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 (4S,5S)-4,5-dihydroxy-2-methylcyclohex-2-enone (1) and (3aS,4R,5S,7aR)-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.

**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$_7$H$_{10}$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 1H- and 13C-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 1H–1H spin coupling system of H-4, H-5, and H-6 was deduced by COSY correlations between H-4/H-5 and H-5/H-2. Considering the molecular formula, IR spectral data, and the carbon

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The absolute structure of Cotton effects at 239.0 and 221.5 nm, respectively. 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 between H-4/H-5 and H-5/H-6b were deduced as (3a,4,5,7a-tetrahydrobenzo[1,3]dioxol-2-one). The proton chemical shift values for the oxymethine H-3a,4,5,7a-tetrahydrobenzo[1,3]dioxol-2-one. 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 (45,5S)-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_{8}H_{10}O_{2} by HREI-MS data, which is the same as that of thebromine-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.\(^{6}\) 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 dimethylaminobenzate 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 \(^3\)J (H-3a/C-7) and \(^3\)J (H-7a/C-4) were small and large, respectively (Fig. 3). The \(^3\)J (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 (3aS,4R,5S,7aR)-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...
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 carbonyl-dioxy 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 \(\text{Na}_2\text{CO}_3\). 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 \(\text{CO}_3^{2-}\), which was enzymatically formed from carboxylic acids such as an acetate within the culture of the fungus. As shown in Fig. 2, 2 and 4 had weaker activity than 3, and the 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 2071 spectrophotometer; CD, Jasco J-720W spectropolarimeter.

Isolation. \(L.\ theobromae\) 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 x 2), and concentrated in vacuo to give an aqueous residue. This was extracted with \(\text{EtOAc}\) (350 ml x 5). The extract was dried over \(\text{Na}_2\text{SO}_4\) 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, 0:90, 0:10, 20:80, v/v). The fraction from MeOH:CHCl\(_3\) = 10:90 (v/v) was applied to silica gel column chromatography (Kanto Chemical, silica gel 60N, 30 g; MeOH:CHCl\(_3\) = 10:90), and then purified by HPLC (Inertsil ODS, 20 x 250 mm; MeOH:\(\text{H}_2\text{O}\) = 10:90, v/v, 5 ml/min, \(\lambda_{210\text{nm}}\)) to afford 1 (1.5 mg) and 2 (6.1 mg).

Compound 1: \([\alpha]_{D}^{22} +128^\circ\) (c 0.21, MeOH); EI-MS \(m/z\) (rel. int., %): [142\(\text{M}^+\)]\(^+\) (8), 124 (5), 98 (100), 70 (38), 69 (35), 43 (17), 41 (15); HREI-MS \(m/z\) = 142.0616; \[^13\]C-NMR (125 MHz, CD\(_2\)OD) \(\delta\) ppm: 185.8 (C-2), 140.4 (C-1), 124.3 (11), 113.6 (9), 100.3 (8), 95.5 (33), 71 (54), 55 (33), 41 (32); HREI-MS \(m/z\) = 186.0525 (rel. int., %): [186\(\text{M}^+\)]\(^+\) (8), 124 (11), 113 (9), 100 (100), 95 (33), 71 (54), 55 (33), 41 (32); HREI-MS \(m/z\) = 186.0525 [\(\text{M}^+\)]\(^+\) (calc. for \(\text{C}_{10}\text{H}_{10}\text{O}_{5}\): 186.0528); \(^1\)H-NMR (500 MHz, CD\(_2\)OD) \(\delta\) 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\(_2\)OD) \(\delta\) 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 \(\nu_{\max }\) cm\(^{-1}\): 3406, 2924, 1784, 1440, 1365, 1078, 982, 898; UV (MeOH) \(\lambda_{\max }\) nm (\(\varepsilon\)) = 231 (5980).

Compound 2: \([\alpha]_{D}^{22} -12.1^\circ\) (c 0.49, MeOH); EI-MS \(m/z\) (rel. int., %): [\(\text{M}^+\)]\(^+\) (8), 124 (11), (13%); 100 (100), 95 (33), 71 (54), 55 (33), 41 (32); HREI-MS \(m/z\) = 186.0525 (rel. int., %): [186\(\text{M}^+\)]\(^+\) (8), 124 (11), 113 (9), 100 (100), 95 (33), 71 (54), 55 (33), 41 (32); HREI-MS \(m/z\) = 186.0525 [\(\text{M}^+\)]\(^+\) (calc. for \(\text{C}_{11}\text{H}_{13}\text{O}_{5}\): 186.0528); \(^1\)H-NMR (500 MHz, CD\(_2\)OD) \(\delta\) 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\(_2\)OD) \(\delta\) 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 \(\nu_{\max }\) 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 \(\mu\)l, 0.2 mmol) were dissolved in pyridine (1 ml). The reaction mixture was stirred at room temperature for 22 h. The reaction mixture was concentrated in vacuo, dissolved in MeOH (100 \(\mu\l\), 100 \(\mu\l\) of 1b was added, and the mixture was stirred at room temperature for 1 h. The mixture was passed through a silica gel column (MeOH:H\(_2\)O = 10:90, v/v, 5 ml/min, \(\lambda_{210\text{nm}}\)) to afford 1a (1.5 mg) and 1b (6.1 mg).
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:H₂O = 80:20, v/v, 5 ml/min, A₂₈₀nm) to give the dibenzoate of 1 (1a; 3.1 mg, 89%).

Preparation of the dimethylbenzoyl 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, the residue was purified by HPLC (Inertsil ODS, 20 × 250 mm; CH₃CN:H₂O = 70:30, v/v, 5 ml/min, A₂₈₀nm) to give the dibenzoate of 2 (2a; 0.6 mg, 25%).

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 CH₃CN (5 ml). 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:CHCl₃ = 3:97, v/v) and HPLC (Inertsil ODS, 20 × 250 mm; CH₃CN:H₂O = 30:70, v/v, 8 ml/min, A₂₁₀nm) 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 BF₃·OEt₂/CH₂Cl₂ (0.2 ml, 0.5 ml, 0.10 mmol) were dissolved in CH₂Cl₂ (3 ml). The reaction mixture was stirred at −40 °C for 30 min. The reaction solution was poured into saturated NaHCO₃ aq. (6 ml) and successfully extracted with CH₂Cl₂ and EtOAc (6 ml × 3). Each extract was washed with saturated brine (5 ml × 1) and dried over Na₂SO₄. The EtOAc extract was evaporated and the resulting residue (5.7 mg) was purified by HPLC (Inertsil ODS, 20 × 250 mm; MeOH:H₂O = 10:90, v/v, 5 ml/min, A₂₁₀nm) 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 ¹H-NMR spectral data for 2 and 4 were identical with those of both compounds isolated from L. theobromae. Compound 3a: FD-MS m/z, 242 [M⁺]; ¹H-NMR (500 MHz, CDCl₃) 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(CH₃)₃]. Compound 3b: FD-MS m/z, 242 [M⁺]; ¹H-NMR (500 MHz, CDCl₃) 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(CH₃)₃].

In vitro conversion of 3 into 2 and 4. Frozen mycelia of L. theobromae (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 Na₂CO₃ 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:H₂O = 10:90, 5 ml/min, A₂₁₀nm). 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

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References


