2-Hydroxy-5-(ethanolamino)-3-(10′-*Z*-pentadecenyl)-1,4-benzoquinone, New Microbial Phase II Metabolite of Maesanin

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The use of microbial models for biotransformation of the natural benzoquinone, maesanin (1), resulted in the isolation of an ethanolamine conjugate (5) from the culture broth of *Debaryomyces polymorphus* ATCC 20280. Metabolite 5 was characterized as 2-hydroxy-5-(ethanolamino)-3-(10′-*Z*-pentadecenyl)-1,4-benzoquinone. The production of 5 represents a new type of phase II conjugation reaction in microbial systems. The results of preliminary mammalian metabolism of 1 in rats were inconclusive.

Key words biotransformation; microbial transformation; benzoquinone; maesanin; ethanolamine conjugation

Maesanin (**1**) is a naturally occurring benzoquinone isolated from the fruits of *Maesa lanceolata* and other Myrsinaceous plants.[1,2] A number of biological activities have been attributed to **1**, such as 5-lipoxygenase and aldose reductase inhibition, as well as non-specific immunostimulation.[2—4] The use of **1** in folk medicine as an antiinflammatory and cholera prophylactic agent has also been reported.[4,5] In an attempt to acquire a better understanding of the metabolic fate of **1** in biological systems, and in order to facilitate its development as a lead compound, microbes (fungi) were utilized as prospective models for predicting the mammalian metabolism of **1**.[6] In an earlier report, three microbial metabolites of **1** had been isolated and characterized.[7] The first metabolite, characterized as (*Z*)-15-(2′-hydroxy-5′-methoxy-3′,6′-dioxocyclohexa-1′,4′-dienyl)pentadec-5-enoic acid (**2**), was produced by *Lipomyces lipofer* ATCC 10742. The second and third metabolites, 7-(2′-hydroxy-5′-methoxy-3′,6′-dioxocyclohexa-1′,4′-dienyl)heptanoic acid (**3**), and 5-(2′-hydroxy-5′-methoxy-3′,6′-dioxocyclohexa-1′,4′-dienyl)pentanoic acid (**4**), were produced by *Rhodotorula rubra* ATCC 20129 (Chart 1). The isolation and structural elucidation of a new type of microbial phase II conjugate, which was characterized as 2-hydroxy-5-(ethanolamino)-3-(10′-*Z*-pentadecenyl)-1,4-benzoquinone (**5**), are herein reported. Also reported is the utilization of **1** and its microbial metabolites as standards for HPLC qualitative analysis of rat urine for mammalian metabolites of **1**.

Results and Discussion

After screening 62 fungal cultures for their ability to biotransform **1**, the yeast *Debaryomyces polymorphus* ATCC 20280 was found to produce a single metabolite that was different from **2**, **3** and **4**. Thus, preparative scale fermentation was conducted on 200 mg of **1** utilizing *D. polymorphus* and following a standard two-stage procedure.[8] At the end of the incubation period, metabolite **5** was isolated and purified in 16% yield. The low and high resolution mass data for **5** suggested the molecular formula $C_{23}H_{37}NO_4$, corresponding to a molecular weight of 391 (392-H^+). The ^{1}H-NMR spectrum of **5** (in CD_3OD) was identical with that of **1** except for the disappearance of the methoxy singlet at δ 3.82 and the emergence of two new methylene triplets at δ 3.38 and 3.75. ^{1}H–^{1}H COSY correlations showed an isolated A_2X_2 system for the two new triplets, and HMQC correlations were utilized in assigning them to the carbons at δ 45.7 and 60.1, respectively. These chemical shifts suggested that the carbon at δ 60.1 was oxygenated while the carbon at δ 45.7 was attached to a nitrogen, giving rise to an $-OCH_2CH_2N-$ moiety. There also seemed to be an overlap at δ 5.32, as evidenced by the presence of two correlations corresponding to the two olefinic carbons at δ 130.0, as well as to another carbon around δ 90.0. The acquired data suggested that the molecule had an ethanolamine unit in place of the original methoxy group at C-5. Thus, the structure of **5** was assigned as 2-hydroxy-5-(ethanolamino)-3-(10′-*Z*-pentadecenyl)-1,4-benzoquinone.

Further evidence for the structure of **5** was established through its acetylation to compound **6**. The ^{13}C-NMR spectrum of **6** (in $CDCl_3$) showed four new signals at δ 20.9, 21.2, 168.6 and 170.0, corresponding to the two acetyl groups. The ^{1}H-NMR spectrum showed acetyl methyl singlets at δ 2.10 and 2.35, and a 1-H triplet at δ 6.01. The triplet at δ 3.75 shifted downfield to δ 4.29, while the other triplet at δ 3.37 appeared as a quartet. In addition, the multiplet at δ 5.33 resolved into a singlet at δ 5.44 (1H) and a triplet at δ 5.34 (2H). ^{1}H–^{1}H COSY showed that the quartet at δ 3.37 was coupled to both the triplets at δ 4.29 and 6.01.

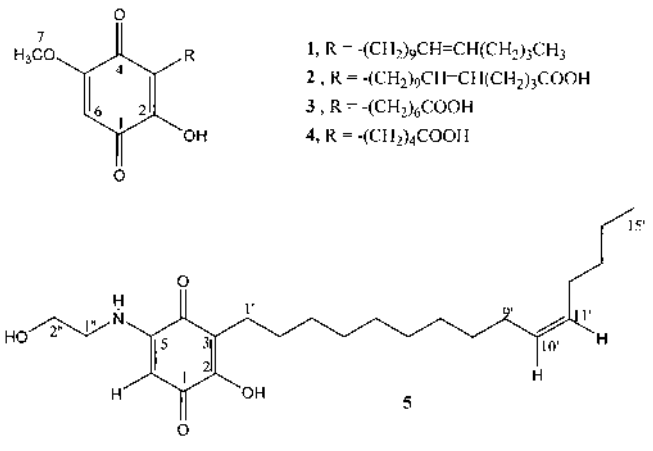

Chart 1. Structures of Maesanin (**1**) and Its Microbial Metabolites (**2**—**5**)

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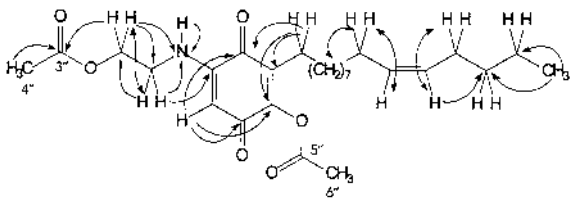

Chart 2. Structure and Selected 2D Correlations of **6**

Single-headed and double-headed arrows indicate HMBC and COSY correlations, respectively.

HMQC revealed that the triplet at δ 6.01 was not attached to any carbon and, when ^{1}H-NMR was run in CD_3OD, the signal at δ 6.01 disappeared and the methylene quartet at δ 3.37 collapsed into a triplet, suggesting an exchangeable proton at δ 6.01. A three-bond HMBC correlation existed between the protons at δ 3.37 and the quaternary ring carbon C-5 at δ 147.1. Also, another three-bond correlation was shown between the protons at δ 4.29 and the acetyl carbonyl at δ 170.0 (Chart 2). Finally, ^{15}N-HMBC revealed one-, two- and three-bond correlations between the protons at δ 6.01, 3.37 and 4.29, respectively, and a single nitrogen atom at δ 79.6 (Chart 2). Compound **6** was thus characterized as the diacetyl derivative of the ethanolamine conjugate of 5-desmethoxymaesanin.

The reported types of conjugation reactions (also referred to as phase II reactions) performed by microorganisms include acetylation, methylation, glycosidation, sulfation, phosphorylation, and amino acid conjugation. The amino acids involved in conjugation are usually glycine, lysine, and serine.[9,10] In addition, the amination of hydroxyl groups has also been reported.[11,12] Consequently, a metabolic route for the production of **5** by *D. polymorphus* can be speculated. The first step would be an *O*-demethylation at C-5, a common reaction catalyzed by cytochrome P450. The following step would be conjugation with ethanolamine to produce **5**. The source of ethanolamine is most probably the amino acid serine, abundantly present in the complex growth medium. This is the first report of a phase II conjugation in microbial systems in which ethanolamine is the substrate-conjugated entity.

With the availability of four microbial metabolites, as possible mimics of mammalian ones, probing the metabolic fate of **1** in mammals was attempted. When **1** was administered to male Wistar rats *via* intraperitoneal (i.p.) injection, the urine acquired a brownish color within 2 h of administration to the test group. Incubation of the collected urine with β-glucuronidase for 16 h, followed by extraction with EtOAc and subsequent HPLC analysis of the EtOAc extract failed to detect metabolites that correlated with the microbial ones. Instead, two minor peaks were detected that eluted at different retention times. The retention times of **1** and its microbial metabolites, **2**—**5**, were 26.4, 18.4, 12.2, 7.2 and 24.0 min, respectively, while those of the two minor components were 5.0 and 13.2 min. Due to their low yields, these compounds could not be isolated and their structures were not determined. Also, an attempt to characterize them *via* GC/MS was not successful due to the thermal instability of the standards **2**—**5** under various conditions. Thus, this study does not establish conclusively that these minor components are mammalian metabolites of **1**. Subsequent urine analysis by the Ames Multistix® strips showed a high level of blood and bilirubin which offered an explanation for the intense reddish brown color of test urine and suggested that **1** may be nephrotoxic.

Experimental

General Experimental Procedure Melting points were determined in open capillary tubes using a Thomas–Hoover capillary melting point apparatus and are uncorrected. The IR spectra were recorded with an ATI Mattson Genesis Series FT-IR spectrophotometer. UV spectra were obtained with a Hitachi 2000 dual beam spectrophotometer. The one dimensional (1D) ^{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 two dimensional (2D) inverse detection experiments: correlation spectroscopy (COSY), heteronuclear multiple quantum correlation (HMQC) and heteronuclear multiple bond correlation (HMBC) were also run on the same machine under the specified pulse sequences for each type of experiment, except for the ^{15}N-HMBC experiments which were run on a Bruker® Avance DRX 500 FT spectrometer. The chemical shift values are reported as parts per million (ppm) units relative to tetramethylsilane (TMS) for ^{1}H- and ^{13}C-, and relative to nitromethane for ^{15}N; the coupling constants are in Hz (in parentheses).[13] Abbreviations for NMR signals are as follows: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet. For the ^{13}C-NMR spectra, multiplicities were determined by the distortionless enhamncement by polarization transfer (DEPT) experiment.[13] Low-resolution electrospray ionization mass spectra (LR-ESI-MS) were obtained using a thermospray Vestec® Model 201 mass spectrometer operating in the filament-on mode. High-resolution fast atom bombardment mass spectral (HR-FAB-MS) analysis (Fisons/VG Autospec Q) was carried out at the University of Kansas. Analysis of urine components was done by Ames Multistix® Reagent Strips (Miles Inc., Elkhart, IN).

Chromatographic Conditions TLC: precoated Si 250F plates (Baker); developing system: EtOAc–methanol (9 : 1); visualization: visible light, UV light, and/or 50% H_2SO_4. Column chromatography: silica gel 230—400 mesh (Merck). HPLC: Waters® LC-1 module equipped with an automatic injector, dual-pump, gradient controller, a variable wavelength UV detector and controlled by a Millennium® 2001 software package operating under Microsoft Windows 3.1; Method: Prodigy® C8 column (250×4.5 mm, Phenomenex, Torrance, CA), step gradient elution going from 25% aqueous CH_3CN to 100% CH_3CN over 30 min, flow rate: 1.2 ml/min, detection wavelength: 280 nm, injection volume: 20 μl.

Microorganisms and Media *Debaryomyces polymorphus* was obtained from The University of Mississippi, Department of Pharmacognosy Culture Collection, and was originally obtained from the American Type Culture Collection (ATCC), Rockville, Maryland. Stock fungal cultures were maintained on agar slants of media recommended by the ATCC and were stored at 4 °C. All fermentations were run in complex culture media of the following composition: 5 g yeast extract (Difco Labs, Detroit, MI), 5 g bacto-peptone (Difco Labs), 5 g NaCl, 5 g Na_2HPO_4, 20 g dextrose, and distilled H_2O to 1 l (Medium α).

Substrate In addition to 200 mg of the isolated natural product, additional amounts of **1** were prepared according to the synthetic scheme reported by Kubo *et al*.[1,4]

Biotransformation of 1 A *D. polymorphous* inoculum was grown for 3 d in 25 ml of complex medium α held in 125 ml steel-capped Belco flasks and incubated on a rotary shaker (New Brunswick Model G10-21) at 250 rpm at room temperature (stage I). Eight ml of stage I culture was equally divided on 4 1-l steel-capped Belco flasks each containing 250 ml of fresh medium α and incubated under the same conditions as stage I (stage II). After one day, 2 ml of 10% ethanolic solution of **1** was equally divided among the four flasks (50 mg/flask) and incubated for 14 d. At the end of the fermentation period the media were filtered. Both the filtrate and biomass were exhaustively extracted with EtOAc (3×300 ml). The combined extracts were dried over Na_2SO_4 and concentrated *in vacuo* at 40 °C to yield a viscous dark violet residue (*ca*. 200 g). The residue was chromatographed twice on a silica gel column using gradient elution (hexane–EtOAc (3 : 1, v/v), EtOAc (100%), EtOAc–methanol (2 : 3, v/v)). The obtained metabolite was further purified on a C_{18} cartridge to yield a purplish brown powder, **5** (0.032 g, 16%).

Acetylation of 5 Three mg of **5** were dissolved in 0.6 ml of Ac_2O and one drop of pyridine was added. The mixture was allowed to stir for 0.5 h at room temperature under nitrogen atmosphere. The reaction was quenched with H_2O (5 ml) and the aqueous phase was extracted with EtOAc. The or-

Table 1. ^{1}H- and ^{13}C- Assignments of Compounds **5** and **6** (in CD_3OD; at 400 and 100 MHz, Respectively)

C	**5**		**6**[a]	
	δ_C	δ_H (J Hz)	δ_C	δ_H (J Hz)
1	180.4 s	—	178.4 s	—
2	157.2 s	—	151.9 s	—
3	114.3 s	—	133.2 s	—
4	178.0 s[b]	—	183.9 s	—
5	158.0 s[b]	—	147.1 s	—
6	90.0 d[b]	5.33 s	97.0 d	5.44 s
7	—	—	—	—
1′	23.7 t	2.32 t (6.7)	24.0 t	2.35 t (6.7)
2′	27.9 t	1.31 m	28.6 t	1.41 m
3′—8′	30.2—30.8 t	1.31 m	29.7—30.2 t	1.30 m
9′	27.9 t[c]	2.00 m	27.6 t[d]	2.00 m
10′	130.5 d[d]	5.33 t (4.6)	130.25 d[e]	5.34 t, (5.2)
11′	130.6 d[d]	5.33 t (4.6)	130.29 d[e]	5.34 t (5.2)
12′	27.7 t[c]	2.00 m	27.3 t[d]	2.00 m
13′	23.2 t	1.31 m	22.7 t	1.31 m
14′	33.0 t	1.31 m	32.4 t	1.31 m
15′	14.2 q	0.90 q (7.1)	14.2 q	0.90 q (6.3)
1″	45.7 t	3.38 t (5.3)	42.2 t	3.37 q (5.3)
2″	60.1 t	3.75 t (5.3)	61.9 t	4.29 t (5.3)
3″	—	—	170.0 s	—
4″	—	—	20.9 q[c]	2.35 s
5″	—	—	168.6 s	—
6″	—	—	21.2 q[c]	2.10 s
NH	—	—	—	6.01 t (5.3)

a) In $CDCl_3$. *b*) Indirectly detected by HMBC and/or HMQC. *c*—*e*) Assignments are interchangeable within the same column.

ganic phase was dried over Na_2SO_4, concentrated *in vacuo* and purified on a C_{18} cartridge to afford an orange red oily residue, **6** (3 mg, 82%).

Detection of Mammalian Metabolites Six male Wistar rats (Charles Rivers, Portage, MI) were grown for one week to a weight of 300—350 g each. The rats were housed at a room temperature of 22 to 23 °C on a 12:12 h light/dark cycle, were provided Rodent Laboratory Chow (Purina) and water *ad libitum*, and were transferred to metabolic cages during experiment times. The first rat, serving as a vehicle control, was administered i.p. 0.40 ml of plain corn oil. The remaining five rats were each i.p. administered **1** in corn oil at a dose of 80 mg/kg. Urine was collected from all rats in polyethylene Falcon® tubes over a period of 72 h, pooled and frozen for later analysis.

Analysis of Urine All procedures were simultaneously conducted on collected test and control urine. For standard urine composition, one Ames Multistix® strip was dipped in each solution and both strips were examined against the supplied reference color chart. For metabolite analysis, pooled urine was extracted with EtOAc (3×30 ml). The organic extracts were pooled, dried over Na_2SO_4 and concentrated at 40 °C *in vacuo*, redissolved in 1 ml of MeOH and analyzed by TLC and HPLC. The aqueous phases of the test and control groups were lyophilized, and half-gram samples were each dissolved in 10 ml of NaOAc buffer (pH 5.0) and incubated with 1 ml of β-glucuronidase (*ca*. 5000 Sigma units, Sigma Chemical, St. Louis, MO) at 37 °C for 16 h.[14] Solutions were extracted with EtOAc and the extracts were dried, concentrated and reconstituted in 1 ml MeOH. The methanolic solutions were subjected to TLC and HPLC analysis.

Metabolite **5**, 2-Hydroxy-5-(ethanolamino)-3-(10′-*Z*-pentadecenyl)-1,4-benzoquinone: *Rf* 0.21; UV (MeOH) λ_{max} (log ε) 332 (4.24) and 518 (2.50) nm; IR (KBr) ν_{max} 3422, 3268, 2924, 2852, 1533, 1490 cm^{-1}. ^{1}H- and ^{13}C-NMR: see Table 1. LR-ESI-MS *m/z* $[M+1]^+$ 392. HR-FAB-MS *m/z* $[M+1]^+$: 392.2782 (Calcd for $C_{23}H_{38}NO_4$: 392.2801).

Compound **6**, 2-Hydroxy-5-(ethanolamino)-3-(10′-*Z*-pentadecenyl)-1,4-benzoquinone diacetate: *Rf* 0.70 ; IR (NaCl disc) ν_{max} 3349, 2925, 2854, 1777, 1735, 1671, 1603, 1513 cm^{-1}. ^{1}H- and ^{13}C-NMR: see Table 1. HR-FAB-MS *m/z* $[M+1]^+$: 476.3010 (Calcd for $C_{27}H_{42}NO_6$: 476.3012).

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