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Epoxidationof Ferutinin by Different Fungi and Antibacterial Activity of its Metabolite

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ABSTRACT

Structural transformation of the sesquiterpene ester, ferutinin (**1**), by suspended fungal cultures of *Aspergillus niger*, *Rhizopus stolonifer*, and *Fusarium lini*, resulted in the formation of a metabolite. This metabolite was identified as ferutinin α-epoxide (**2**), by different spectroscopic techniques. Metabolite **2** showed a good antibacterial activity against *Staphylococcus aureus*compared to ferutinin (**1**).

Keywords: Sesquiterpene ester, ferutinin, fungal transformation, *Aspergillus niger*, *Rhizopus stolonifer*, *Fusarium lini*, ferutinin α-epoxide, epoxidation, *Staphylococcus aureus*, antibacterial activity.

INTRODUCTION

The ability of microorganisms to oxidize chemical compounds has an immense syntheticand commercial importance. The hydroxylation of a large number of substances, including terpenes, has been studied by employing a variety of microorganisms.[1] However, no report concerning the transformation of ferutinin (**1**)by microorganisms or plant cell cultures has been found in the literature.

Ferutinin (**1**),$C_{22}H_{30}O_4$, is a p-hydroxybenzoyl ester and a potent naturally occurring non-steroid estrogenic compound. It is an agonist for an estrogen receptor (ER), and it opens the mitochondrial permeability transition pore (MPTP).[2]Ferutinin (**1**) showed good antibacterial activity against *Staphylococcus aureus*.[3]

Ferutinin (**1**) was initially isolated from the roots of *Ferula harmonis*, a*Ferula tenuisecta*Eug. Kor.[4]which is a native plant of Syria and Lebanon that grows in particular at more than 2,500 meters on Mount Hermon.

In continuation of our biotransformation studies on bioactive compounds,[5-8]the synthesis of the derivative of ferutinin (**1**) by transformation from *Aspergillus niger*, *Rhizopus stolonifer*, and *Fusarium lini*is described here within. This resulted in the formation of compound **2** which was identified as ferutinin α-epoxide (**2**),and its spectroscopic data has been reported here for the first time.Metabolite **2** was tested for antibacterial activity against *S. aureus*, and it showed the same level of antibacterial activity as substrate **1**.

EXPERIMENTAL

General

Ferutinin (**1**) was isolated from the roots of *Ferula harmonis*[3]and was obtained from the Department of Pharmaceutical Sciences, Faculty of Pharmacy, University of Jordan, Amman-11942, Jordan. Silica gel pre-coated plates (Merck, PF_{254}; 20×20, 0.25mm) were used for thin layer chromatography (TLC) while silica gel (70-230 mesh, Merck) was used for the column chromatography. The UV Spectra (in nm) were recorded in methanol with a Hitachi U-3200 spectrophotometer. The infrared (IR) spectra were recorded in KBr discs with an FT-IR-8900 spectrophotometer. ^{1}H- and ^{13}C-NMR

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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 (δvalues) werereported in parts per million, relative to TMS at 0 ppm. The coupling constants (*J*values) were reported in Hertz. The electron impact mass spectra (EI-MS) and high resolution electron impact mass spectra (HREI-MS) were recorded on a Jeol JMS-600H mass spectrometer in *m/z* (rel. %).

Microorganisms and Culture Medium

Aspergillus niger(ATCC 10549)was purchased from the American Type Culture Collection,while *Rhizopus stolonifer* (NRRL 1392) and *Fusarium lini* (NRRL 68751) were purchased from the Northern Regional Research Laboratories (NRRL), Peoria, Illinois, U.S.A., 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 (1.5L): glucose (30.0 g), peptone (7.5 g), yeast extract (7.5 g), KH_2PO_4 (7.5 g), NaCl (7.5 g), and glycerol (7.5 mL).The medium for *R. stolonifer* was prepared by mixing the following ingredients into H_2O (1.5L): glucose (30.0 g), peptone (7.5 g), yeast extract (7.5 g), KH_2PO_4 (7.5 g), and NaCl (7.5 g).The medium for *F. lini* was prepared by mixing the following ingredients into H_2O (1.5L): glucose (30.0 g), peptone (7.5 g), yeast extract (7.5 g), KH_2PO_4 (7.5 g), and NaCl (7.5 g).

Fermentation and Extraction Conditionsfor Substrate 1

The fungal medium was transferred into 250 mL conical flasks (100 mL each) and autoclaved at 121°C. Mycelia of *A. niger*, *R. stolonifer,* and *F. lini* were transferred to all the flasks and incubated at 28°C for three days with rotary shaking (128 rpm). After three days, compound **1** (0.25 g, 0.70 mmol) for each fungus was dissolved in 6 mL acetone and added to each flask (20 mg/0.5 mL acetone),and the 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 hours,and the degree of transformation was analyzed by TLC. After 10 days, the culture medium was filtrated and extracted with dichloromethane (12 L) in three portions. The extract was dried over anhydrous $Na_2SO_4$and evaporated under reduced pressure, and the brown gummy crude residue around (0.30 g) for each experiment was analyzed by TLC.

Isolation of Transformed Products

The crude extract was dissolved in dichloromethane and methanol (8:2 v/v), absorbed on silica (2.0 g) and subjected to column chromatography. The eluent system consisted of gradient mixtures of dichloromethane and methanol. Compound **1** (150 mg) was eluted in CH_2Cl_2 and MeOH (9.8:0.2 v/v), while compound **2**(20 mg) was eluted inCH_2Cl_2 and MeOH (9.6:0.4 v/v)from the *A. niger* extract.Compound **1** (200 mg) was eluted in CH_2Cl_2 and MeOH (9.8:0.2 v/v), while compound **2**(5 mg) was eluted inCH_2Cl_2 and MeOH (9.6:0.4 v/v) from the *R. stolonifer* extract.Compound **1** (220 mg) was eluted in CH_2Cl_2 and MeOH (9.8:0.2 v/v), while compound **2**(5 mg) was eluted inCH_2Cl_2 and MeOH (9.6:0.4 v/v) from the *F. lini* extract.

Ferutinin α-epoxide (**2**)

UV (MeOH): λ_{max} (logε) 249 nm (1.2). IR (MeOH): 3915, 3786, 3699, 3661, 2926, 2855, 1730, 1666, 1587, 1553, 1445, 1352, 1242, 1165, 1118, 964, 851 cm^{-1}. ^{13}C- and ^{1}H-NMR: Table 1. EI-MS: *m/z* 331 (3, $[M\text{-}CH(CH_3)_2]^+$), 244 (2), 193 (11), 175 (6), 151 (8), 121 (100), 99 (15), 93(25), 56 (29). HREI-MS: *m/z* 331.1531 ($[M\text{-}CH(CH_3)_2]^+$, $[C_{19}H_{23}O_5]^+$; calc. 331.1545).

Antimicrobial Activity Assay

Cultures of *Staphylococcus aureus*, ATCC 25923,weregrown in a nutrient broth (Oxoid) overnight at 37 ^{o}C and maintained on nutrient agar (Oxoid) plates at 4 ^{o}C.Ferutinin (**1**) and its metabolite **2** were screened for their antimicrobial activity using the diffusion technique on solid media. Cups of 8 mm diameter on seeded nutrient agar plates were filled with the saturated

ethanolic solution of ferutinin (**1**) and its transformed product **2**. The plates were incubated for 24 hours at 37 ^{o}C for *S. aureus*. Control cups containing the solvent alone were also included. Minimum inhibitory concentration was determined for compounds **1** and **2** using the broth dilution method. Chloramphenicol (Pliva) was tested as a standard. The experiment was carried out intriplicate.

RESULTS AND DISCUSSION

The screening scale experiment showed that*Aspergillus niger*(ATCC 10549),*Rhizopus stolonifer*(NRRL 1392), and *Fusarium lini*(NRRL 68751) all have a capacity to transform compound **1**into its derivative **2**, thus a large scale experiment was performed.Incubation of ferutinin (**1**) with these fungiyielded one metabolite **2** (Figure1). Metabolite **2** was obtained as a major product with 7.7 %, 1.9 % and 1.9 % yield by *A. niger*, *R. stolonifer* and *F. lini*, respectively. A time course analysis of the transformation of **1** revealed that metabolite **2** wasformed after 48 hour of incubation. The structure of the metabolite **2** was elucidated through spectroscopic studies.

The HREI-MS of metabolite **2** exhibited an $[M\text{-}CH(CH_3)_2]^+$ at *m/z* 331.1531, ($C_{19}H_{23}O_5$, calc. 331.1545), 16 a.m.u. higher than **1**($[M\text{-}CH(CH_3)_2]^+$ at *m/z* 315.1605, calc. 315.1596,$C_{19}H_{23}O_4$), indicating the incorporation of an oxygen atom.The ^{1}H-NMR spectrum showed an additional epoxide-bearing methine proton signal at δ 2.86 (t, *J* = 7.5 Hz), while the ^{13}C-NMR spectrum showed an epoxide-bearing methine carbon at δ 61.0 and quaternary carbon at δ 56.2, and disappearance of C-9 and C-8 signals at δ 125.3 and 133.5, respectively, in comparison to compound **1**. HMBC spectrum of metabolite **2** showed correlations of H_3-15 (δ_H 1.24), H_3-11 (δ_H 1.47), and H-7 (δ_H 2.25) with C-9 (δ_C 61.0), while H-9 (δ_H 2.86) showed correlations with C-1 (δ_C 44.4), C-7 (δ_C 44.3), and C-8 (δ_C 56.2), which further supported epoxidation at C-8 and C-9.A COSY 45^{o} spectrum showed correlations of H-9 withboth H_2-10. The *α* stereochemistry of the epoxide was inferred by NOESY correlations of □H-6 (δ 5.41) and □Me-15 (δ 1.24) with □Me-11 (δ1.47), and H-9 (δ_H 2.86) (Figure2).The structure of compound **2** wasdeduced as ferutinin α-epoxide.

Compound**2** was tested for its antimicrobial activity against *S. aureus*, incomparison with ferutinin (**1**) and chloramphenicol as a standard. Results indicate that ferutinin α-epoxide (**2**) has no change in antimicrobial activity against *S. aureus*, in comparison with ferutinin (**1**) (Table2). This explains that endocyclic double bond has no contribution against antimicrobial activity. Moreover, Al-Sha'eret al.[3] has reported that the phenolic group in ferutinin is responsible for the above mentioned activity which was further supported by our results.

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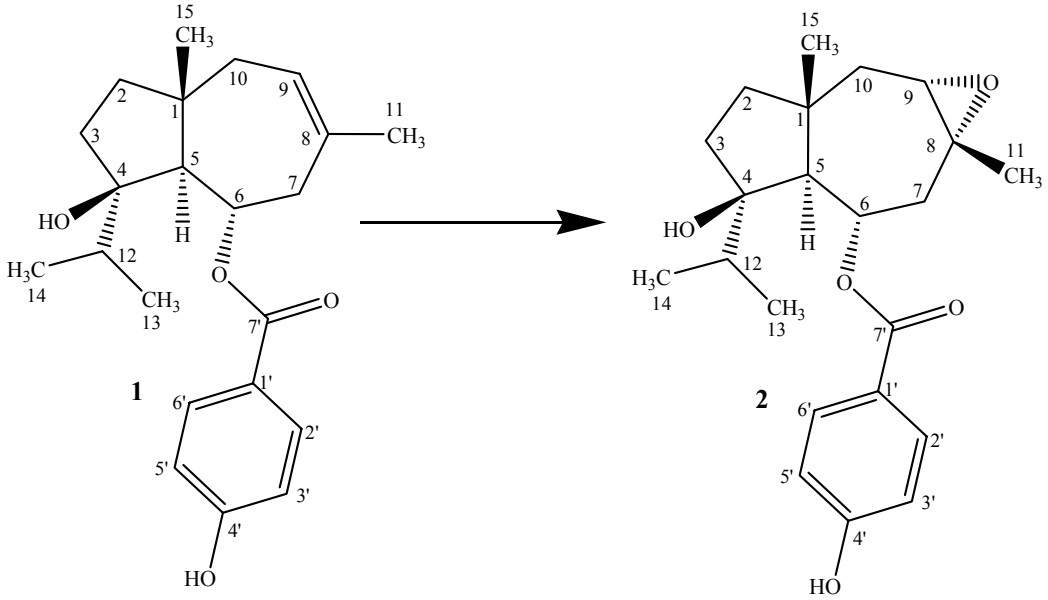

Figure 1: Biotransformation of ferutinin (1) by *A. niger*, *R. stolonifer*, and *F. lini.*

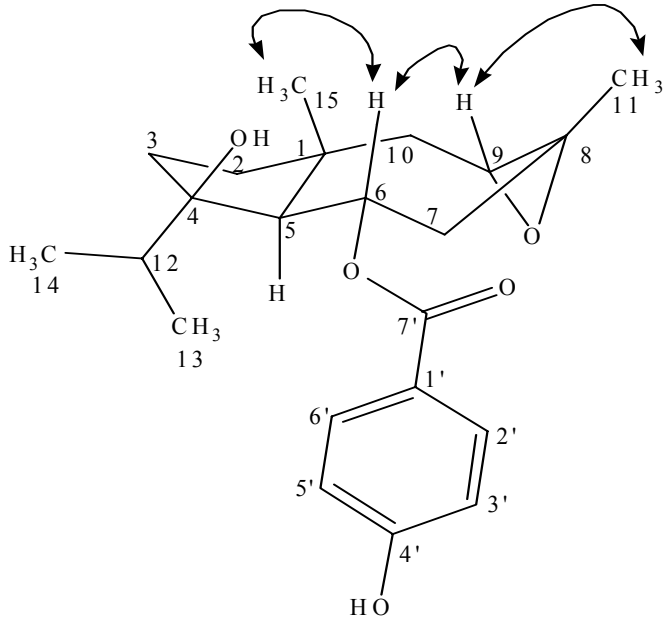

Figure 2: Key NOESY correlations in compound 2.

Table 1: ^{13}C-NMR and ^{1}H-NMR chemical shift data of compound 1and its metabolite2.

C. No.	1 ^{13}C-NMR	1 ^{1}H-NMR	2 ^{13}C-NMR	2 ^{1}H-NMR
1	44.1 (*s*)	-	44.4 (*s*)	-
2	31.5 (*t*)	1.61 (1H, m), 1.93[a] (1H, m)	31.7 (*t*)	1.62 (1H, m), 1.93[a] (1H, m)
3	41.2[a] (*t*)	1.24 (1H, m), 1.53 (1H, m)	40.5[a] (*t*)	1.21 (1H, m), 1.54 (1H, m)
4	87.0 (*s*)	-	86.0 (*s*)	-
5	60.1 (*d*)	1.99 (1H, d, *J* = 11.1 Hz)	60.8[b] (*d*)	1.84 (1H, d, *J* = 11.0 Hz)
6	71.3 (*d*)	5.26 (1H, td, *J* = 10.5, 10.5, 2.7 Hz)	70.0 (*d*)	5.41 (1H, td, *J* = 11.5, 11.5, 2.0 Hz)
7	41.4[a] (*t*)	2.27 (1H, dd, *J* = 14.1, 2.4 Hz), 2.55 (1H, t, *J* = 12.0 Hz)	44.3 (*t*)	1.91[a] (1H, m), 2.25[b] (1H, m)
8	133.5 (*s*)	-	56.2 (*s*)	-
9	125.3 (*d*)	5.53 (1H, m)	61.0[b] (*d*)	2.86 (1H, t, *J* = 7.5 Hz)
10	41.0[a] (*t*)	1.96[a](1H, m), 2.20 (1H, m)	41.2[a] (*t*)	1.29 (1H, m), 2.24[b] (1H, m)
11	26.4 (*q*)	1.80 (3H, s)	23.4 (*q*)	1.47 (3H, s)
12	37.1 (*d*)	1.83 (1H, m)	37.2 (*d*)	1.88 (1H, m)
13	18.6 (*q*)	0.92 (3H, d, *J* = 6.6 Hz)	18.5 (*q*)	0.89 (3H, d, *J* = 6.5 Hz)
14	17.6 (*q*)	0.83 (3H, d, *J* = 6.6 Hz)	17.4 (*q*)	0.82 (3H, d, *J* = 6.5 Hz)
15	20.2 (*q*)	1.08 (3H, s)	19.4 (*q*)	1.24 (3H, s)
1'	122.0 (*s*)	-	122.6 (*s*)	-
2', 6'	132.1 (*d*)	7.90 (2H, d, *J* = 8.7 Hz)	132.0 (*d*)	7.90 (2H, d, *J* = 9.0 Hz)
3', 5'	115.5 (*d*)	6.87 (2H, d, *J* = 9.0 Hz)	115.4 (*d*)	6.86 (2H, d, *J* = 8.5 Hz)
4'	161.1 (*s*)	-	160.2 (*s*)	-
7'	167.3 (*s*)	-	166.1 (*s*)	-

Multiplicities were determined by DEPT experiments.

a, b: These values are interchangeable.

Table 2:The antimicrobial activity and MIC (µg/mL) against *S. aureus* for ferutinin (1), ferutinin α-epoxide (2), and chloramphenicol.

Compound	Zone of inhibition (mm)	MIC (µg/mL)
1	22	15
2	22	15
Chloramphenicol	15	8

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إبكسدة مركب الفيروتينين عن طريق فطريات مختلفة ودراسة أيضه كمضاد للبكتيريا

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ملخـص

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