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New monoterpenoid by biotransformation of thymoquinone using *Aspergillus niger*

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ABSTRACT

Microbial transformation of thymoquinone (5-isopropyl-2-methyl-cyclohexa-2,5-diene-1,4-dione) (**1**) by suspended cell-cultures of the plant pathogenic fungus *Aspergillus niger* resulted in the production of three metabolites. These metabolites were identified as 5-isopropyl-2-methyloxepin-1-one (**2**), 3-hydroxy-5-isopropyl-2-methylcyclohexa-2,5-diene-1,4-dione (**3**), and 5-isopropyl-2-methylbenzene-1,4-diol (**4**) by different spectroscopic methods. Metabolite **2** was found to be a new compound. Compound **4** showed a potent antioxidant activity.

1. Introduction

Microorganisms have been used extensively for the hydroxylation of terpenoids and steroids. Their enzymes catalyze reactions with high regio- and stereo-specifity. Microorganisms have the ability to oxidize terpenoidal compounds and produce derivatives of immense synthetic and commercial importance. Therefore hydroxylation of a large number of substances, including terpenoids, has been studied by employing a variety of microorganisms [1]. In particular, *Aspergillus niger* has performed a variety of reactions on terpenoidal compounds, including oxidations [2], reductions [3], and lactonizations [4]. However, no studies on the transformation of thymoquinone (**1**), a monoterpene, by fungi have been reported in the literature.

Thymoquinone (2-Isopropyl-5-methyl cyclohexa-2,5-diene-1,4-dione, $C_{10}H_{12}O_2$) (**1**) was isolated from the seeds of *Nigella sativa*. It has been shown to have anti-tumor activity against liver, prostate, colon, breast, lung and pancreatic cancers [5–7]. Thymoquinone (**1**) has also shown antioxidant [8], analgesic [9], and anticonvulsant properties [10].

In continuation of our studies on the biotransformation of bioactive compounds and drugs [11–16], we synthesized derivatives of thymoquinone (**1**) using a plant pathogenic fungus *Aspergillus niger*. Transformation of **1** by *A. niger* resulted in the formation of three metabolites, 5-isopropyl-2-methyloxepin-1-one (**2**), 3-hydroxy-5-isopropyl-2-methylcyclohexa-2,5-diene-1,4-dione (**3**), and 5-isopropyl-2-methylbenzene-1,4-diol (**4**). Metabolites **2–4** were tested for their antioxidant activity in comparison to substrate **1**, and ascorbic acid, and a potent activity was observed in metabolite **2**.

2. Experimental

2.1. General

Thymoquinone (**1**) was obtained from the Sigma-Aldrich. Silica gel precoated plates (Merck, PF_{254}; 20 × 20, 0.25 mm) were used for chromatography. Silica gel (70–230 mesh, Merck) was used for column chromatography. UV Spectra (in nm) were recorded in methanol with a Hitachi U-3200 spectrophotometer. Infrared (IR) spectra were recorded in KBr discs on a FT-IR-8900 spectrophotometer. ^{1}H- and ^{13}C-NMR spectra were recorded in $CDCl_3$ on a Bruker Avance-300 NMR spectrometer at 300 and 75 MHz, respectively, with tetramethylsilane (TMS) as the internal standard. Standard pulse sequences were used for distortionless enhancement by polarization transfer (DEPT) and 2D-NMR experiments. The chemical shifts (δ values) were reported in parts per million, relative to TMS at 0 ppm. The coupling constants (J values) were reported in Hertz (Hz). High resolution mass spectrometry was performed using LC Mass Bruker Apex-IV mass spectrometer utilizing an electrospray interface.

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2.2. Microorganisms and culture medium

Aspergillus niger (ATCC 16404) was purchased from the American Type Culture Collection (ATCC), and grown on Sabouraud-4% potato dextrose-agar (Merck) at 28 °C and stored at 4 °C. The medium for *A. niger* was prepared by mixing the following ingredients into distilled H_2O (3.0 L): glucose (60.0 g), peptone (15.0 g), yeast extract (15.0 g), KH_2PO_4 (15.0 g), and NaCl (15.0 g) [17].

2.3. Fermentation and extraction conditions for compound 1

The fungal medium was transferred into 250 mL conical flasks (100 mL each), and autoclaved at 121 °C. Mycelia of *A. niger* were transferred to all the flasks, and incubated at 28 °C for three days with rotary shaking (128 rpm). After three days, compound **1** (1.00 g, 6.1 mmol) was dissolved in 40 mL acetone, and added to each flask (25 mg/1.0 mL acetone). These flasks were placed on a rotatory shaker (128 rpm) at 28 °C for fermentation. Parallel control experiments were conducted which included an incubation of the fungus without sample **1**, and another incubation of **1** in a medium without fungus. Time course studies were carried out after every 24 h, and the degree of transformation was analyzed by TLC. After 7 days, the culture medium was filtered and extracted with ethyl acetate (9 L) in three portions. The extract was dried over anhydrous Na_2SO_4, evaporated under reduced pressure, and the brown gummy crude residue (1.5 g) was analyzed by thin layer chromatography.

2.4. Isolation of transformed products

The crude extract was dissolved in chloroform: methanol (8:2 v/v), absorbed on silica gel (2.0 g), and subjected to column chromatography. The eluent system consisted of gradient mixtures of chloroform and methanol. Compounds **1** (50 mg), and **2** (20 mg) were eluted with $CHCl_3$, while compounds **3** (80 mg), and **4** (280 mg) were eluted in $CHCl_3$/MeOH (9.5:0.5 v/v).

5-Isopropyl-2-methyloxepin-1-one (**2**), oily. UV (MeOH): λ_{max} (log ε) 244 nm (2.1). IR (MeOH): 2951, 1643, 1384, 1017 cm^{-1}. ^{1}H- and ^{13}C-NMR: Tables 1 and 2.

Hydroxythymoquinone (3-Hydroxy-5-isopropyl-2-methylcyclohexa-2,5-diene-1,4-dione) (**3**), white solid, m.p.: 120–122 °C. UV (MeOH): λ_{max} (log ε) 248 nm (2.4). IR (MeOH): 3417, 2952, 1640, 1384, 1019 cm^{-1}. ^{1}H- and ^{13}C-NMR: Tables 1 and 2.

5-Isopropyl-2-methylbenzene-1,4-diol (**4**), white solid, m.p.: 138–140 °C. UV (MeOH): λ_{max} (log ε) 289 nm (2.3). IR (MeOH): 3340, 2966, 1426, 1382, 1035, 818, 738 cm^{-1}. ^{1}H- and ^{13}C-NMR: Tables 1 and 2.

2.5. Stock and sample solutions

The stock solutions of the test compounds **1–4** (1.5 mg/mL) were prepared in methanol, and serially diluted with the methanol to obtain lower dilutions (1.95–250, 5.47–700 and 0.5–32.0 μg/mL for **4**, **1**, and ascorbic acid, respectively).

Table 2
^{13}C NMR data of compound **1**, and its metabolites **2–4** (300 MHz; $CDCl_3$).

C. No.	1	2	3	4
1	188.5	167.8	188.2	147.7
2	145.1	132.5	151.4	121.6
3	133.8	130.9	116.7	113.0
4	187.4	128.8	183.2	146.3
5	154.9	154.9	150.4	133.1
6	130.3	68.2	132.4	117.7
7	21.4	11.0	7.9	15.4
8	26.5	26.5	26.7	26.8
9	15.3	14.1	21.3	22.7
10	15.3	14.1	21.3	22.7

Multiplicities were determined by DEPT experiments.

2.6. Antioxidant activity (1,1-diphenyl-2-picryl-hydrazyl (DPPH) free radical scavenging activity)

Antioxidant activity of the test samples and the standard was assessed based on radical scavenging effect against stable DPPH free radical, using a modified method [18]. DPPH solution (0.002% w/v) was prepared in methanol. Ascorbic acid was used as the standard in solutions ranging between 0.5 and 32 μg/mL. 1 mL of this solution was mixed with either 1 mL of sample solution or the solution of standard to be tested separately. These solution mixtures were kept in the dark for 20 min, and optical density was measured at 517 nm using a spectrophotometer against methanol. The blank was used as 1 mL of methanol with 1 mL of DPPH solution (0.002%). The optical density was recorded and percent of inhibition was calculated using the formula given below [19]:

% Inhibition of DPPH activity = A–B/A × 100, where A is optical density of the blank, and B is optical density of the sample.

3. Results and discussion

Screening scale experiment showed that *Aspergillus niger* (ATCC 16404) has the capacity to transform compound **1** into its derivatives, thus a large scale experiment was performed. Incubation of thymoquinone (**1**) with *A. niger* yielded three metabolites **2–4** (Fig. 1). Metabolite **4** was obtained as a major product with 28.0% yield, while compounds **2**, and **3** were obtained as minor products with 4.9 and 7.3% yields, respectively. A time course analysis of the transformation of **1** revealed that metabolite **4** was formed after 24 h of incubation, while metabolites **2**, and **3** were detected only after 72 h. Structures of the metabolites were identified through comparative spectroscopic studies with thymoquinone (**1**).

The HRESI-MS of metabolite **2** revealed a protonated molecular ion peak $[M+H]^+$ at *m/z* 167.1028, refers to the formula $C_{10}H_{14}O_2+H$ (calc. 167.1072), 2 a.m.u. higher than **1**. The IR spectrum showed the

Table 1
^{1}H NMR data of compound **1**, and its metabolites **2–4** (300 MHz; $CDCl_3$).

C. No.	1	2	3	4
1	–	–	–	–
2	–	–	–	–
3	6.59 (1H, q, *J* = 1.6 Hz)	7.53 (1H, d, *J* = 8.9 Hz)	–	6.64 (1H, s)
4	–	7.71 (1H, d, *J* = 9.0 Hz)	–	–
5	–	–	–	–
6	6.52 (1H, d, *J* = 1.2 Hz)	4.22 (2H, m)	6.46 (1H, s)	6.55 (1H, s)
7	2.04 (3H, d, *J* = 1.6 Hz)	0.93 (3H, s)	1.94 (3H, s)	2.18 (3H, s)
8	3.03 (1H, d, *J* = 6.8 Hz, *J* = 1.2 Hz)	2.14 (1H, m)	3.02 (1H, m)	3.14 (1H, m)
9	1.13 (3H, d, *J* = 6.8 Hz)	0.99 (3H, d, *J* = 6.7 Hz)	1.15 (3H, d, *J* = 6.9 Hz)	1.22 (3H, d, *J* = 6.9 Hz)
10	1.13 (3H, d, *J* = 6.8 Hz)	0.99 (3H, d, *J* = 6.7 Hz)	1.15 (3H, d, *J* = 6.9 Hz)	1.22 (3H, d, *J* = 6.9 Hz)

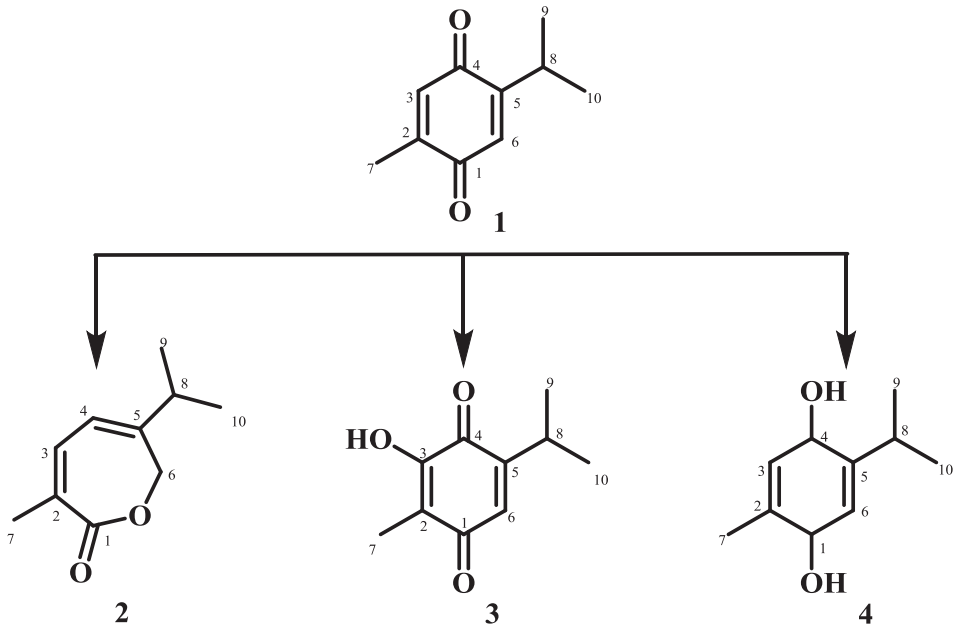


Fig. 1. Biotransformation of thymoquinone (**1**) by *Aspergillus niger*.

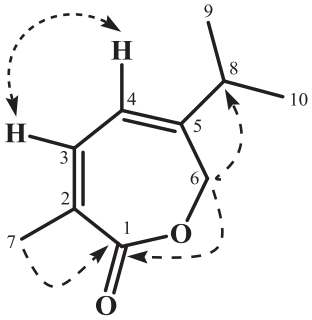


Fig. 2. HMBC (- - - ➔) and COSY (⋰⋱) correlations in metabolite **2**.

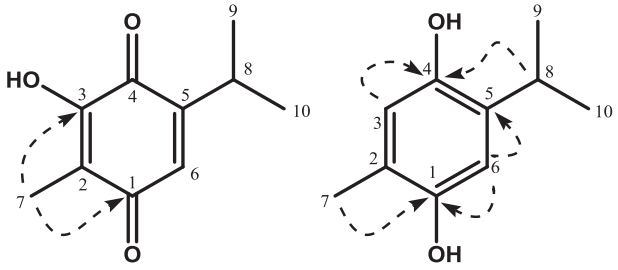
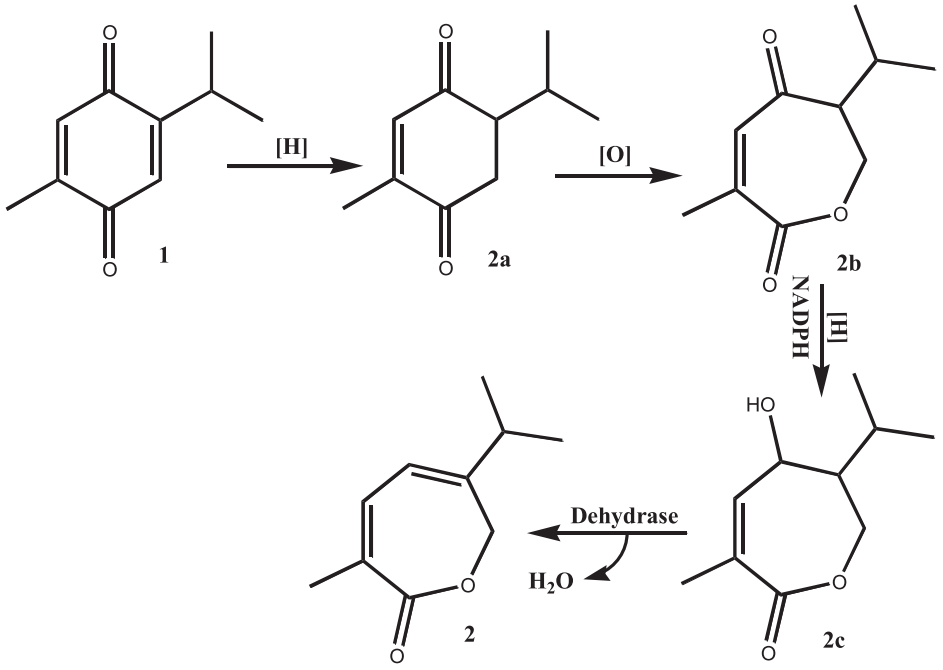


Fig. 3. HMBC (- - - ➔) correlations in metabolites **3** and **4**.



Scheme 1. Proposed pathway for the synthesis of new metabolite **2**.

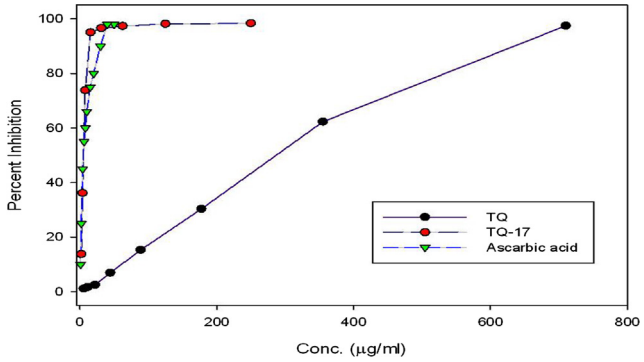


Fig. 4. Percent inhibition of metabolite **4** (TQ-17) in comparison with thymoquinone (**1** = TQ) and ascorbic acid.

presence of a lactone carbonyl functionality (1643 cm^{-1}). The ^{1}H NMR spectrum showed a methylene protons signal at δ 4.22 (m), while the ^{13}C NMR spectrum showed a methylene carbon at δ 68.2, along with disappearance of C-6 olefinic methine signal at δ 130.3, in comparison to compound **1**. Moreover the upfield shifts of C-1 (δ 167.8), and C-4 (δ 128.8) and disappearance of keto carbonyl carbons at C-1 (δ 188.5), and C-4 (δ 187.4), in comparison to compound **1**, suggested the conversion of ketone into a lactone along with the reduction of C-4 carbonyl into a methine carbon. HMBC spectrum of metabolite **2** showed correlations of H_2-6 (δ 4.22) with C-8 (δ 26.5) and H-8 (δ 2.14) with C-6 (δ 68.2) (Fig. 2) which supported the oxidative cleavage of C-6 – C-1 bond (Baeyer–Villiger type oxidation). COSY 45° spectrum showed correlations of H-3 with H-4 (Fig. 2) which supported the reduction of C-4 carbonyl. The structure of compound **2** was thus deduced as 5-isopropyl-2-methyloxepin-1-one (Scheme 1). Compound **2** may arise from Baeyer–Villiger type oxidation of partially reduced benzoquinone, followed by elimination of water (Scheme 1).

The IR spectrum of metabolite **3** showed the presence of OH functionality (3417 cm^{-1}). The ^{1}H NMR spectrum of compound **3** was similar to that of **1** but the ^{13}C NMR spectrum showed an additional quaternary carbon signal at δ 116.7, and the disappearance of methine C-3 (δ 133.8), in comparison to compound **1**, which indicated the hydroxylation at C-3. HMBC spectrum showed correlations of H_3-7 (δ 1.94) with C-3 (δ 116.7) suggested that the hydroxylation occurred at C-3 (Fig. 3). These data supported the structure of compound **3** as 3-hydroxy-5-isopropyl-2-methylcyclohexa-2,5-diene-1,4-dione.

The IR spectrum of metabolite **4** showed the presence of OH functionality (3340 cm^{-1}). The ^{13}C NMR spectrum showed quaternary carbon signals at δ 146.3 and 147.7 along with disappearance of C-4 (δ 187.4), and C-1 (δ 188.5) keto carbonyl signal, in comparison to compound **1**. HMBC Spectrum of metabolite **4** showed correlations of H-3 (δ 6.64) with C-4 (δ 146.3), and H-6 (δ 6.55) with C-1 (δ 147.7) which supported the conversion of **1** to *p*-disubstituted phenol (Fig. 3). The structure of compound **4** was deduced as 5-isopropyl-2-methylbenzene-1,4-diol.

Metabolite **4** was tested for its antioxidant activity in comparison to **1** and ascorbic acid, and a potent antioxidant activity was observed (Fig. 4).

In conclusion, biotransformation of thymoquinone (**1**) by *A. niger* proved to be an effective procedure for lactonization and hydroxylation of this monoterpene. Compound **2** was reported for the first time, whereas metabolite **4** was obtained as a major product. This procedure can be used to synthesize new derivatives of monoterpene with interesting biological activities.

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