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Isolation of an α-methylene-γ-butyrolactone derivative, a toxin from the plant pathogen *Lasiodiplodia theobromae*

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Abstract

Lasiodiplodia theobromae is known as a multi-infectious microorganism that causes considerable crop damage, particularly to tropical fruits. When the fruits are infected by *L. theobromae*, the typical symptom is the appearance of black spots on the surface of the infected fruit. When injected in to the peel of banana, the culture filtrate of *L. theobromae* induced formation of black spots. The structure of the isolated compound responsible for this effect was determined to be (3*S*,4*R*)-3-carboxy-2-methylene-heptan-4-olide on the basis of analysis of MS, IR, and ^{1}H and ^{13}C NMR spectroscopic data, including HMQC, HMBC, and ^{1}H–^{1}H COSY experiments. The active compound was not only isolated from the culture filtrate derived from potato dextrose medium, but also from the extract of infected peels of bananas.

Keywords: *Lasiodiplodia theobromae*; Banana; *Musa acuminata*; Musaceae; α-methylene-γ-butyrolactone; Pathogenic toxin

1. Introduction

Lasiodiplodia theobromae spoils many farm products in tropical regions and is one of the main pathogens responsible for the decay of fruits, and as a result has a serious economical impact on the agricultural industry (Yaguchi, 1996). The search for toxins produced by *L. theobromae* may allow for a better understanding of disease pathogenesis and facilitate screening programs for disease-resistant plants. In this paper, we report the isolation and structural elucidation of a novel toxin, (3*S*,4*R*)-3-carboxy-2-methylene-heptan-4-olide (**1**), together with decumbic acid (**2**) from the culture filtrate of *L. theobromae* (Figs. 1 and 2).

2. Results and discussion

The fungus isolated from rotted mango branches in Miyako islands, Okinawa, Japan, was identified as *L. theobromae* based on the spore shape. The ethyl acetate extract of the culture filtrate of this fungus resulted in a rot on the surface of banana (*Musa acuminata*) when injected with a micro syringe. Separation of the active compounds was performed by a series of silica gel column chromatographic steps, with final purification performed by HPLC to afford an active compound **1** and an inactive compound **2**. When the purified **1** was applied to HPLC under the same condition **2**, the HPLC chromatograms again showed two peaks at $t_R = 17.4$ min and $t_R = 13.4$ min corresponding to **1** and **2**, respectively, indicating that isomerization of **1** to **2** had occurred during the HPLC analysis; **1** was further purified by recrystallization.

Compound **2** had an optical rotation value of +44.3° (*c* 0.47, $CHCl_3$), but did not exhibit rot-inducing

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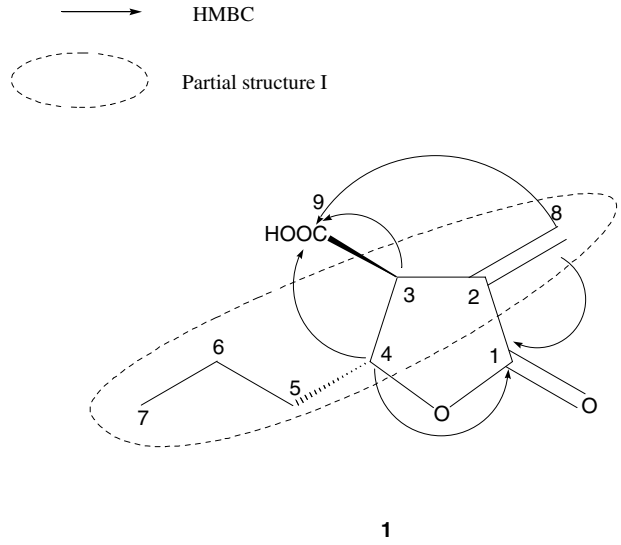


1

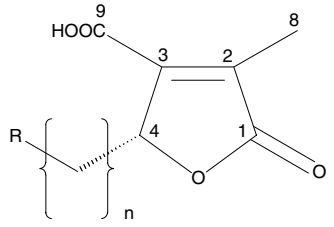
Fig. 1. Partial structure of I and important HMBC correlations of compound **1**.

activity. If chemical structure **2** was determined to be decumbic acid (Fig. 1) on the basis of analysis of the MS, IR, ^{1}H and ^{13}C NMR spectroscopic data, including HMQC, HMBC, and ^{1}H–^{1}H COSY experiments. Decumbic acid (**2**) was first identified as a metabolite of *Penicillium decumben* (McCorkindale et al., 1978). The absolute configuration of C-4 of decumbic acid (**2**) was anticipated to be *R*, because 13-acetoxylichesterinic acid (**3**), isomuronic acid (**4**), and neuropogolic acid (**5**), the homologues of **2** which possess the *R*-configuration (Fig. 2), exhibited a positive specific rotation value (Ghogomu and Bodo, 1982), whereas (*S*)-(−)-lichesterinic acid (**6**) exhibited a negative specific rotation value (Boll, 1968). Recently, the synthesis of optically active (4*R*)-decumbic acid was reported using (*R*)-1,2-pentanediol as a starting material, and the synthetic and natural decumbic acid had the same (+)-sign of specific rotation value (Toshima, 2001).

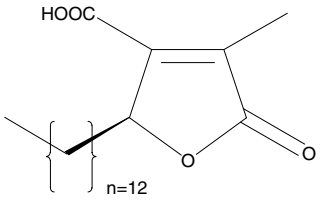
Compound **1** was obtained as white crystals, whose molecular formula was determined to be $C_9H_{12}O_4$ $[M]^+$ by FD-HR-MS. The IR spectrum showed absorption of carbonyl at 1748 cm^{-1}, indicating the presence of a γ-lactone structure. The absorption of the carbonyl at 1702 cm^{-1} and hydroxyl at 3300 cm^{-1} attributed to the carboxyl group was also observed. The analysis of ^{1}H, ^{13}C NMR, DEPT, and HMQC spectra revealed one terminal methyl (δ_H 0.91 and δ_C 14.1), two methylenes (δ_H 1.43 and δ_C 18.6; δ_H 1.19 and δ_C 38.2), two methines (δ_H 3.56 and δ_C 50.0; δ_H 4.76 and δ_C 79.1), two olefinic carbons (–C=CH_2, δ_H 5.96, 6.40 and δ_C 126.4; δ_C 132.8) and, two carbonyl carbons (δ_C 168.5; 174.7) (Table 1). The ^{1}H–^{1}H COSY spectrum established the partial structure (I, Fig. 1) from the cross peaks of CH_3/H-6/H-5/H-4/H-3/H-8. The partial structure (I) and two carbonyl groups were connected by HMBC correlations to give **1** as in Fig. 3.

1 is an α-methylene-γ-butyrolactone derivative and has the similar structure with that of **2**. The only difference between **1** and **2** is that the double bond in the former is exocyclic whereas the latter is endocyclic; **2** is thought to be derived from the isomerization of **1**, since α-methylene-γ-butyrolactones are slowly isomerized when they are kept in solution for a long time or easily isomerized by heating in Ac_2O under reflux (Ghogomu and Bodo, 1982; Huneck et al., 1986). We did convert **1** into **2** by heating a solution of **1** in Ac_2O under reflux (Scheme 1).

In order to determine whether the two methine protons in the lactone ring are in the *trans* or *cis*-position, the ^{1}H NMR spectrum of **1** was compared with those of *dl*-protolichesterinic acid (**7**) (Martin et al., 1974), nephrosterinic acid (**8**) (Carlson and Oyler, 1976), methylenolactocin (**9**) (Kongsaeree et al., 2001; Park et al., 1987), **10** (Kuhajda et al., 2000), (−)-allo-pertusaric acid (**11**) (Huneck et al., 1986), **12** (Carlson and Oyler, 1976), and **13** (Carlson and Oyler, 1976) (Fig. 3). The ^{1}H NMR resonances of **1** were in excellent agreement with the corresponding data of *trans*-conformational compounds (**7**–**10**) (Table 2). Thus, the two methine protons of **1** were determined to be in the *trans*-position. For the determination of the absolute configuration, **1** was con-



decumbic acid (**2**): n=2, R=Me
13-acetoxylichesterinic acid (**3**): n=12, R=CH_2OAc
isomuronic acid (**4**): n=12, R=CH_2Ac
neuropogolic acid (**5**): n=12, R=$CH_2CH(OH)Me$



lichesterinic acid (**6**)

Fig. 2. Structures of decumbic acid (**2**) and other homologous compounds, **3**–**6**.

Table 1
NMR spectral data (^{1}H, 270 MHz and ^{13}C, 67.5 MHz, $CDCl_3$) for compound **1**[a]

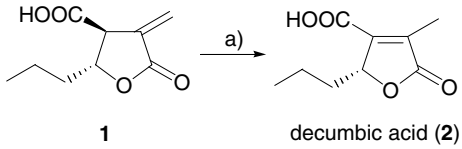
		C-1	168.5
H-3	3.56 (1H, *m*)	C-2	132.8
H-4	4.76 (1H, *m*)	C-3	50.0
H-5	1.69 (2H, *m*)	C-4	79.1
H-6	1.43 (2H, *m*)	C-5	38.2
H-7	0.91 (3H, *t*, *J* = 7.4)	C-6	18.6
H-8a	5.96 (1H, *d*, *J* = 3.0)	C-7	14.1
H-8b	6.40 (1H, *d*, *J* = 2.8)	C-8	126.4
		C-9	174.7

[a] Assignments confirmed by two-dimensional experiments (COSY, HMQC and HMBC).

verted to decumbic acid according to Scheme 1, and the specific rotation value of the converted decumbic acid from **1** was in good accordance with that of decumbic acid (**2**). Thus, the absolute configuration of C-3 and -4 were determined to have (*S*) and (*R*) stereochemistry, respectively (Fig. 3).

Black spot inducing activities of the isolated compounds were examined using bananas. Compound **1** was found to cause clearly visible rot on the surface of the peel of banana when 18 μg of the compound was injected, while an identical quantity of compound **2** did not produce any rot when injected (Fig. 4). The main structural difference between **2** and **1** was the position of olefin as exocyclic or endocyclic type, and it is reasonable to assume that the α-methylene exocyclic double bond is essential for inducing toxic activity.

In order to obtain information on the ability of this pathogen to produce the toxin in vivo, bananas were inoculated with *L. theobromae* by placing mycelial



Scheme 1. Conversion of compound **1** into decumbic acid (**2**): (a) acetic anhydride, reflux (29%).

discs on the surface in contrast with the controls which were subjected to the same treatment but inoculated with PDA-disc instead of mycelium. After incubation for 3 days at 25 °C, *L. theobromae* exhibited severe pathogenicity, and bananas were softened along with spreading decay together with the occurrence of hyphae on the surface of bananas. The fungal metabolite, compound **1** was detected in rotted regions of the infected bananas, indicating that *L. theobromae* did accumulate this toxin in the fruits, and this toxin is likely responsible for the rot of fruits. Although we checked the peels of control, compound **1** could not be detected by TLC-analyses.

Disubstituted α-methylene-γ-butyrolactones (Fig. 3) are noted in certain cases for their antibacterial (Cavallito et al., 1948), antifungal, and antitumor activities, and as growth regulating agents (Huneck and Schreiber, 1972). It is known that the olefinic double bonds are the active center of α-exomethylene lactone-class antibiotics, which may react with the sulfhydryl group of the receptor protein. To the best of our knowledge, however, this is the first report on the isolation and toxic activity of compound **1**, a new α-methylene-3γ-butyrolactone derivative. We observed that toxic activity disappeared when compound **1** was converted

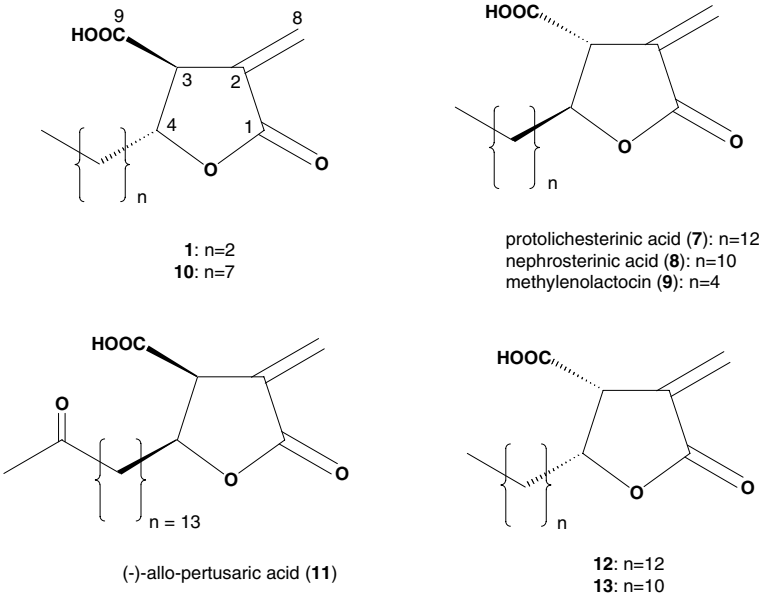


Fig. 3. Structures of compound **1** and other homologous compounds, **7**–**13**.

Table 2
^{1}H NMR arguments of H-3 and H-4 for compounds **1** and **7**–**13**

	H-3	H-4
1[a]	3.56 (1H, *m*)	4.76 (1H, *m*)
7[b]	3.64 (1H, *m*)	4.80 (1H, *m*)
8[c]	3.65 (1H, *m*)	4.80 (1H, *m*)
9[d]	3.65 (1H, *ddd*, *J* = 5.6, 3.0, 2.7 Hz)	4.83 (1H, *ddd*, *J* = 7.2, 7.2, 5.6 Hz)
10[e]	3.59 (1H, *dt*, *J* = 12.8, 5.6, 2.8 Hz)	4.77 (1H, *q*, *J* = 12.8, 6.0 Hz)
11[d]	4.02 (1H, *ddd*, *J* = 8.0, 2.0 Hz)	4.69 (1H, *ddd*, *J* = 11.5, 8.0 Hz)
12[c]	4.08 (1H, *dt*, *J* = 7.6, 2.0 Hz)	4.70 (1H, *m*)
13[c]	4.05 (1H, *dt*, *J* = 8.0, 2.1 Hz)	4.65 (1H, *m*)

[a] CD_3OD, 270 MHz.
[b] $CDCl_3$, 60 MHz.
[c] $CDCl_3$, 360 MHz.
[d] $CDCl_3$, 400 MHz.
[e] $CDCl_3$.

to decumbic acid, and this suggests that activity depends on the α-exomethylene lactone structure. This result coincides with the fact that the exomethylene unit is the active center of α-exomethylene lactone-class antibiotics. It is hoped that our preliminary experiments will provide the basis for a greater understanding of the pathogenesis of *L. theobromae* infection and facilitate the development of strategies to prevent attacks by this pathogen.

3. Experimental

3.1. General

Spectra were obtained with the following instruments: IR, Hitachi 285 spectrometer; optical rotations, JASCO DIP-4 polarimeter; NMR, Bruker AM-500FTNMR spectrometer and JEOL JNM-EX 270 FT-NMR system; FD-and EI-MS, JEOL JMS01SG-2 and JMS-DX-300 mass spectrometers, respectively. Melting points were measured with a Yanaco micro melting point apparatus.

3.2. Isolation of pathogen

Tissue specimens were collected from the rotted mango branches in Miyako islands, Okinawa, Japan. After surface sterilization with 1% antiformin for 3–5 min, pieces of the infected branch were placed on PDA medium and incubated at 25 °C until the fungus sporulated. Then, the fungus was identified as *L. theobromae* according to the spore shape.

3.3. Bioassay

Banana (*M. acuminata*) was used for monitoring the activities causing the rot. Initially, the surface of the banana peel was sterilized with EtOH–H_2O (7:3) for 5 min and washed with tap water. An aliquot (usually 10 μl) of the EtOH-solution containing the extract was injected into the peel of bananas using a micro-syringe, and the bananas were incubated with the extract at 25 °C for 3 days. The toxicity was evaluated by comparing the resulting rot spot diameters with those of bananas that were injected with EtOH (without extract) as a control. Each test was performed in triplicate.

3.4. Cultures and isolation

The isolated fungus was stationary cultured using a potato–glucose medium for 21 days at 25 °C, and the culture filtrate was concentrated in vacuo to one-fifth of its volume. The concentrated culture filtrate was extracted with EtOAC, with the extract evaporated in vacuo. The resulting residue was then subjected to silica gel CC, which was successively eluted with $CHCl_3$, MeOH–$CHCl_3$ (3:97, v/v), MeOH–$CHCl_3$ (20:80), and MeOH. The activity was found in the MeOH–$CHCl_3$ (3:97) elute and the active fraction was subjected to further silica gel CC (C-200 Wako gel, Wako, 20 g, hexane:EtOAc = 1:1, v/v) to give active Fr. A. Fr. A was purified by HPLC (Wakosil 5C8, Wako, 10 × 300 mm, MeOH:H_2O:AcOH = 60:40:0.1, v/v, 2.0 ml/min, UV detector 254 nm), followed by further purification by HPLC (Mightysil RP-18 GP Aqua, Kanto Chemical, 4.6 × 250 mm,

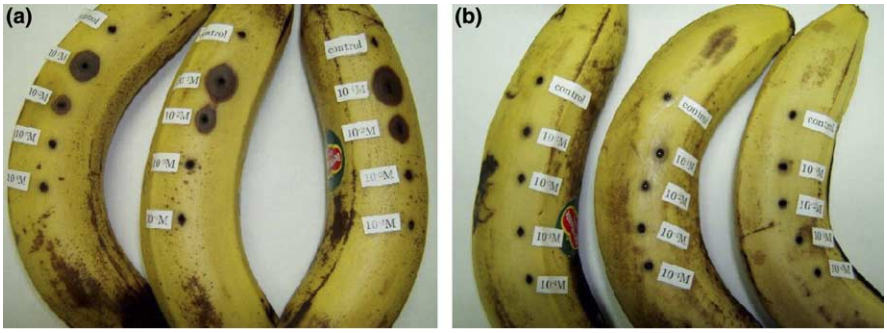


Fig. 4. Black spots inducing activities of **1** and **2**: (a) activity test for compound **1**; (b) activity test for decumbic acid (**2**).

CH_3CN:H_2O:AcOH = 20:80:0.1, v/v, 1.0 ml/min, UV detector 254 nm) to afford two fractions, Fr. B (t_R: 17.4 min) and C (t_R: 13.4 min). Compounds **2** and **1** were obtained by recrystallization of Fr. B and C from hexane–$CHCl_3$, respectively.

*3.4.1. (3S,4R)-3-carboxy-2-methylene-heptan-4-olide (**1**)*

M.p. 77–80 °C; $[\alpha]_D^{25}$ + 18.2° (*c* 0.22, $CHCl_3$); EI-MS *m/z* (rel. int.): 166 $[M - H_2O]^+$ (12), 155 (16), 141 (100), 123 (27), 113 (67), 96 (76), 85 (69), 71 (56), 67 (19), 39 (52); FDMS *m/z* (rel. int.): 185 $[M + H]^+$ (100); FD-HR-MS *m/z*: 185.0795 $[M + H]^+$ (calcd for $C_9H_{13}O_4$: 185.0814); IR. ν_{max}^{KBr} cm^{-1}: 3440, 3100, 2963, 2876, 1748, 1702, 1660, 1253; For ^1H and ^{13}C NMR spectroscopic data, see Table 1.

*3.4.2. Decumbic acid (**2**)*

M.p. 125–127 °C; $[\alpha]_D^{25}$ + 44.3° (*c* 0.47, $CHCl_3$); EI-MS *m/z* (rel. int.): 155 (81), 142 (100), 124 (40), 123 (20), 96 (54), 84 (27), 71 (38), 44 (65); FAB-MS *m/z* (rel. int.): 183 $[M - H]^-$ (100); FAB-HR-MS *m/z* (rel. int.): 183.0658 $[M - H]^-$ (calcd for $C_9H_{11}O_4$: 183.0658); IR. ν_{max}^{KBr} cm^{-1}: 3424, 3100, 2964, 2876, 1743, 1702, 1660, 1426, 1215, 1151, 1133, 1023, 959, 879, 708, 626; ^1H NMR (270 MHz, $CDCl_3$): δ 5.13 (1H, *m*, H-4), 2.22 (3H, *d*, *J* = 2.0 Hz, H-8), 2.09 (1H, *m*, H-5a), 1.56 (1H, *m*, H-5b), 1.45 (2H, *m*, H-6), 0.94 (3H, *t*, *J* = 7.6 Hz, H-7); ^{13}C NMR (67.5 MHz, $CDCl_3$): δ 172.6 (CO, C-1), 166.1 (COOH, C-9), 146.6 (C, C-3), 140.0 (C, C-2), 81.2 (CH, C-4), 34.8 (CH_2, C-5), 18.2 (CH_2, C-6), 13.7 (CH_3, C-7), 11.1 (CH_3, C-8).

*3.5. Conversion of 1 to decumbic acid (**2**)*

A stirred solution of **1** (10 mg, 0.05 mmol) in acetic anhydride (5 ml) was refluxed for 1 h, and the reaction mixture was poured into the mixture of EtOAc (10 ml) and distilled water (10 ml). The organic layer was dried (Na_2SO_4) and evaporated to give an oil, which was purified by a preparative TLC to afford decumbic acid (**2**, 2.9 mg, 0.02 mmol, 29%).

3.6. Isolation of 6 from bananas infected by L. theobromae

The fungus *L. theobromae* obtained from rotted mango branches was cultured on PDA slants and used for this experiment. Initially, six bananas were surface-sterilized with EtOH–H_2O (7:3) for 5 min and rinsed with tap water. Five inoculum discs (∅: 5 mm), cut form PDA slants, were placed onto tangential wounded surface of each banana. After incubation in the dark for 3 days at 25 °C, the fungal mycelium were scraped off, and the rotted peels were excised and extracted with EtOAc. The extracts were filtered and then subjected to silica gel CC (C-200 Wako gel, Wako, 15 g, hexane:EtOAc = 1:1, v/v) to give five fractions (Fr. A–E). Fr. C was further purified by preparative TLC on silica gel. Plates were developed twice with hexane–EtOAc–AcOH (10:10:0.1, v/v/v). The bands were collected and eluted from the silica gel with $CHCl_3$ to give **1** (0.9 mg).

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