Carboxylic Acid Microbial Metabolites of the Natural Benzoquinone, Maesanin

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Maesanin (**1**) is a naturally occurring bioactive benzoquinone isolated from the fruits of *Maesa lanceolata* (Myrsinaceae). Three carboxylic acid metabolites of maesanin were isolated in the course of a prospective microbial transformation study. The first metabolite, **2**, was produced by *Lipomyces lipofer* ATCC 10742 and was characterized as (*Z*)-15-(2′-hydroxy-5′-methoxy-3′,6′-dioxocyclohexa-1′,4′-dienyl)pentadec-5-enoic acid. Metabolites **3** and **4** were produced by *Rhodotorula rubra* ATCC 20129 and were characterized as 6-(2′-hydroxy-5′-methoxy-3′,6′-dioxocyclohexa-1′,4′-dienyl)hexanoic acid and 4-(2′-hydroxy-5′-methoxy-3′,6′-dioxocyclohexa-1′,4′-dienyl)butanoic acid, respectively.

Maesanin, 2-hydroxy-5-methoxy-3-(10′-pentadecenyl)-1,4-benzoquinone (**1**), is a natural *p*-benzoquinone isolated from the fruits of *Maesa lanceolata* and *Ardisia japonica* (Myrsinaceae).[1,2] The biological activities of **1** include nonspecific immunostimulation,[1] 5-lipoxygenase inhibition,[2,3] aldose reductase inhibition,[4] in addition to potentiation of the anticandidal effect of the sesquiterpene dialdehyde, polygodial.[5]

In the course of a prospective study utilizing microbes as models of mammalian metabolism, three carboxylic acid metabolites of **1** were isolated from the fermentation broths of the two yeasts *Lipomyces lipofer* ATCC 10742 and *Rhodotorula rubra* ATCC 20129 and are hereby reported.

Results and Discussion

Sixty-two fungal cultures were screened for their ability to biotransform maesanin (**1**) according to a standard two-stage procedure.[6] Two yeasts, *L. lipofer* and *R. rubra*, were among the organisms selected for preparative-scale microbial transformation after exhibiting a high efficiency of substrate bioconversion. The maesanin used was obtained either by isolation from *M. lanceolata* or by total synthesis following the procedure of Kubo et al.[3]

Preparative scale fermentation of **1** by *L. lipofer* ATCC 10742 provided metabolite **2** in an 18% yield. The ^{1}H NMR spectrum of **2** differed from that of **1** in the disappearance of the triplet at δ 0.89 and the appearance of another triplet at δ 2.35 which integrated for 2 protons. The two olefinic protons, originally showing as a triplet in **1**, now became split into two multiplets at δ 5.31 and 5.40 and each integrated for one proton. Also, the four allylic protons, originally showing in **1** as a multiplet at δ 2.0, became split into two equal multiplets at δ 1.99 and 2.08. The ^{13}C NMR spectrum also showed the disappearance of the upfield signal, originally found in **1**, at δ 14.0 and the appearance of a downfield quaternary signal at δ 179.2. At that point, it was apparent that the terminal methyl of **1** has been replaced by another moiety containing a carbonyl group, as suggested by the appearance of the triplet at δ 2.35 and the reduction in the integration of the aliphatic side chain methylenes around δ 1.2−1.4 by 2 protons. Apart from this observation, the rest of the molecule resembled that of **1**. Results of the COSY and HMBC experiments supported the observations obtained from the ^{1}H and ^{13}C experiments and are shown in Figure 1.

Preparative-scale fermentation of **1** by *R. rubra* ATCC 20129 yielded metabolites **3** and **4**. The ^{1}H NMR spectrum of **3** resembled that of **1** in that it had downfield singlets at δ 3.75 and 5.56 corresponding to the methoxy protons (H-7) and the olefinic ring proton (H-6), respectively. Signals for the terminal methyl protons (H-15′), the allylic methylene protons (H-9′ and -12′), and the olefinic protons (H-10′ and -11′) of the side chain were completely missing. On the other hand, a new methylene triplet at δ 2.04 emerged in the vicinity of the original methylene triplet at δ 2.12. The total integration of all the methylene protons at δ 1.2−2.1 was less by 12 protons (6 CH_2's), suggesting a maesanin analogue with a shorter aliphatic side chain. The ^{13}C NMR spectrum looked much simpler than that of **1** in the upfield methylene region where the number of signals was indeed reduced by 6 in the region around δ 29. Signals for the two olefinic carbons around δ 130 were missing and a new quaternary carbonyl signal emerged at δ 184.8. Structure **3** was further established via 2D NMR correlations (Figure 1), as well as electrospray high-resolution MS supporting a molecular formula of $C_{14}H_{18}O_6$.

Metabolite **4** was the major metabolite isolated from the culture broth of *R. rubra* (10% yield). The ^{1}H NMR spectrum was almost identical with that of **3** but contained two fewer CH_2 units. The other observation was that instead of two separate triplets, a single triplet integrating for four protons showed at 2.10 ppm. The ^{13}C NMR spectrum showed only four methylene signals around δ 22.4−37.0 (DEPT 135), and a total of 12 signals in all. Comparing this set of data with that of **3**, a molecular formula of $C_{12}H_{14}O_6$ corresponding to structure **4** was proposed. COSY correlations (Figure 1) further established the side chain proton couplings, which were much simplified as compared to **1** and the earlier carboxy analogues **2** and **3**. Although the HMBC experiment did not establish any correlation between the terminal carboxy carbon and its neighboring methylene protons, all other correlations were established in such a way that eliminated any doubt about the assignment of that carbon at δ 187.4.

While this work represents the first microbial transformation study of a substituted *p*-benzoquinone, it is note worthy that similar cases of microbial ω-oxidation of long chain hydrocarbons have been previously reported and can

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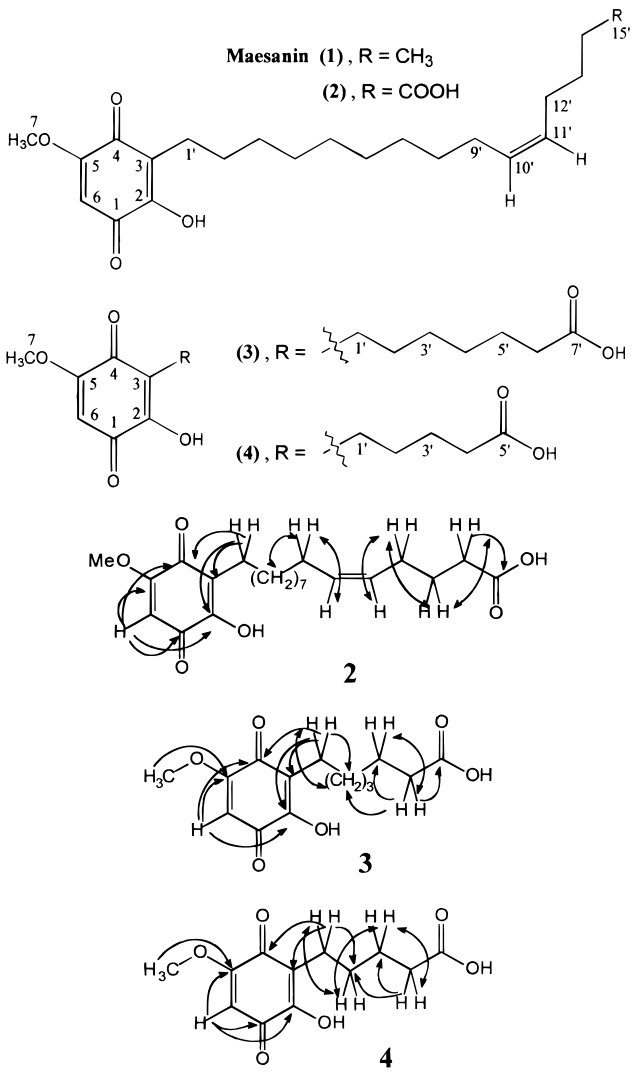

Figure 1. Structures and major 2D NMR correlations of maesanin metabolites. Double-headed arrows indicate COSY, and single-headed arrows indicate HMBC correlations.

be used as basis for speculating the biotransformational pathway of **1**.[7−10]

Experimental Section

General Experimental Procedures. Melting points were determined on a Thomas-Hoover capillary melting point apparatus and are uncorrected. The IR spectra were recorded with an ATI Mattson Genesis Series FTIR spectrophotometer. UV spectra were obtained with a Hitachi U2000 dual-beam spectrophotometer. The ^{1}H and ^{13}C NMR spectra were obtained on a Bruker Avance DRX-400 FT spectrometer operating at 400 and 100 MHz, respectively. The low-resolution mass analysis (thermospray, Vestec model 201) was run by Dr. J. K. Baker at Alcon Laboratories, Inc. (Fort Worth, TX). HRESIMS analysis (Fisons/VG Autospec Q) was conducted at the University of Kansas. TLC analyses were carried out on precoated silica gel Si 250F plates (Baker). The developing systems used were as follows: A, hexanes−EtOAc (1:3), and B, EtOAc−methanol (9:1). Visualization of plates was performed using visible light, UV light (254 nm), and 50% H_2SO_4 spray reagent. For column chromatography, the adsorbent used was Si gel 230−400 mesh (Merck).

Substrate. In addition to 200 mg of the isolated natural product, the main bulk of maesanin (**1**) was prepared according to the total synthetic scheme reported by Kubo et al.[1,3]

Organisms and Metabolism. Fungi were obtained from The University of Mississippi, Department of Pharmacognosy Culture Collection, and were originally obtained from the American Type Culture Collection (ATCC), Rockville, Maryland, or from Northern Regional Research Laboratories (NRRL, Peoria, IL). Stock cultures were maintained on agar slants of media recommended by the ATCC and were stored at 4 °C. All screening and scale-up fermentations were run in complex culture media (Medium α) of the following composition: 5 g of yeast extract (Difco Labs, Detroit, MI), 5 g of bacto-peptone (Difco Labs), 5 g of NaCl, 5 g of Na_2HPO_4, 20 g of dextrose, and distilled H_2O to 1 L. This composition always gave a starting medium pH of 7.0−7.2. For screening, cultures were grown in 25 mL of complex medium α held in 125-mL Erlenmeyer flasks and equipped with stainless steel caps. Maesanin (**1**) was added to 1-day-old stage II culture media as a 5% solution in ethanol (0.1 mg of substrate/mL of culture medium). Cultures were incubated on a rotary shaker (New Brunswick Model G10-21) at 250 rpm and room temperature for a maximum period of 14 days with sampling and TLC monitoring at three-day intervals. Preparative-scale fermentations followed the same general procedure with the difference that, for each organism selected for scale-up, 2 mL of 10% ethanolic solution of **1** was equally divided on 4 1-liter Erlenmeyer flasks each containing 250 mL of stage II culture of the respective organism (50 mg/flask). Incubation periods for all the conducted preparative-scale fermentations were 14 days. Workup followed a routine procedure[11] that comprised exhaustive EtOAc extraction of both culture filtrate and residue as determined by TLC. The combined residue and filtrate extracts were concentrated at 40 °C, under vacuum, to yield residues that were subsequently subjected to column chromatography for metabolite isolation and purification. Appropriate substrate and culture controls were run simultaneously with each preparative-scale fermentation. In the substrate control experiment, **1** (3 mg in 30 μL of EtOH) was added to 25 mL of autoclaved medium α and allowed to shake at room temperature for 14 days alongside of another medium α flask that contained no **1**. At the end of that time, the two media samples were extracted with EtOAc and analyzed by TLC. Results of the analysis showed that **1** was stable under culture conditions over the maximum period for which the fermentations were conducted.

Biotransformation of Maesanin (1) with *L. lipofer*. At the end of the fermentation period, the media were filtered. Both the filtrate and residue were exhaustively extracted with EtOAc to yield a viscous dark red liquid residue (ca. 0.350 g). The residue was chromatographed on a silica gel column using gradient elution with hexanes−EtOAc (3:1, v/v), through EtOAc (100%), and increasing in polarity to EtOAc−MeOH (2:3, v/v). A dark violet residue was obtained from the polar fractions which was rechromatographed with the same solvent system to yield metabolite **2** (0.039 g, 18%), R_f 0.37 (system A).

(*Z*)-15-(2′-Hydroxy-5′-methoxy-3′,6′-dioxocyclohexa-1′,4′-dienyl)pentadec-5-enoic acid (2): violet amorphous residue; mp >250 °C; UV (MeOH) λ_{max} (log ϵ) 292 (4.01) and 534 (2.91) nm; IR (KBr) ν_{max} 3422, 2924, 2855, 1688, 1655, 1548 cm^{-1}. ^{1}H and ^{13}C NMR assignments, Table 1. Low-resolution thermospray MS *m*/*z* [M + 1]$^+$ 393 and [M + 18]$^+$ 410. HRESIMS *m*/*z* calcd for $C_{22}H_{32}O_6Na$ [M + Na]$^+$ 415.2097, found 415.2087.

Biotransformation of Maesanin (1) with *R. rubra*. Fermentation media were filtered at the end of the 14-day fermentation period. Both the filtrate and residue were exhaustively extracted with EtOAc to yield an orange red residue (ca. 0.300 g) which was chromatographed on a silica gel column using gradient elution with EtOAc−hexane (1:1, v/v), through EtOAc (100%) and increasing in polarity to EtOAc−methanol (1:4, v/v). Two violet residues of semipure metabolites were collected. The first residue was rechromatographed with the same solvent system to yield metabolite **3** (6.0 mg, 4%), R_f 0.66 (system B). The second residue was also rechromatographed with the same solvent system to yield metabolite **4** (12.0 mg, 9%), R_f 0.50 (system B).

6-(2′-Hydroxy-5′-methoxy-3′,6′-dioxocyclohexa-1′,4′-dienyl)hexanoic acid (3): amorphous violet residue; mp >250

Table 1. ^{1}H and ^{13}C NMR Data of the Microbial Metabolites (**2**−**4**)[a] (^{13}C Multiplicities Were Determined by DEPT 135 Experiment)

	2[b]		**3**[c]		**4**[c]	
carbon	δ_C	δ_H (m, *J* Hz)	δ_C	δ_H (m, *J* Hz)	δ_C	δ_H (m, *J* Hz)
1	181.7 s		188.5 s		180.3 s	
2	151.7 s		172.4 s		168.6 s	
3	119.3 s		113.5 s		117.0 s	
4	181.7 s		179.0 s		183.1 s	
5	161.2 s		165.1 s		164.1 s	
6	102.1 d	5.83 (s)	101.7 d	5.56 (s)	102.1 d	5.53 (s)
7	56.7 q	3.85 (s)	56.8 q	3.75 (s)	56.8 q	3.67 (s)
1′	22.6 t	2.43 (t, 7.7)	22.7 t	2.12 (t, 7.1)	22.4 t	2.10 (t, 7.3)
2′	27.9 t	1.45 (m)	29.1 t[e]	1.19 (m)	28.1 t	1.19 (m)
3′	29.2 t[d]	1.3 (m)	28.5 t[e]	1.19 (m)	25.8 t	1.41 (m)
4′	29.3 t[d]	1.3 (m)	29.3 t	1.19 (m)	37.0 t	2.10 (t, 7.3)
5′	29.4 t[d]	1.3 (m)	26.4 t	1.40 (m)	187.4 s	
6′	29.4 t[d]	1.3 (m)	38.1 t	2.04 (t, 7.5)		
7′	29.5 t[d]	1.3 (m)	184.8 s			
8′	29.6 t[d]	1.3 (m)				
9′	27.1 t	1.99 (q, 6.9)				
10′	131.3 d	5.40 (m)				
11′	128.1 d	5.31 (m)				
12′	26.4 t	2.08 (q, 7.2)				
13′	24.6 t	1.69 (m)				
14′	33.3 t	2.36 (t, 7.3)				
15′	179.2 s					

[a] NMR data of **1** previously reported.[1,3] [b] In $CDCl_3$. [c] In D_2O. [d,e] Assignments may be interchanged within the same column.

°C; UV (MeOH) λ_{max} (log ϵ) 296 (3.85) and 520 (2.77) nm; IR (KBr) ν_{max} 3448, 2924, 2845, 1694, 1655, 1635, 1602, 1550 cm^{-1}. ^{1}H and ^{13}C NMR assignments, Table 1. Low-resolution thermospray MS *m*/*z* $[M + 1]^+$ 283 and $[M + 18]^+$ 300. HRESIMS *m*/*z* calcd for $C_{14}H_{18}O_6Na$ $[M + Na]^+$ 305.1001, found 305.1007.

4-(2′-Hydroxy-5′-methoxy-3′,6′-dioxocyclohexa-1′,4′-dienyl)butanoic acid (4): amorphous violet residue; mp >250 °C; UV (MeOH) λ_{max} (log ϵ) 290 (3.91) and 520 (2.84) nm; IR (KBr) ν_{max} 3448, 2935, 2845, 1654, 1635, 1522 cm^{-1}. ^{1}H and ^{13}C NMR assignments, Table 1. Low-resolution thermospray MS *m*/*z* $[M + 1]^+$ 255 and $[M + 18]^+$ 272. HRESIMS *m*/*z* calcd for $C_{12}H_{14}O_6Na$ $[M + Na]^+$ 277.0688, found 277.0688.

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