A Polyketide Metabolite from an Endophytic *Fusarium equiseti* in a Medicinal Plant

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A new polyketide, fusaequisin A (**1**), was isolated from rice cultures of *Fusarium equiseti* SF-3-17, an endophytic fungus previously discovered during screening of medicinal plants in Cameroon. The structure of **1** was determined using spectroscopic methods. Fusaequisin A (**1**) exhibited moderate antimicrobial activity against *Staphylococcus aureus* NBRC 13276 and *Pseudomonas aeruginosa* ATCC 15442 at a concentration of 200 μg per disk.

Key words: Endophyte, *Fusarium equiseti*, Antimicrobial Activity

Introduction

The endophytic fungus *Fusarium equiseti* SF-3-17 has been isolated from the herbaceous plant, *Ageratum conyzoides* L., which is traditionally used for the treatment of fever, rheumatism, headache, and colic as a traditional folk medicine in Cameroon and the Congo [1 – 3]. Our ongoing search for biologically active metabolites from fungi [1] revealed that the crude culture extract of *F. equiseti* SF-3-17 exhibits good antifungal activity against *Aspergillus clavatus* and antibacterial activity against *Pseudomonas aeruginosa*, which prompted further investigation of the chemical constituents of this fungus. Bioassay-guided fractionation *via* extensive column chromatography of the methanol extract of this fungal culture afforded a new compound, fusaequisin A (**1**), the structure of which was elucidated using spectroscopic and chemical methods, along with two known antifungal compounds, deoxyneofusapyrone (**2**) [4] and neofusapyrone (**3**) [5], as the active components. We report herein the isolation and structure determination of the secondary metabolites of *F. equiseti* SF-3-17.

Results and Discussion

The producing strain *F. equiseti* SF-3-17 was grown on steamed brown rice, under static conditions, for three weeks, and the culture was then extracted with methanol. The organic extract was concentrated, and the aqueous residue was extracted with ethyl acetate. This extract was subjected to silica gel and ODS column chromatography to afford fusaequisin A (**1**), along with two known antimicrobial compounds, deoxyneofusapyrone (**2**) and neofusapyrone (**3**) (Fig. 1).

Compounds **2** and **3** were positively identified as deoxyneofusapyrone and neofusapyrone based on their spectral data (MS, UV, IR, and ^{1}H and ^{13}C NMR), which were indistinguishable from the literature data for deoxyneofusapyrone [4] and neofusapyrone [5] (Fig. 1).

Fusaequisin A (**1**) was obtained as a colorless oil. The molecular formula of **1** was determined to be $C_{28}H_{46}O_4$ on the basis of HRMS (ESI-TOF), suggesting the presence of six double-bond equivalents. The IR spectrum exhibited an absorption at 1708 cm^{-1}, characteristic of the carbonyl functionality. The UV spectrum of **1** was typified by an absorption band

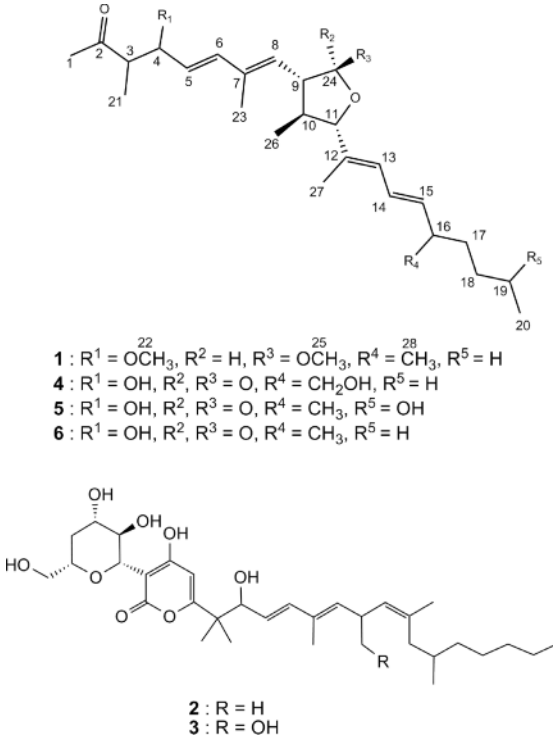

Fig. 1. Structures of compounds **1**–**6**.

at 244 nm, which suggested the presence of a chromophoric group comprising conjugated double bonds. The ^{13}C NMR spectrum (C_5D_5N) showed 28 resolved peaks (Table 1), which were classified into nine methyls, three sp^3 methylenes, seven sp^3 methines, six sp^2 methines, two sp^2 quaternary carbons, and one carbonyl carbon. The signals corresponding to four olefinic carbon atoms indicated the presence of four double bonds in **1**. The ^{1}H NMR spectrum revealed six olefinic signals at $\delta_H = 5.67$ (dd, $J = 15.0$, 8.1 Hz, 1H, 5-H), 6.58 (d, $J = 15.0$ Hz, 1H, 6-H), 5.57 (d, $J = 9.7$ Hz, 1H, 8-H), 6.25 (d, $J = 11.0$ Hz, 1H, 13-H), 6.46 (dd, $J = 15.0$, 11.0 Hz, 1H, 14-H), and 5.92 (dd, $J = 15.0$, 8.0 Hz, 1H, 15-H), which were attributed to the protons of four distributed double bonds, as well as seven methine signals, including three protons linked to oxygenated carbons at $\delta_H = 4.67$ (t, $J = 8.1$ Hz, 1H, 4-H), 4.27 (d, $J = 11.0$ Hz, 1H, 11-H), and 4.94 (d, $J = 4.1$ Hz, 3H, 24-H), two olefinic methyl singlets at $\delta_H = 1.88$ (d, $J = 1.2$ Hz, 3H, 23-Me) and 1.85 (s, 3H, 27-H_3), and two methoxy signals at $\delta_H = 3.29$ (s, 3H, 22-OCH_3) and 3.40 (s, 3H, 25-OCH_3), a methyl singlet at $\delta_H = 2.33$ (3H, s, 3H, 1-H_3), three methyl doublets at $\delta_H = 1.09$ (d, $J = 7.1$ Hz, 3H, 21-H_3), 0.89 (d, $J = 6.6$ Hz, 3H, 26-H_3) and 0.99 (d, $J = 6.6$ Hz, 3H, 28-H_3), a methyl triplet at $\delta_H = 0.81$ (t, $J = 7.1$ Hz, 3H, 20-H_3), and three aliphatic methylenes. Three spin systems were clearly defined in the analysis of the ^{1}H–^{1}H COSY spectrum, and are shown as bold lines in Fig. 2. The HMBC spectrum of **1** shows correlations between the 27-Me signal and those of C-11, C-12, and C-13, and correlation of the 11-H signal with that of C-13, indicative of a link between C-11 and the 6-methyl-deca-2,4-dien-2-yl moiety (C-12 to C-20, C-27, and C-28). The linkages of C-7 to C-6, C-8, and C-23 were deduced based on the HMBC correlations of 23-Me to C-6, C-7, and C-8. The HMBC correlations of 1-H_3 and 21-H_3 to C-2 and C-3 revealed that the methyl ketone moiety was located at C-2. The methoxy group ($\delta_H = 3.40$) showed HMBC correlations with the acetal carbon C-24 (Fig. 2), indicating the presence of the methyl acetal. The acetal proton at $\delta_H = 4.94$ showed HMBC correlations with the carbons at $\delta_C = 133.0$ (C-8), 45.3 (C-10) and 90.5 (C-11). These data clearly confirmed the presence of the 2-methoxytetrahydrofuran moiety (C-9, C-10, C-11, C-24, and C-25). The relative stereochemistry in the tetrahydrofuran ring was established by means of

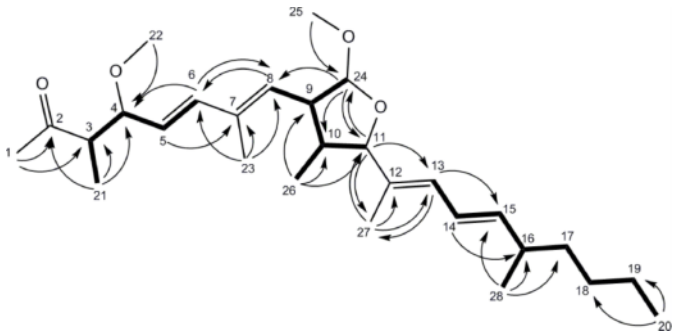

Fig. 2. ^{1}H-^{1}H COSY (bold lines) and HMBC (arrows) correlations observed for **1**.

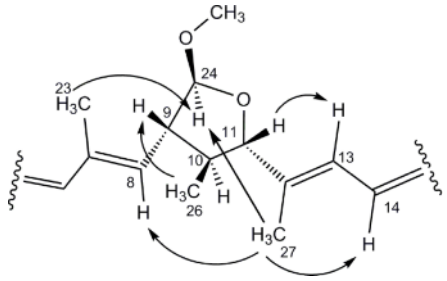

Fig. 3. Selected NOE correlations observed for **1**.

1D NOE experiments (Fig. 3). NOEs from 27-H_3 to 8-H, 14-H and 24-H, from 11-H to 13-H, and from 26-H_3 to 9-H, determined by NOE analysis of **1**, indicated that the relative configurations of the tetrahydrofuran ring carbons (C-9, C-10, C-11, and C-24) are as shown.

The relative configurations of C-3 and C-4 in **1**, as well as those in **4**, **5** and **6**, remain unsettled and will have to be determined in future work, for example by crystal structure determinations of suitable derivates.

The closest structurally similar microbial polyketide metabolites having two long side-chain-substituted γ-lactone ring systems are curvicollides A (**4**), B (**5**) and C (**6**), isolated from a culture of *Podospora curvicolla* [6]. Disk assay demonstrated that curvicollide A (**4**) exhibited weak antifungal activity against *Aspergillus flavus* and *Fusarium verticillioides* at a concentration of 200 μg per disk [6]. Neither **5** nor **6** has been tested for antifungal activity because the compounds were isolated only in trace quantities [6]. The activities of compound **1** against Gram-positive and Gram-negative bacteria, yeast, and fungal strains were evaluated using the agar diffusion method. Compound **1** exhibited moderate activity against *Staphylococcus aureus* NBRC 13276 and *Pseudomonas aeruginosa* ATCC 15442, with corresponding zones of inhibition of diameter 13 mm and 12 mm, respectively, at a concentration of 200 μg per disk. Compound **1** showed no activity against *Aspergillus clavatus* F 318a or *Candida albicans* ATCC 2019 ($>$ 200 μg per disk). The lack of antifungal activity in the case of **1** suggested that the γ-lactone moiety may be a requisite for, or the compound's polarity may determine, the antifungal activity of **4**. The acetal functional group of **1** did not prove to be prone to hydrolysis in this case. Nonetheless, we were interested in establishing that the functional group was not an artifact of the isolation procedure. The fungal material was extracted with ethanol, and no material having an ethoxy group was found in the extract. In addition, **2** and **3** showed activities with observed zones of inhibition of 12 mm and 13 mm in diameter, respectively, at 6.25 μg per disk against *Aspergillus clavatus*.

Table 1. NMR data of compound **1**[a].

No	δ_C	δ_H	HMBC
1	30.0 q	2.33 (3H, s)	2, 3
2	211.6 s		
3	53.7 d	2.68 – 2.79 (1H, m)	4, 21
4	75.4 d	4.67 (1H, t, 8.1)	
5	128.8 d	5.67 (1H, dd, 15.0, 8.1)	4, 7
6	136.1 d	6.58 (1H, d, 15.0)	4, 8, 23
7	136.0 s		
8	133.0 d	5.57 (1H, d, 9.7)	6, 9, 23
9	54.2 d	3.00 – 3.05 (1H, m)	8
10	45.3 d	1.88 – 1.93 (1H, m)	
11	90.5 d		13, 24, 27
12	132.0 s	4.27 (1H, d, 11.0)	
13	130.5 d	6.25 (1H, d, 11.0)	15, 27
14	124.7 d	6.46 (1H, dd, 15.0, 11.0)	16
15	141.9 d	5.92 (1H, dd, 15.0, 8.0)	14, 16, 17, 28
16	37.4 d	2.17 – 2.23 (1H, m)	
17	36.7 t	1.21 – 1.26[b]	
18	29.6 t	1.21 – 1.26[b]	
19	22.8 t	1.21 – 1.26[b]	
20	14.3 q	0.81 (3H, t, 7.1)	18, 19
21	14.2 q	1.09 (3H, d, 7.1)	2, 3, 4
22	49.6 q	3.29 (3H, s)	4
23	13.2 q	1.88 (3H, d, 1.2)	6, 7, 8
24	110.1 d	4.94 (1H, d, 4.1)	8, 10, 11, 25
25	55.6 q	3.40 (3H, s)	24
26	13.6 q	0.89 (3H, d, 6.6)	9, 10, 11
27	11.9 q	1.85 (3H, s)	11, 12, 13
28	20.8 q	0.99 (3H, d, 6.6)	15, 16, 17

[a] Measured in C_5D_5N, values in parentheses are coupling constants in Hz; [b] overlapping signals.

Screening of the fungal extract isolated from *F. equiseti* SF-3-17 resulted in the discovery of fusaequisin A (**1**) and two previously reported compounds, deoxyneofusapyrone (**2**) and neofusapyrone (**3**). The current finding of the antimicrobial compounds **1**, **2** and **3** produced by *F. equiseti* SF-3-17 suggests that the endophytic fungus *F. equiseti* SF-3-17 might be involved in protecting host plants from invasion by phytopathogens. Further pharmacological studies of **1**, **2** and **3** are currently in progress.

Experimental Section

General experimental procedures

Optical rotation was measured with a Horiba model SEPA-300 polarimeter, IR spectra were recorded with

a JASCO J-20A spectrophotometer, and UV spectra were recorded with a Shimadzu UV mini-1240 instrument. Mass spectra were obtained with a Synapt G2 mass spectrometer and ^{1}H and ^{13}C NMR spectra were obtained with a Jeol EX-400 spectrometer. Chemical shifts are given on a δ (ppm) scale with TMS as an internal standard. Column chromatography was conducted on ODS (Fuji Silysia, Japan) and silica gel 60 (Kanto Chemical Co., Inc.). TLC was done on a precoated silica gel plate (Merck), and spots were detected by spraying 10 % vanillin in H_2SO_4 followed by heating.

Isolation of the producing strain

The medicinal plant, *Ageratum conyzoides* L., has been collected at a suburb of Yaunde, Cameroon, in August 2009. To obtain the endophytic fungi, the stem parts of the plant were successively surface-sterilized with 70 % EtOH for 1 min, 5 % sodium hypochlorite for 5 min and 70 % EtOH for 1 min, and then rinsed twice in sterile water. The surface-sterilized segments were dried on sterilized paper and cut into 1-cm pieces. The pieces were placed on plates of potato dextrose agar (PDA) containing chloramphenicol (100 mg L^{-1}). After incubating at 25 °C for 7 days, the hyphal tips of the fungus on the plates were removed from the agar plates and transferred to PDA slants. The SF-3-17 strain was isolated and grew on the PDA slants as a white-colored culture. This strain was identified to be *Fusarium equiseti* by BEX. Co. Japan, using a DNA analysis of the 18S rDNA regions. This fungus has been deposited at our laboratory in the Faculty of Agriculture of Yamagata University.

*Cultivation of the endophytic fungus, extraction and isolation of fusaequisin A (**1**), deoxyneofusapyrone (**2**) and neofusapyrone (**3**)*

F. equiseti SF-3-17 was cultivated on sterilized unpolished rice (1000 g) at 25 °C for 3 weeks. The moldy unpolished rice was extracted with MeOH, and the MeOH extract was concentrated. The resulting aqueous concentrate was partitioned into *n*-hexane and EtOAc layers. The purification of the EtOAc layer was guided by the intense blue characteristic coloration with vanillin-sulfuric acid solution on TLC plates. The EtOAc layer was chromatographed on a silica gel column using a gradient of *n*-hexane-EtOAc (100 : 0 – 0 : 100) to give fractions 1 – 11 (Fr. 1-1 to 1-11). Fr. 1-7 (*n*-hexane-EtOAc, 40 : 60, 1.0 g) was subjected to silica gel column chromatography by eluting with $CHCl_3$ and an increasing ratio of MeOH. Eleven fractions (Fr. 2-1 to 2-11) were obtained. Fr. 2-6 ($CHCl_3$-EtOAc, 50 : 50, 0.20 g) was subjected to ODS column chromatography using a gradient of MeOH-H_2O (0 : 100 – 100 : 0) to afford crude **1**, which was finally purified by silica gel flash column chromatography with a mixture $CHCl_3$-MeOH (90 : 10) to yield fusaequisin A (**1**, 12.0 mg). Fr. 2-11 ($CHCl_3$-EtOAc, 0 : 100, 0.50 g) was subject to ODS column chromatography using a gradient of MeOH-H_2O (0 : 100 – 100 : 0) to afford deoxyneofusapyrone (**2**, 76 mg) and neofusapyrone (**3**, 50 mg).

*Fusaequisin A (**1**).* Colorless oil. – $[\alpha]_D^{20} = -200^\circ$ (c = 0.40, MeOH). – UV (MeOH): $\lambda_{max}(\lg \varepsilon)$ = 199 nm (4.3), 244 nm (4.3). – IR (KBr): ν = 2871, 1708, 1457, 1375, 964 cm^{-1}. – ^{1}H NMR (400 MHz, C_5D_5N) and ^{13}C {^{1}H} NMR (100 MHz, C_5D_5N) data see Table 1. – HRMS ((+)-FAB): m/z = 469.3208 (calcd. 469.3294 for $C_{28}H_{46}NaO_4$, $[M+Na]^+$). – MS ((+)-FAB): m/z = 447 $[M+H]^+$.

Antimicrobial activity

Test organisms were *Staphylococcus aureus* NBRC 13276, *Pseudomonas aeruginosa* ATCC 15442, *Aspergillus clavatus* F 318a, *Pyricularia oryzae* NBRC 31178 and *Candida albicans* ATCC 2019. Antimicrobial assays were carried out by the paper disk diffusion method using a published protocol [7].

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