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Biosynthesis of theobroxide and its related compounds, metabolites of *Lasiodiplodia theobromae*

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Abstract

Administration of ^{13}C labeled acetates ([1-^{13}C], [2-^{13}C] and [1,2-$^{13}C_2$] to *Lasiodiplodia theobromae* showed the tetraketide origins of both theobroxide, a potato-tuber inducing substance {**1**, (1*S*, 2*R*, 5*S*, 6*R*)-3-methyl-7-oxa-bicyclo[4.1.0]hept-3-en-2,5-diol}) and its carbonyldioxy derivative {**2**, (1*S*, 4*R*, 5*S*, 6*R*)-7,9-dioxa-3-methyl-8-oxobicyclo [4.3.0]-2-nonene-4,5-diol}. The incorporation of acetate-derived hydrogen into **1** and **2** was studied using [2-$^{2}H_3$, 2-^{13}C]acetate. Three and one deuterium atoms were incorporated at one methyl and epoxy carbons, respectively. The observed loss of deuterium atoms from the methyl group suggests a considerable amount of exchange from the methyl group of [2-$^{2}H_3$, 2-^{13}C]acetate during biosynthesis of **1** and **2**. Incorporation of [1-^{13}C]- and [1,2-$^{13}C_2$]acetates indicates the carbonyl carbon of the carbonyldioxy derivative is derived from the carboxy carbon of the precursor.

Keywords: *Lasiodiplodia theobromae*; Fungus biosynthesis; ^{2}H NMR; ^{13}C NMR; Tetraketide; Theobroxide; Potato-tuber inducing substance

1. Introduction

Theobroxide **1** {(1*S*, 2*R*, 5*S*, 6*R*)-3-methyl-7-oxa-bicyclo[4.1.0]hept-3-en-2,5-diol}, a compact epoxy cyclohexanoid, is a metabolite of the tropical fungus, *Lasiodiplodia theobromae* (Nakamori et al., 1994), which strongly induces flower bud formation in morning glory (*Pharbitis nil*), and tuber formation in potato (*Solanum tuberosum*) under non-inducing conditions (Yoshihara et al., 2000). We subsequently encountered co-metabolite **2** {(1*S*, 4*R*, 5*S*, 6*R*)-7,9-dioxa-3-methyl-8-oxobicyclo [4.3.0]-2-nonene-4,5-diol} while continuing a survey on potato-tuber inducing substances from a culture of *L. theobromae* (Matsuura et al., 1998). Compound **1** was proven to inhibit stem elongation in morning glory and spinach through the suppression of gibberellin biosynthesis and stimulation of jasmonic acid biosynthesis (Kong et al., 2005a; Kong et al., 2006; Gao et al., 2006). Immunoblot analysis of key enzymes in JA biosynthesis showed that lipoxygenase, allene oxide synthase and allene oxide cyclase activity, and endogenous levels of JA in *Pharbitis nil* were induced by **1** (Kong et al., 2005b).

A range of a compact epoxyquinones derived from motif **5** have been encountered among diverse sources such as bacteria, fungi and higher plants (Yoshihara et al., 2000; Scott et al., 1973; Nagasawa et al., 1978; Sakamura et al., 1970; Closse et al., 1966). In addition to induction of certain plant organs, these polyoxygenated cyclohexanoids and their derivatives show a wide range of biological activities from phytotoxicity, anti-microbial, and anti-tumor activities to various kind of enzyme inhibition. The biosynthesis of such polyoxygenated cyclohexanoids derived from a tetraketide intermediate has been studied using ^{13}C and ^{2}H labeled acetates (for example, Nabeta et al., 1973; Cuppels et al., 1986; Yoshizawa et al., 1990; Avent et al., 1990; Gould et al., 1996; Li and Piel, 2002; Hu and Floss, 2004). The cyclohexene structure of theobroxide **1** includes two hydroxy groups and one epoxide. Co-metabolite **2** includes

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four oxygen atoms and a carbonyldioxy group at the cyclohexene ring. Such structures have an advantage in studying the origin of the carbons and hydrogens in polyketide biosynthesis (for reviews of the polyketide pathway for example, see Simpson, 1991; Rawlings, 1997; Rawlings, 1999; Staunton and Weissman, 2001). Our present interest in the biosynthesis of theobroxide **1** and co-metabolite **2** were as follows: to demonstrate the tetraketide origins of **1** and **2**; to define the origin of hydrogen at the cyclohexene rings of **1** and **2**, and to establish the origin of the carbonate carbon in **2** by administering ^{2}H and ^{13}C-labeled acetates ([1-^{13}C], [2-^{13}C], [1,2-$^{13}C_2$], and [2-$^{2}H_3$, 2-^{13}C]-).

2. Results and discussion

L. theobromae was grown by a static culture on liquid potato-1%-D-glucose medium supplemented with 1×10^{-2}M ^{13}C- and ^{2}H-labeled acetates. Chromatography of the EtOAc extracts of the culture filtrate gave labeled **1** and **2** in the ranges of 7.0–40 mg and 0.8–8.0 mg/100 ml of 7 day-old culture, respectively (Table 1). Compound **2** was isolated as the main metabolite from a 14 day-old culture (yields of compound **2**: 5.0 mg/100 ml and **1**:2.5 mg/100 ml).

The $^{13}C\{^{1}H\}$ NMR spectrum of the [1-^{13}C]acetate-derived theobroxide **1** showed three enhanced signals, attributable to C-1, -3 and -5 (Table 2 and Fig. 1), whereas the spectrum of **1** derived from the [2-^{13}C]acetate showed enhanced signals for C-2, -4, -6 and -8. Specific incorporation of ^{13}C was generally obtained for the [1-^{13}C] and [2-^{13}C]acetate-derived carbon atoms ranging 4.3–5.1 and from 4.7–5.4 at.% present, respectively. The specific incorporation was calculated on the relative intensity to one of the acetate methyl carbons at δ1.0 ppm and is referred to as 1.1% of the synthesized theobroxide diacetate **3**.

The arrangement of intact acetate units in **1** was studied by administering [1, 2-$^{13}C_2$]-acetate. Intense ^{13}C-^{13}C coupling signals were observed between C-1 and C-2, between C-3 and C-8 and between C-5 and C-6, whereas C-4 was observed as an intense singlet, indicating the presence of three intact acetate units, *viz*., C-1-C-2, C-3-C-8 and C-5-C-6. These labeling patterns confirmed that the skeleton of **1** was formed via a tetraketide intermediate and this C-8-C-3 is the starter unit.

Table 1
Administration experiments using ^{13}C- and/ or ^{2}H-labeled precursors and yields of compounds **1** and **2**

Precursors	Atom% (^{13}C)	Atom% (^{2}H)	Amount mg/100ml	Yields (mg/100 ml)[a]	
				1	**2**
Sodium[1-^{13}C]acetate	99	–	83	1.2	0.5
Sodium[2-^{13}C]acetate	99	–	83	4.3	0.3
Sodium[1,2-$^{13}C_2$]acetate	99	–	84	7.3	1.3
Sodium[2-$^{2}H_3$,2-^{13}C]acetate	97	97	86	6.7 (2.5)	0.1 (5.0)

[a] Yields from 7 day-old culture. Figures in parenthesis are yields from 14 day-old culture.

Table 2
Incorporation of sodium [1-^{13}C]-[2-^{13}C]-and [1,2-$^{13}C_2$] acetate into compound **1**[a]

C	δ_C (δ_C)[b]	^{13}C-atom%[c]			
		[1-^{13}C]acetate	[2-^{13}C]acetate	[1,2-$^{13}C_2$]acetate	
1	53.0 (50.8)	5.12	1.26	2.97	Doublet[d]
2	66.3 (66.7)	1.26	4.66	2.79	Doublet[d]
3	135.1(133.1)	4.56	1.17	3.00	Doublet[e]
4	121.5(120.1)	1.28	5.15	4.03	Singlet
5	63.0 (64.5)	4.31	1.16	2.82	Doublet[f]
6	51.9 (49.9)	1.67	5.37	2.88	Doublet[f]
8	21.2 (20.9)	1.22	5.09	3.01	Doublet[e]
	$\underline{C}H_3CO$(21.1)				
	$\underline{C}H_3CO$(21.0)	1.1	1.1	1.1	
	$CH_3\underline{C}$=O (170.1)				
	$CH_3\underline{C}$=O (170.3)				

[a] Compound **1** from a 7 day-old culture.
[b] ppm of diacetate **3** in $CDCl_3$.
[c] The methyl signal of the acetyl group of **3** at 21.0 ppm was chosen as a reference signal. All other signal intensities are given as the percent abundance relative to the methyl signal of the acetyl group = 1.1% natural abundance.
[d] $Jc_1,c_2 = 52.2$ Hz.
[e] $Jc_3,c_8 = 40.0$ Hz.
[f] $Jc_5,c_6 = 52.6$ Hz.

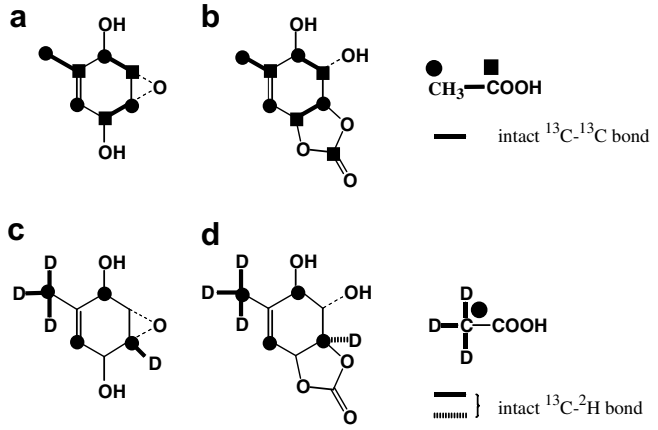

Fig. 1. Labeling patterns of a: theobroxide **1** following incorporation of [1-^{13}C], [2-^{13}C], [1,2-$^{13}C_2$]acetate; b: its carbonyldioxy derivatives **2** following incorporation of [1-^{13}C], [2-^{13}C], [1,2-$^{13}C_2$]acetate, c: **1** following incorporation of [2-$^{2}H_3$, 2-^{13}C]acetate and d: **2** from [2-$^{2}H_3$, 2-^{13}C]acetate.

The presence of an acetate starter unit in **1** was unambiguously demonstrated by administering [2-$^{2}H_3$,2-^{13}C]acetate to 7 day-old cultures. The ^{13}C signals of the 6- and 8-^{13}C-^{2}H species appeared as multiplets shifted to higher field (α-isotope effect) (Vederas, 1987) than the ^{13}C resonance due to the corresponding ^{13}C-^{1}H species (Table 3). A heptet ($J =$ 19.7 Hz) at 20.36 ppm [0. 83 ppm upfield of the normal chemical shift value for C-8] was assigned to a tri-deuterated methyl, confirming that C-8 and C-3 came from acetyl CoA. However, the 8-^{13}C signal of the ^{13}C-^{2}H species of **1**, from a 14 day-old culture, was observed as a heptet with a quintet at δ20.91 ppm and a triplet at 20.64 ppm, which correspond to di- and mono-deuterated methyls, respectively. The loss of deuterium atoms and an enriched CH_3 singlet suggest a considerable level of exchange of hydrogen from the methyl group in the biosynthesis of **1** at later stages of growth of *L. theobromae*. The observed triplet ($J = 23.1$ Hz) at 52.2 ppm ($\Delta\delta$: -0.28 ppm) corresponded to mono-deuterated C-6 methine. The intense singlet of C-4 at δ21.5 ppm demonstrated that a deuterium atom at C-4 was completely lost during biosynthesis of **1**. The ^{2}H{^{1}H} NMR spectrum of compounds **1** also indicated the retention of three deuterium atoms at C-8 ($\delta 2_H$: 1.70 ppm, $J = 19.7$ Hz) and one deuterium atom at C-6 ($\delta 2_H$: 3.28 ppm, $J = 23.1$ Hz).

As expected from the labeling pattern of compound **1**, the ^{13}C{^{1}H} NMR spectrum of compound **2** formed in the presence of [2-^{13}C]-acetate and its diacetate (**4**) showed that four ^{13}C signals corresponding to C-2, C-4, C-6 and C-10 (Table 4 and Fig. 1) had an enhanced intensity with ^{13}C atoms (4.0–4.9% of specific incorporation). With

Table 3
Incorporation of sodium [2-$^{2}H_3$, 2-^{13}C]acetate into compound **1**[a]

C	^{13}C-atom%[b]	^{2}H:^{1}H[c]	Isotope shift $\Delta\delta$ ppm
1	1.12	–	–
2	4.97	–	–
3	1.00	–	–
4	4.86	–	–
5	1.07	–	–
6	3.36(t, $J = 23.1$ Hz)	64:100	−0.28
8	3.65(sep,$J = 19.7$ Hz)	79:100	−0.83[d]

[a] Compound **1** from 7 day-old culture.
[b] The methyl signal of the acetyl group of the diacetate **3** at 21.0 ppm was chosen as a reference. All other signal intensities are given as percent abundance relative to the methyl resonance of the acetyl group 1.1% natural abundance.
[c] Ratio of carbon peak intensity of protonated ^{13}C to deuterated ^{13}C.
[d] C-8 of compound **1** from 14 day-old culture appeared as heptet with quintet ($\Delta\delta$,−0.55 ppm) and triplet ($\Delta\delta$, −0.28 ppm).

Table 4
Incorporation of sodium [1-^{13}C]-[2-^{13}C]-and [1,2-$^{13}C_2$] acetate into compound **2**[a]

C	$\delta_C(\delta_C)$[b]	^{13}C-atom%[c]			
		[1-^{13}C]acetate	[2-^{13}C]acetate	[1,2-$^{13}C_2$]acetate	
1	75.6 (72.5)	3.56	1.12	2.35	doublet[d]
2	116.1 (118.1)	1.04	4.44	2.13	singlet
3	147.0(141.1)	3.03	0.98	2.17	doublet[e]
4	71.8(69.1)	1.21	4.49	3.25	doublet[f]
5	73.9 (69.9)	4.09	1.08	2.28	doublet[f]
6	79.9(74.3)	1.09	4.87	2.41	doublet[d]
8	156.3 (153.2)	3.79	1.47	2.73	singlet
10	19.2 (19.4)	1.31	4.03	2.98	doublet[e]
	$\underline{C}H_3CO$(20.6)				
	$\underline{C}H_3CO$(20.7)	1.1	1.1	1.1	
	$CH_3\underline{C}$=O (169.2)				
	$CH_3\underline{C}$=O (170.3)				

[a] Compound **2** from a 7 day-old culture.
[b] ppm of diacetate **4** in $CDCl_3$.
[c] The methyl signal of the acetyl group of **4** at 20.7 ppm was chosen as a reference. All other resonance intensities are given as percent abundance relative to the methyl signal of the acetyl group = 1.1% natural abundance.
[d] $Jc_1,c_6 = 31.6$ Hz.
[e] $Jc_3,c_{10} = 43.9$ Hz.
[f] $Jc_4,c_5 = 42.2$ Hz.

[1-^{13}C]-acetate as precursor, the ^{13}C NMR spectrum displayed intense ^{13}C signals for C-1 (3.6%), C-3 (3.0%) and C-5 (4.1%). The ^{13}C resonance of the carbonyl carbon (C-8) also had an increased intensity (3.8%), indicating the carbonyl carbon in **2** originates from the carboxyl carbon of acetate (Fig. 2). With [1,2-$^{13}C_2$]acetate as precursor, the ^{13}C NMR spectrum of **2** showed three pairs of intense ^{13}C-^{13}C coupled resonances (2.1–3.3%), while the enrichment factor for C-8 in **2** was 2.7%. The considerable exchange of hydrogen from the methyl group in a 14 day-old culture was also observed in the biosynthesis of **2** incorporating [2-$^{2}H_3$, 2-^{13}C]acetate (Table 5). The ^{2}H:^{1}H ratio of the C-6 of **2** was significantly lower for comparison of that in C-10 of **2** and that in C-6 of **1** (see Table 3). At present, there is no explanation for such differences. Except for **2** incorporating [^{2}H,^{13}C]acetate, biosynthetically labeled compounds **1** and **2** were isolated from 7 day-old cultures, while **2** incorporating [^{2}H, ^{13}C]acetate was isolated from 14 day-old cultures. Thus, long-term culture may result in an extensive loss of intact ^{2}H from [^{2}H, ^{13}C] acetate and a predominant incorporation of low-^{2}H labeled acetate in the malonyl CoA derived portion of **2**.

Concluding remarks: In this study, the tetraketide origin of theobroxide was established as depicted in Fig. 2. In an upcoming report, we will address the biotransformation mechanism of theobroxide **1** to the carbonyldioxy derivative **2**.

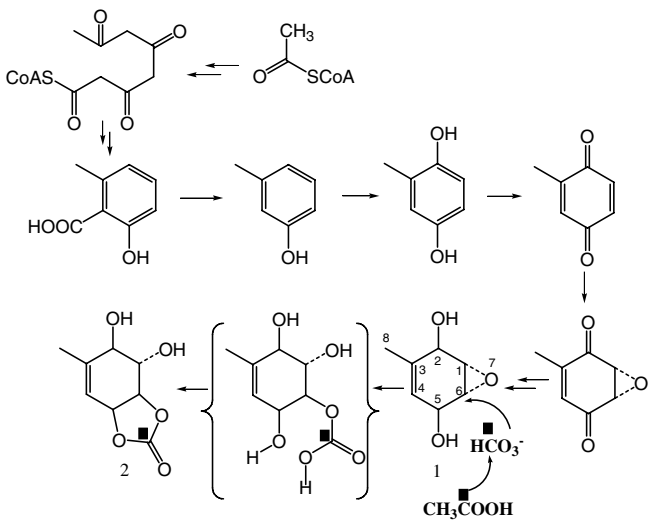

Fig. 2. Possible biosynthetic pathway to theobroxide **1** and its carbonyldioxy derivative **2**.

Table 5
Incorporation of sodium [2-$^{2}H_3$, 2-^{13}C]acetate into compound **2**[a]

C	^{13}C-Atom%[b]	^{2}H:^{1}H[c]	Isotope shift $\triangle\delta$ ppm
1	1.27	–	–
2	4.44	–	–
3	0.85	–	–
4	5.24	–	–
5	1.39	–	–
6	3.27(t, $J = 23.9$ Hz)	28:100	−0.36
8	1.18	–	–
10	2.78(m,$J = 19.0$ Hz)[d]	61:100	-0.28,-0.55,-0.83

[a] Compound **2** from a 14 day-old culture.
[b] The methyl signal of the acetyl group of the diacetate **4** at 20.7 ppm was chosen as a reference. All other intensities are given as the percent abundance relative to the methyl resonance of the acetyl group = 1.1% natural abundance.
[c] Ratio of carbon peak intensity of protonated ^{13}C to deuterated ^{13}C.
[d] Heptet with quintet and triplet.

3. Experimental

3.1. General

All ^{1}H NMR (270 MHz and 500 MHz), ^{13}C{^{1}H} NMR (67.8 and 125.8 MHz), ^{2}H{^{1}H}(41.3 and 76.5 MHz)spectra of compounds **1**, **2** and their acetates (**3** and **4**, respectively) incorporating ^{2}H and ^{13}C labeled acetates were recorded on JEOL JMN-EX 27-FT-NMR and Bruker AMX-500 FT-NMR spectrometers, respectively. Chemical shift values were referenced to residual solvent signals as follows ($\delta 1_H$ and $\delta^2H/\delta 13_C$): $CHCl_3$ and $CDCl_3$ (δ7.24/77.0), and CH_3OD and CD_3OD (δ 3.30/49.0}. Numbers of scans; 1500–57,600 for ^{13}C NMR and 610–3600 for ^{2}H NMR.

3.2. Culture conditions

Spores of *Lasiodiplodia theobromae* were maintained on the agar (1.5%) of a 2%-potato D-glucose medium (Nissui Seiyaku) at 30 °C and transferred at intervals of 6 months.

A piece (1 cm^2) of agar bearing the spore-formed culture of *L. theobromae* OCS 71 was used to inoculate three 500 ml Erlenmeyer flasks containing 200 ml of a potato D-glucose medium (1% glucose) individually administered with 10^{-2} M isotopically labeled acetates. The flasks were statically cultured at 25 °C in the dark for 7 days or 14 days.

3.3. Extraction and isolation of biosynthetically labeled *1* and *2*

The culture broths from each administration experiment for 7 day-old or 14 day-old cultures were individually combined, filtered and concentrated (to a 100 ml) with the filtrate extracted with equal volumes of EtOAc (5×100 ml). The combined EtOAc extracts were concentrated to dryness under reduced pressure, and the residues subjected to a silica gel cc (50 g) eluted with MeOH–$CHCl_3$ (1:19). Recrystallized of fractions of interest from EtOAc-*n*-hexane gave pure compound **1** (1.2–7.3 mg/100 ml from 7 day-old culture and 2.5 mg from 14 day-old culture) and compound **2** (0.1–1.3 mg and 5.0 mg, respectively).

3.4. Acetylation of compound *1*

Biosynthetically labeled **1** (10 mg) was added to a solution of (30 μl) Ac_2O in dry pyridine (90 μl) and kept at room temperature for 24 h. The mixture was then poured into iced H_2O (3 ml) and extracted with Et_2O (3×3 ml). The combined Et_2O extracts were dried (anhydr. Na_2SO_4) and concentrated under reduced pressure to give diacetates **3** of compound **1** in yields of 90–95%. ^1H NMR spectroscopic data (270 MHz, $CDCl_3$): δ1.66 (3 H, *s*, 8-CH_3), 2.06 (3H, *s*, CH_3CO), 2.10 (3H, *s*, CH_3CO), 3.21 (1H, *m*, H-1), 3.23 (1H, *m*, H-6), 5.45 (2H, *br s*, H-2 and H-5), 5.52 (1H, *br s*, H-4).

3.5. Acetylation of compound 2

Biosynthetically labeled compound **2** (8–3 mg) was added a solution of dry Ac_2O/dry pyridine as above. After 24 h at room temperature, the reaction mixture was concentrated to dryness to give the diacetate **4**. ^1H NMR spectroscopic data (270 MHz, $CDCl_3$): δ 1.84 (3 H, *s*, 10-Me), 2.12 (6H, *s*, 2 x CH_3CO). 4.78 (1H, *t*, $J = 7.3$ Hz, H-6), 5.16 (1H, *m*, H-1), 5.34 (1H, *dd*, $J = 6.8$ Hz and 7.3 Hz, H-5), 5.47 (1H, *br d*, $J = 6.8$ Hz, H-4), 5.80 (1H,*br s*, H-7).

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