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ORIGINAL ARTICLE

A New Antifungal Aminobenzamide Derivative from the Endophytic Fungus *Fusarium* sp.

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ABSTRACT

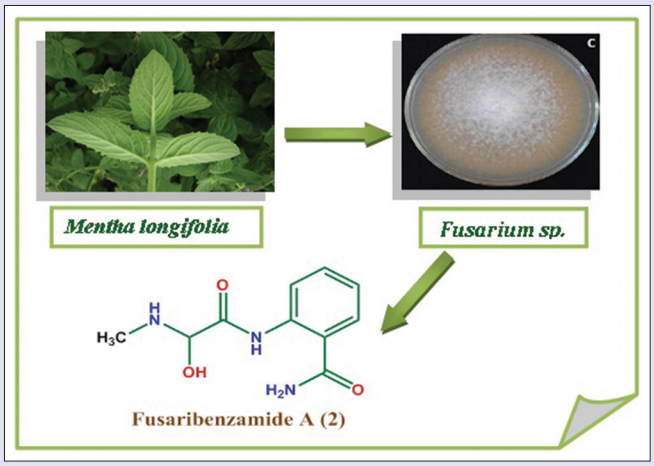
Background: Endophytic fungi attracted attention as a prolific source of bioactive natural products with a potent pharmaceutical activity and unique structure. **Objective:** The main goal of the study is to separate and identify the bioactive constituents from the endophytic fungus *Fusarium* sp. as well as to evaluate the antimicrobial of the new metabolites. **Materials and Methods:** The fungus was cultured on a rice medium, and then, the cultures were extracted with ethyl acetate (EtOAc). The EtOAc extract was chromatographed utilizing different chromatographic methods to give five metabolites. The structural determination of these metabolites was carried out by the analyses of various spectroscopic data, in addition to comparison with the formerly reported data. The antifungal and antibacterial potentials were evaluated toward various microbial strains using disc diffusion assay. **Results**: A new aminobenzamide derivative, namely fusaribenzamide A (2), and four known metabolites: (22*E*,24*R*)-stigmasta-5,7,22-trien-3-β-ol (1), adenosine (3), *p*-hydroxyacetophenone (4), and tyrosol (5) were isolated. Fusaribenzamide A (2) possessed significant antifungal activity toward *Candida albicans* with minimum inhibitory concentration (MIC) value 11.9 μg/disc compared to nystatin (MIC 4.9 μg/disc). **Conclusion:** The endophytic fungus *Fusarium* sp. could be considered as a wealthy pool for the isolation of aminobenzamide derivatives. Fusaribenzamide A may be a candidate for the discovery of a promising antifungal agent.

Keywords: Aminobenzamide derivative, antimicrobial, endophytes, fusaribenzamide A, *Fusarium* sp., *Mentha longifolia*

SUMMARY

- A new aminobenzamide derivative, fusaribenzamide A (2), and four known compounds, (22*E*,24*R*)-stigmasta-5,7,22-trien-3-*β*-ol (1), adenosine (3), *p*-hydroxyacetophenone (4), and tyrosol (5), were separated from *Fusarium* sp. Their structural assignment was done with the assist of various spectral analyses. Compound 2 had a prominent antifungal effect.

Abbreviations Used: CC: Column chromatography; $CHCl_3$: Chloroform; COSY: Correlations spectroscopy; DBE: double bond equivalent; EtOAc: Ethyl acetate; DMSO: Dimethyl sulfoxide; H_2SO_4: Sulfuric acid; HMBC: Heteronuclear multiple bond correlation experiment; HRMS: High-resolution mass spectrometry; HRESIMS: High-resolution electrospray ionization mass spectrometry; HSQC: Heteronuclear single quantum correlation; IR: Infrared; IZD: Inhibition zone diameter; KBr: Potassium bromide; LTQ: Linear trap quadrupole; MeOH; Methanol; MIC: Minimum inhibitory concentration; NMR: Nuclear magnetic resonance; RP: Reversed phase; SiO_2: Silica gel; TLC: Thin-layer chromatography; UV: Ultraviolet; VLC: Vacuum liquid chromatography.



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INTRODUCTION

Endophytic fungi live inside the plant tissues, without causing damage or disease symptoms.[1-3] Recently, they attracted attention as a prolific source of bioactive natural products with a potent pharmaceutical activity and unique structure.[4,5] Endophytes may contribute to their hosts either by influencing the evolution process and growth to benefit the ecological adaptability of the host via the signal transduction pathway. Furthermore, they produce a wide range of metabolites that may provide protection and ultimately survival value to their hosts.[6]

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Genus *Fusarium* is a widespread fungal group, which can infect fruits, vegetables, and small grain cereals, resulting in vascular wilt, root, stem, and ear rot, with massive reduction in the crops' yields and serious economic losses.[7,8] Our previous phytochemical investigation of this genus led to the identification of integracides, benzamide derivatives, and peptides.[2,3,9-11] In the course of our ongoing efforts to discover biometabolites from *Fusarium* sp., the chemical investigation of its ethyl acetate (EtOAc) extract resulted in the separation and characterization of a new aminobenzamide derivative: fusaribenzamide A (2) and four known metabolites: (22*E*,24*R*)-stigmasta-5,7,22-trien-3-*β*-ol (1), adenosine (3), *p*-hydroxyacetophenone (4), and tyrosol (5). Their structures were verified by various spectroscopic methods. The antimicrobial activity of the new compound was assessed.

MATERIALS AND METHODS

General experimental procedure

A spectrophotometer 1601 ultraviolet visible (UV/VIS) Shimadzu was utilized to get the UV spectra (Shimadzu, Kyoto, Japan). Electrothermal 9100 Digital Melting Point instrument was used for measuring the melting points (Electrothermal Engineering Ltd., Essex, England). Optical rotation was measured on a JASCO DIP-370 digital polarimeter (Jasco Co., Tokyo, Japan) at 25°C at the sodium D line (589 nm). High-resolution electrospray ionization mass spectrometry (HRESIMS) was performed on a Thermo Finnigan linear trap quadrupole Orbitrap (Thermo Finnigan, Bremen, Germany). A spectrophotometer infrared (IR)-400 Shimadzu was used to assess the IR spectra (Shimadzu, Kyoto, Japan). An 850 MHz Bruker AVANCE DRX spectrometer was used in measuring nuclear magnetic resonance (NMR) spectra (Bruker BioSpin, Billerica, MA, USA). Compounds separation was performed on silica gel (SiO_2) 60 (0.063–0.200 mm), Sephadex LH-20 (0.25–0.1 mm), and reverse phase (RP)-18 (0.04-0.063 mm, Merck, Darmstadt, Germany). Six-milliliter standard extraction tube (RP-18, 40–63 mm, Merck, Darmstadt, Germany) was used for compounds purification. Thin-layer chromatography (TLC) was done on precoated SiO_2 60 F_{254} TLC plates (0.2 mm, Merck, Darmstadt, Germany). The compounds were detected by UV absorption at λ_{max}255 and 366 nm followed by spraying with anisaldehyde:sulfuric acid (H_2SO_4) and heating at 110°C.

Fungal material

The fungal strain *Fusarium* sp. was isolated from the internal tissue of *Mentha longifolia* roots collected in March 2014 from Al Madinah Al Munawwarah, Saudi Arabia, as previously described.[2,3,9] The fungus was deposited at the Department of Microbiology, College of Pharmacy, Taibah University, Al Madinah Al Munawwarah, Saudi Arabia (FS No. MAR2014).

Cultivation of the fungal material

For isolation and identification of secondary metabolites, the fresh fungal culture was transferred into 15 Erlenmeyer flasks (1 L each), containing a rice solid culture (100 mL of distilled water was added to 100 g commercially available a rice and kept overnight prior to autoclaving). The cultures were then incubated at room temperature for 30 days under septic conditions.

Metabolites isolation

The culture was extracted at room temperature using EtOAc and concentrated under vacuum. The obtained extract was suspended in distilled water (200 mL) and fractionated between *n*-hexane and methanol (MeOH). The MeOH extract (5.1 g) was separated on vacuum liquid chromatography (VLC), utilizing *n*-hexane, EtOAc, and MeOH, which were independently concentrated to get FH (0.9 g), FE (2.7 g), and FM (1.2 g), respectively. Fraction FE (2.7 g) was subjected to Sephadex LH-20 column chromatography (CC) using $CHCl_3$:MeOH (70:30); 100 mL fractions were collected and monitored by TLC to obtain five sub-fractions: FE-1 to FE-5. Sub-fraction FE-3 (369 mg) was chromatographed over SiO_2 CC (40 g × 50 × 2 cm) using *n*-hexane: EtOAc (98:2–85:15) as an eluent to give impure 1, which was purified on RP-18 column (0.04–0.063 mm; 40 g, 50 × 2 cm), using H_2O:MeOH gradient to get 1 (22.6 mg). SiO_2 CC (60 g × 50 × 3 cm) of sub-fraction FE-4 (728 mg) using $CHCl_3$:MeOH (98:2–80:20) afforded impure 2 and 3. Separately, each one was purified on RP-18 column (0.04–0.063 mm; 40 g, 50 × 2 cm) using H_2O:MeOH gradient to get 2 (6.2 mg) and 3 (14.9 mg). Sub-fraction FE-5 (858 mg) was chromatographed on a SiO_2 CC (70 g, 50 cm ×3 cm) using $CHCl_3$:MeOH (96:4–85:15) to obtain impure 4 and 5. Each one was purified separately on a LiChrolut RP-18 solid-phase extraction tube, eluting with H_2O:acetonitrile gradient to yield 4 (9.1 mg) and 5 (7.9 mg).

Spectral data

Fusaribenzamide A (2-(2-hydroxy-2-(methylamino)acetamido) benzamide) (2): A white amorphous powder; $[\alpha]_D^{25}$ + 89.7 (MeOH). UV (MeOH) λ_{max} (log ε) 213 (3.79), 262 (3.15), 294 (2.98) nm. IR (potassium bromide): V_{max}3423, 3214, 1668, 1582, 1516, 763 cm^{-1}; NMR [Table 1]: HRESIMS m/z 224.1029 [M + H] + (calcd for $C_{10}H_{14}N_3O_3$, 224.1035).

Antimicrobial activity

The antimicrobial effect of 2 was assessed by agar disc diffusion assay toward Gram-positive bacteria (*Staphylococcus aureus* ATCC 29213 and *Bacillus subtilis* ATCC 6633), Gram-negative bacteria (*Escherichia coli* ATCC 35218 and *Pseudomonas aeruginosa* ATCC 27853 14153), and yeast (*Candida albicans* ATCC 76615) as previously outlined.[12] The microorganisms were obtained from the Microbiology Laboratory, King Abdulaziz University Hospital, Jeddah, KSA. Control discs impregnated with dimethyl sulfoxide were used to determine the solvent activity. All experiments were performed in triplicate. The minimal inhibitory concentrations (MICs) of 2 were assessed against all tested strains as previously outlined.[3,10] Ciprofloxacin (30 μg/disc) and nystatin (10 μg/disc) were used as the standard antibacterial and antifungal, respectively.

RESULTS AND DISCUSSION

The endophytic fungi *Fusarium* sp. was cultured on a rice medium. The EtOAc extract of the fermented a rice was subjected to various chromatographic techniques (SiO_2, Sephadex LH-20, and RP-18) to

Table 1: Nuclear magnetic resonance spectral data of compound 2 (dimethyl sulfoxide -d_6, 850 and 214 MHz)

Number	δH (mult., J [Hz])	δC (mult.)	HMBC
1	-	120.9 C	-
2	8.55 dd (8.5, 1.7)	119.9 CH	4, 6, 7
3	7.46 dt (8.5, 1.7)	131.9 CH	2, 5, 6
4	7.10 dt (8.5, 1.7)	122.4 CH	1, 5, 6
5	7.73 dd (8.5, 1.7)	128.5 CH	1, 3, 6
6	-	138.7 C	-
7	-	170.3 C	-
8	8.15 brs 7.58 brs	-	1, 7
9	9.86 s	-	5, 6, 10
10	-	174.2 C	-
11	5.97 brd (2.6)	91.1 CH	10, 13
11-OH	11.97 s	-	10
12	4.08 dq (2.6, 6.8)	-	10, 11, 13
13	3.28 d (6.8)	31.7 CH3	11

HMBC: Heteronuclear multiple bond correlation experiment

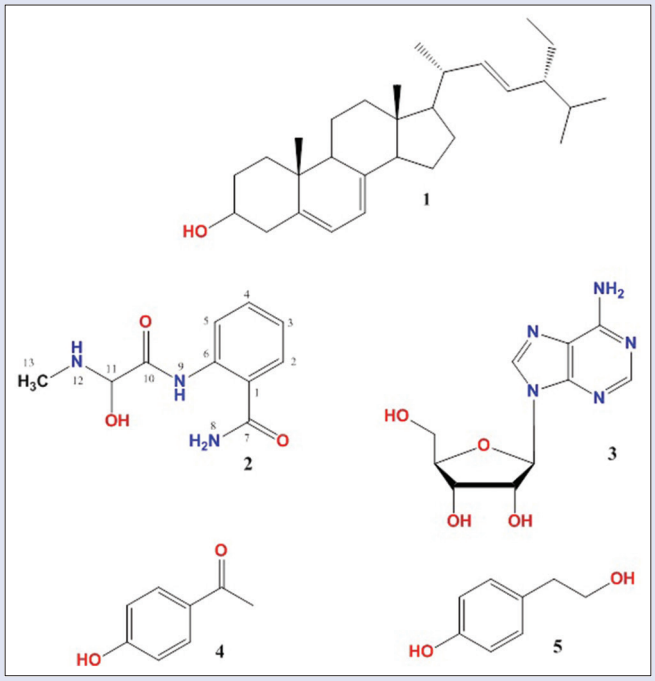

Figure 1: Structures of isolated compounds: (22*E*,24*R*)-stigmasta-5,7,22-trien-3-*β*-ol (1), fusaribenzamide A (2), adenosine (3), *p*-hydroxyacetophenone (4), and tyrosol (5)

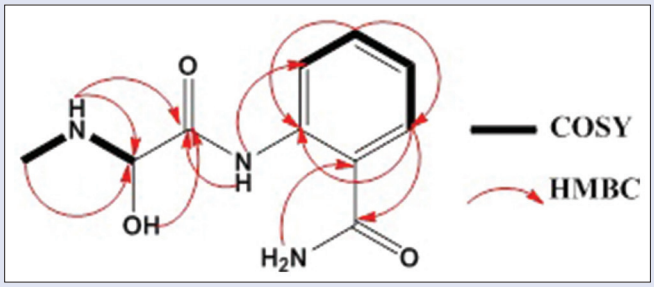

Figure 2: Some key ^{1}H-^{1}H correlations spectroscopy and heteronuclear multiple bond correlation experiment correlations of fusaribenzamide A

yield one new (2) and four known compounds (1 and 3–5) [Figure 1]. Their structures were verified by spectral data analysis, including UV, IR, NMR, and high-resolution mass spectrometry.

Compound 2 was separated as a white amorphous powder and gave positive Dragendorff's and ninhydrin tests, indicating the nitrogenous nature of 2.[3,10] Its molecular formula was assigned to be $C_{10}H_{13}N_3O_3$ based on the HRESIMS pseudo-molecular ion peak at m/z 224.1029 [M + H] + (calcd for $C_{10}H_{14}N_3O_3$, 224.1035) and ^{13}C NMR. This molecular formula required six double bond equivalents accounting for two carbonyls and a phenyl moiety. It displayed UV absorptions at 213, 262, and 294 nm. It showed IR bands at 1582 and 1516 (C-H aromatic), 3214 (NH), 3423 (OH), and 1668 (C=O) cm^{-1}. The ^{13}C and heteronuclear single quantum correlation (HSQC) revealed 10 carbon resonances: 5 methines: an oxymethine (δ_C 91.1) and four aromatic methines, one methyl, and 4 quaternary carbons, including two nonprotonated aromatic carbons and two amide carbonyls at δ_C 170.3 (C-7) and 174.2 (C-10) [Table 1]. The ^{1}H-^{1}H correlations spectroscopy and ^{1}H spectra of 2 exhibited four correlated aromatic protons for a 1,6-*di*-substituted phenyl moiety at δ_H 8.55 (dd, *J* = 8.5, 1.7 Hz, H-2), 7.73 (dd, *J* = 8.5, 1.7 Hz, H-5), 7.46 (dt, *J* = 8.5, 1.7 Hz, H-3), and 7.10 (dt, *J* = 8.5, 1.7 Hz, H-4). They correlated with the carbons, resonating at δ_C 119.9, 128.5, 131.9, and 122.4, respectively, in the HSQC spectrum. This was confirmed by the heteronuclear multiple bond correlation experiment (HMBC) cross peaks of H-2/C-4 and C-6, H-3/C-5, H-4/C-6, and H-5/C-3 and C-1 [Figure 2]. The two protons signals for NH group at δ_H 8.15 and 7.58 (each brs, H-8) and carbonyl group δ_C (170.3, C-7) revealed the presence a benzamide moiety in 2.[3,10,13] This was assured by the HMBC correlations of H-8 to C-1 and C-7 and the ESIMS fragment peak at m/z 121 $[C_7H_7NO]$+. The ^{1}H and ^{13}C signals at δ_H 9.86 (H-9), δ_C 174.2 (C-10), 5.97 (H-11)/91.1 (C-11), 11.97 (11-OH), 4.08 (H-12), and 3.28 (H-13)/31.7 (C-13) were attributable to a 2-hydroxy-2-(methylamino)acetamido moiety, which was secured by the observed HMBC relations [Figure 2]. Its connectivity at C-6 of the benzamide moiety was proved by the HMBC cross peaks of H-9 to C-5 and C-6. On the basis of these findings and by comparing with literature, 2 was identified as 2-(2-hydroxy-2-(methylamino) acetamido)benzamide and named fusaribenzamide A. It is noteworthy that aminobenzamide derivatives have been previously reported from different fungi belonging to the genus *Fusarium*.[3,10,13]

The known metabolites were assigned as (22*E*,24*R*)-stigmasta-5,7,22-trien-3-*β*-ol (1),[14] adenosine (3),[15] *p*-hydroxy acetophenone (4),[16] and tyrosol (5)[17] by comparing their spectral data to those formerly published.

Compound 2 was evaluated for its antibacterial activity against *S. aureus*, *B. subtilis*, *E. coli*, and *P. aeruginosa*, as well as antifungal effect towards *C. albicans*, using agar disc diffusion assay. It possessed a significant antifungal activity towards *C. albicans* with MIC 11.9 μg/disc compared to nystatin (MIC 4.9 μg/disc). However, it showed moderate activity toward *S. aureus* and *E. coli* with MIC values 62.8 and 56.4 μg/disc, respectively in comparison to ciprofloxacin (MICs 12.5 and 10.4 μg/disc, respectively).

CONCLUSION

A new aminobenzamide derivative (2) and four known metabolites (1 and 3–5) from *Fusarium* sp. Their structural elucidation was achieved with the aid of extensive spectroscopic techniques. Fusaribenzamide A (2) possessed a remarkable antifungal activity. *Fusarium* sp. has been proven to be a rich source of aminobenzamide derivatives and other metabolites of diverse classes. Fusaribenzamide A has a good potential for future use as a lead drug for antifungal agent.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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