Lasiodiplodia theobromae* IFO 31059. *Biosci. Biotechnol. Biochem.*, **62**, 2460–2462 (1998).
5) Peng, L., Takei, R., Takahashi, K., and Nabeta, K., Biosynthesis of theobroxide and its related compounds, metabolites of *Lasiodiplodia theobromae*. *Phytochemistry*, **68**, 819–823 (2007).

6) Matsumori, N., Kaneno, D., Murata, M., Nakamura, H., and Tachibana, K., Stereochemical determination of acyclic structures based on carbon-proton spin-coupling constants. A method of configuration analysis for natural products. *J. Org. Chem.*, **64**, 866–876 (1999).
7) Grubbs, A., Artman III, G., and Williams, R., Concise syntheses of the 1,7-dihydropyrano[2,3-g]indole ring system of the stephacidins, aspergamides and norgeamides. *Tetrahedron Lett.*, **46**, 9013–9016 (2005).
8) McDonald, F. E., Bravo, F., Wang, X., Wei, X., Toganoh, M., Rodriguez, J. R., Do, B., Neiwert, W. A., and Hardcastle, K. I., Endo-oxacyclizations of polyepoxides: biomimetic synthesis of fused polycyclic ethers. *J. Org. Chem.*, **67**, 2513–2523 (2002).
9) Sakamura, S., Nabeta, K., Shoichi, Y., and Ichihara, A., Minor constituents from *Phyllosticta* sp. and their correlation with epoxydon (phyllosinol). *Agric. Biol. Chem.*, **39**, 403–407 (1975).
10) Nabeta, K., Ichihara, A., and Sakamura, S., Biosynthesis of epoxydon and related compounds by *Phyllosticta* sp. *Agric. Biol. Chem.*, **39**, 409–413 (1975).
11) Koda, Y., and Okazawa, Y., Detection of potato tuber-inducing activity in potato leaves and old tubers. *Plant Cell Physiol.*, **29**, 969–974 (1988).