Two novel aliphatic unsaturated alcohols isolated from a pathogenic fungus *Fusarium proliferatum*

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ABSTRACT

Phytopathogenic fungi have attracted great attention as a promising source for new drug discovery. In the progress of our ongoing study for bioactive natural products from an in-house phytopathogenic fungi library, a pathogenic fungus, *Fusarium proliferatum* strain 13294 (FP13294), was selected for chemical investigation. Two novel aliphatic unsaturated alcohols named fusariumnols A and B (**1** and **2**), together with one previously characterized sesquiterpenoid lignoren (**3**) were identified. Structures of **1**–**3** were assigned by mass spectrometry and NMR spectroscopy. Their bioactivities were assessed against *Staphylococcus epidermidis*, *S. aureus*, and Methicillin-resistant *S. aureus* (MRSA). Compounds **1** and **2** exhibited weak antibacterial activity against *S. epidermidis* (MIC = 100 μM).

1. Introduction

Fungi inhabiting special environments are well-known producers of secondary metabolites with diverse biological activities. Phytopathogenic fungi have attracted increasing attention because of frequent discoveries of natural products with diverse structural properties and promising biological activities [1,2]. These are treasure troves for mining novel products especially in phytopathogenic fungi where genes have arisen during the long-term co-evolutionary process with host plants; these gene products have high potential in pharmaceutical and agricultural applications [3,4]. For example, phytotoxins derived from phytopathogenic fungi have been used as ecofriendly tools in the development of safe bioherbicides [5,6]. In addition, fungal phytotoxins exhibited a variety of biological activities, including promising antifungal, anticancer, and anti-inflammatory activities [7–10].

The genus *Fusarium*, often isolated from different plant tissues and from plant debris, can afford diverse mycotoxins, causing reductions of crop quality and harvest, as well as effecting human health [11]. Nevertheless, there is abundant evidence indicating *Fusarium* sp. have the capacity to afford different kinds of natural products, such as

polyketides, terpenoids, alkaloids, and peptides, which show significant biological activities [12–15]. *F. proliferatum* is a widespread plant pathogenic fungi associated with diverse crops, including rice, wheat, maize, garlic, asparagus, date palm, and Chinese chive [16]. *F. proliferatum* has been reported to produce multiple mycotoxins, such as fumonisins, beauvericin, fusaproliferin, moniliformin, and fusaric acid [17].

Staphylococci, which are Gram-positive bacteria, can colonize humans and cause infections as the immune system weakens. Pathogens are introduced through wounds and also associated with the insertion of medical devices [18,19]. They are able to form biofilms in chronic wounds, resulting in doubling of time for recovery. Due to extremely difficult to treat, these infections lead to a serious burden for the public health system [20]. The *Staphylococcus* species usually contain *S. aureus* and *S. epidermidis*. Because of increased bacterial resistance to current antibiotics in clinical use, there is a need to explore natural products to identify novel compounds for drug discovery.

During an ongoing study of novel bioactive natural products in our in-house phytopathogenic fungi library, a strain of *F. proliferatum* (FP13294) was subjected to investigate secondary metabolites based on

bioassay guided strategy. Its crude extract showed weak antibacterial activity against *S. epidermidis* (MIC = 200 μM). Fractionation of crude extracts obtained from a rice fermentation culture yielded two new secondary metabolites, which we named fusariumnols A and B (**1** and **2**), in addition to one known compound lignoren (**3**). We herein provide details of the structure identification and biological activities of compounds **1**–**3**.

2. Materials and methods

2.1. General experimental procedures

1D and 2D NMR spectra were measured on a Bruker Avance DRX 600 MHz spectrometer with TCI cryoprobes. Chemical shifts are expressed as δ (ppm) referenced to the solvent peaks at δ_C 49.0 and δ_H 3.31 for Methanol-d_4. HRESIMS spectra were measured on a Thermo Orbitrap Q Exactive mass spectrometer. Materials including Sephadex LH-20 (GE Healthcare) and ODS-A (YMC) were utilized for column chromatography. Reverse Phase HPLC chromatography was carried out by an Agilent 1100 Series HPLC, and ChemStation Rev.B.02.01 software was used to analysis the data. Semipreparative RP-HPLC was performed equipped with an ACE Excel 5C$_{18}$-AR column (10 × 250 mm, 5 μm).

2.2. Microbial strain culture and identification of F. proliferatum 13,294

F. proliferatum 13294 (FP13294) was prepared on potato dextrose agar (PDA) at 28 °C. Strain FP13294 was identified based on morphology and 18S rRNA gene sequence. The primer pair EF-1a-F (5″-AAGGCTGGTTCCAAGACTGG-3″) and EF-1a-R (5″-TGGTCGTCTCTTTCGCTCCT-3″) were used to amplify the translation elongation factor 1 alpha (EF-1a) gene region of FP13294. The PCR amplification (25 μL in total) contained 1 μL of DNA template, 0.4 μL of r*Taq* polymerase, 0.4 μL of each primer, 2.5 μL of 2.5 nM dNTP, and 2.5 μL of 10 × buffer. The PCR reaction was carried out on an ABI PCR Thermal Cycler with following condition: denaturation at 94 °C for 5 min, 25 cycles of denaturation at 94 °C for1 min, annealing at 55 °C for 1 min, elongation at 72 °C for 45 s, and a final elongation at 72 °C for 10 min. Both forward and reverse primers were used for PCR product sequencing, and the consensus sequence was compared to the GenBank database using BLASTn. Sequences with high similarity were acquired from GenBank. Phylogenetic neighbor-joining tree was constructed by MEGA 6.0 software [21], and rooted on *Trichoderma auranteffusum*.

2.3. Fermentation and isolation of compounds 1–3

FP13294 was prepared on PDA plate medium at 28 °C for 7 days, and agar plugs (0.5 × 0.5 × 0.5 cm^3) were inoculated into six Erlenmeyer flasks (250 mL) each containing 100 mL potato dextrose broth (PDB). Then the Erlenmeyer flasks were incubated for five days at 28 °C on a rotary shaker at 200 rpm as the seed culture. Fermentation was then carried out in 200 aseptic-bags each containing 80 g of autoclaved rice and 120 mL sterilized distilled H$_2$O for 30 days at 28 °C. The fermentation material was extracted with EtOAc three times, and then was evaporated under *in vacuo* to give an extract (18.64 g).

The crude extract was subjected to Sephadex LH-20 eluting with 100% MeOH to yield seven fractions (A–G). Fraction E (11.33 g) was fractionated by Sephadex LH-20 again eluting with100% MeOH to give four sub-fractions (E1–E4). Then Fraction E3 (7.34 g) was subjected to ODS-MPLC with an ACN-H$_2$O gradient (5%–100%) elution for 100 min to get ten sub-fractions (E3A–E3J). Sub-fraction E3C (2.56 g) was further purified by Sephadex LH-20 eluting with 100% MeOH to afford seven fractions (E3C1–E3C16). Fraction E3C4 (641 mg) was chromatographed over ODS-MPLC with an ACN-H$_2$O gradient (10%–100%) elution for 100 min to yield eight sub-fractions (E3C4A–E3C4H). Sub-fraction E3C4D (148.2 mg) was then purified by semipreparative RP-HPLC equipped with an ACE Excel 5C$_{18}$-AR (10 × 250 mm) eluting

with 35% ACN-H$_2$O for 60 min (flow rate: 4 ml/min) to obtain **1** (2.4 mg, t_R = 30.9 min), **2** (0.5 mg, t_R = 52.9 min), and **3** (17.4 mg, t_R = 21.4 min).

2.4. Accession number

Fusarium proliferatum stain 13,294 has been deposited at the China General Microbiological Culture Collection Center with the accession number 21945. The sequence data of the EF-1a gene has been deposited under GenBank accession number MZ382914.

3. Results

3.1. Characterization and identification of strain FP13294

Isolate 13294 was originally obtained from wheat tissue. It appeared as white villous colonies which produce light purple pigment and have a colony diameter of 7 mm after 7 d on potato dextrose agar (PDA) at 28 °C (Fig. 1). The EF-1a gene sequence and neighbor-joining tree (Fig. 1) confirmed it as *Fusarium proliferatum*.

3.2. Structure elucidation

The HRESIMS (Fig. S1a) analysis revealed the molecular formula of **1** as C$_{15}$H$_{28}$O$_2$ (241.2162 [M + H]$^+$, calcd. for 241.2162), implying two degrees of unsaturation. Based on combined analysis of the ^{1}H, ^{13}C, and HSQC spectra of **1** (Table 1 and Figs. S1b, S1c, S1e), 15 carbon resonances could be observed, composed of one olefinic quaternary carbon (δ_C 137.1), three conjugated sp^2 methines [δ_C/δ_H 133.0/5.57 (dt, J = 15.2, 6.9 Hz), 128.3/6.29 (dd, J = 15.2, 10.5 Hz), 126.3/5.83 (d, J = 10.5 Hz)], two oxymethine groups [δ_C/δ_H 73.7/3.44 (m), 73.4/3.47 (m)], six sp^3 methylenes, two terminal methyl triplets [2 × δ_C/δ_H 10.5/0.95 (t, J = 7.4 Hz)], and one methyl singlet [δ_C/δ_H 16.7/1.72 (s)], indicating that the structure of **1** comprises a bishydroxylated alkyl chain with two conjugated olefinic groups. The ^{1}H–^{1}H COSY (Fig. S1d) correlations of **1** revealed the existence of two sub-units C-1–C-5 and C-7–C-14, which were deduced to connect at C-6 based on the key HMBC (Fig. S1f) correlations of H$_3$-15 with C-5/C-6/C-7. Further HMBC analysis revealed that the two hydroxyl groups were substituted at C-3 and C-12, based on the crosspeaks between H$_3$-1/C-3 and H$_3$-14/C-12. The NOESY (Fig. S1g) correlations between H$_3$-15/H-8, H-5/H-7, and H-7/H-9 together with a large coupling constant between H-8/H-9 ($^3J_{H-H}$ = 15.2 Hz) assigned the Δ^{6,7} and Δ^{8,9} double bonds as *E* configuration. To determine the relative configurations between C-3 and C-12, the density functional theory (DFT) based ^{13}C NMR calculation and DP4 analysis of two epimers **1a**/**1b** was conducted, resulting in higher Bayes's theorem probability of **1b** (75%) compared with **1a** (25%) (Table S1). Therefore, the structure of **1** was established as illustrated in Fig. 3 and named fusariumnol A.

The molecular formula of **2** could be deduced as C$_{15}$H$_{26}$O$_2$ with three degrees of unsaturation from its HRESIMS (Fig. S2a) spectrum (239.2005 [M + H]$^+$, calcd. for 239.2006). The ^{1}H and ^{13}C NMR data of **2** (Figs. S2b and S2c) showed high similarity with those of **1**, except for an additional keto-carbonyl group (δ_C 214.0) replacing one of the original *O*-substituted methines in **1**. Further HMBC (Fig. S2f) analysis revealed that compound **2** is the 3-oxo derivative of **1** based on crosspeaks of H$_3$-1 with C-2/C-3, and H$_2$-4 with C-3. The Δ^{6,7} and Δ^{8,9} double bonds were also determined to be *E* configuration based on the NOESY correlations (Fig. S2g) between H$_3$-15/H-8, H-5/H-7, and H-7/H-9 combined with $^3J_{H8-H9}$ value (15.1 Hz). Compound **2** was proposed as a new analog of **1** and was named fusariumnol B (Fig. 2).

The structure of one known compound, lignoren (**3**), was determined based on the HRESIMS, ^{1}H and ^{13}C NMR data (Figs. S3a–S3c) and by comparison with those of published work [22].
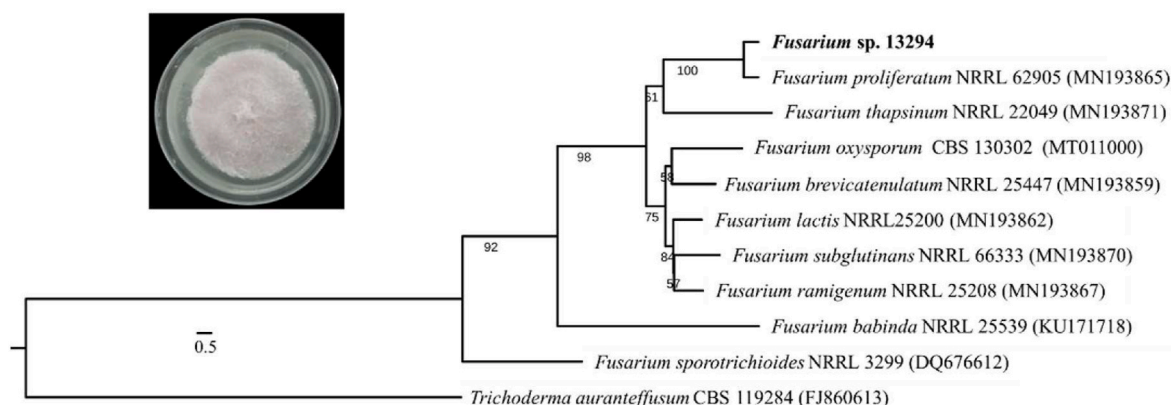
Fig. 1. Morphology and phylogenetic tree of *F. proliferatum* 13294. **a** Morphology characteristic of FP13294 cultured on PDA at 28 °C for 7 days. **b** Phylogenetic neighbor-joining tree of FP13294 based on EF-1a gene sequences. NCBI accession numbers are given in parentheses. Numbers at nodes indicate levels of bootstrap support (percentage) based on 1000 resampled datasets; only values > 50% are presented. The bar indicates 0.5 amino acid substitutions per site. *Trichoderma auranteffusum* was chosen as outgroup.

Table 1
^{1}H and^{13}C NMR Data of compounds **1** and **2**.

Pos.	1			2		
	$\delta_H{}^a$ mult (*J* in Hz)	$\delta_C{}^b$, type		$\delta_H{}^a$ mult (*J* in Hz)	$\delta_C{}^b$, type	
1	0.95, t (7.4)	10.5, CH₃		1.00, t (7.4)	8.0, CH₃	
2	1.44, m	31.2, CH₂		2.47, q (7.3)	35.1, CH₂	
3	3.44, m	73.7, CH			214.0, C	
4a	1.59, m	36.4, CH₂		2.56, t (7.6)	40.2, CH₂	
4b	1.51, m					
5a	2.19, m	37.1, CH₂		2.27, t (7.6)	34.8, CH₂	
5b	2.08, m					
6		137.1, C			135.6, C	
7	5.83, d (10.5)	126.3, CH		5.77, d (10.7)	126.5, CH	
8	6.29, dd (15.2, 10.7)	128.3, CH		6.25, dd (15.2, 10.7)	128.0, CH	
9	5.57, dt (15.1, 6.9)	133.0, CH		5.56, dt (15.1, 7.1)	133.5, CH	
10a	2.24, m	30.3, CH₂		2.21, m	30.1, CH₂	
10b	2.15, m			2.13, m		
11a	1.54, m	38.0, CH₂		1.54, m	37.8, CH₂	
11b	1.49, m			1.49, m		
12	3.47, m	73.4, CH		3.44, m	73.3, CH	
13a	1.51, m	31.2, CH₂		1.48, m	31.1, CH₂	
13b				1.41, m		
14	0.95, t (7.4)	10.5, CH₃		0.93, t (7.5)	10.3, CH₃	
15	1.72, s	16.7, CH₃		1.75, s	16.7, CH₃	

^{a} Recorded at 600 MHz in Methanol-d_4.
^{b} Recorded at 125 MHz in Methanol-d_4.

3.3. Bioactivity tests

The crude extract, purified subfractions, and isolated compounds **1**–**3** were assessed for their anti-bacterial activity against Gram-positive bacterium, including *S. aureus,* MRSA, and *S. epidermidis*. The crude extract and subfraction E3C4D showed anti-bacterial activity against

S. epidermidis (MIC = 200 μM). Finally, we found that compounds **1** and **2** exhibited weak anti-bacterial activity against *S. epidermidis* (MIC = 100 μM).

4. Discussion and conclusion

The discovery of penicillin initiated an era wherein natural products derived from microorganisms revolutionized medicine. Afterwards, human life expectancy was extended by nearly 40 years [23]. Historically, the majority of approved drugs have been found from natural sources or derivatives of natural compounds. However, due to the repeat discovery of known compounds in other microorganisms, the discovery ratio of new natural compounds with bioactivity has declined in recent years. Additionally, many pharmaceutical companies have reduced their investment in natural product research. Thus, to further advance drug discovery, new strategies for the discovery of novel natural products are of great importance.

Recently, poorly tapped biological resources, including rare actinobacteria, endophytic fungi, and even phytopathogenic fungi, have been explored as innovative resources for discovering novel natural products with promising bioactivity [24–26]. Among them, phytopathogenic fungi play an important, but yet rarely explored role. They are classified as biotrophs, hemibiotrophs, and necrotrophs, acquiring nutrition during invading process of their host plants. Between the interactions of phytopathogenic fungi and plants, fungi usually metabolize and produce series of low-molecular weight secondary metabolites, which are not essential for life of phytopathogenic fungi while are known for their versatility. These secondary metabolites always play an important role in ecological defense and biological competition. In association with host plants, phytopathogenic fungi can become rich sources of cyclic peptides, terpenes, alkaloids, aliphatic hydrocarbons, aromatic polyketones, and heterocyclic compounds, showing special values to humans
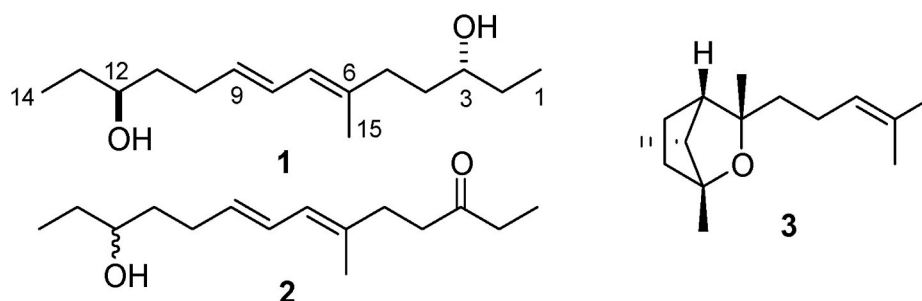

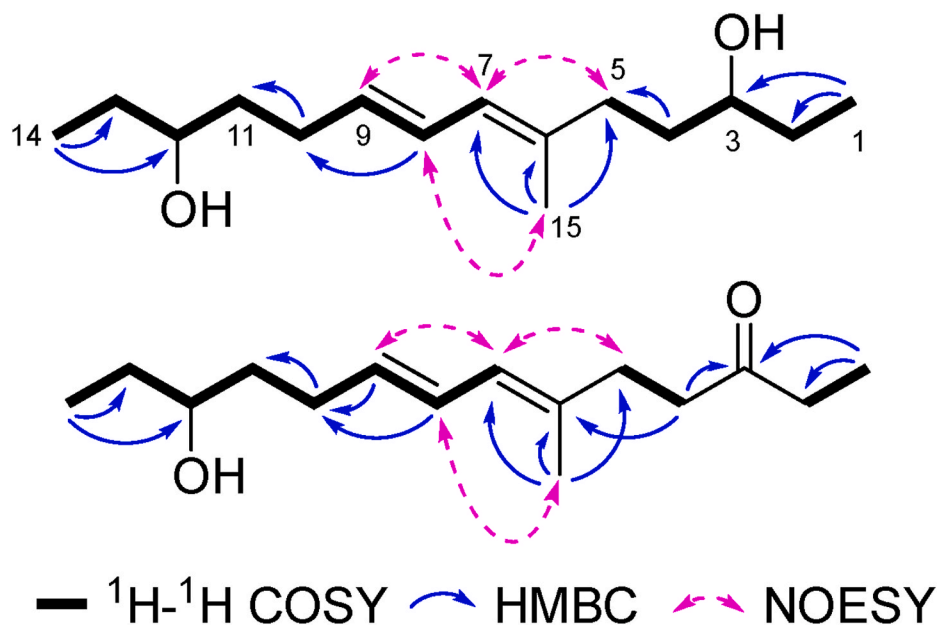**Fig. 2.** Structure of isolated compounds (**1**–**3**) from *F. proliferatum* 13294.

Fig. 3. Key 2D NMR correlations of compounds **1** and **2**.

[27]. Recently, there has been great progress in the research of the secondary metabolites from phytopathogenic fungi. Many natural products they produce e.g., sirodesmin PL [28,29], AF-toxin I/II [30], and cytochalasins B [31] show different promising bioactivities. *Fusarium* species are distributed worldwide, and mainly attack grain crops causing quality and yield reductions; they also produce a variety of mycotoxins, such as corn gibberelenone [11], beauvericin [32], fusaproliferin [33], and fumonisin [34]. In addition, abundant active secondary metabolites have also been isolated from *Fusarium* sp., including antibacterial and anticancer naphthoquinone derivatives [35,36], anticancer and anti-inflammatory alkaloids [37,38], antibacterial terpenoids [39], antifungal and anti-malarial cyclodepsipeptide [40], etc. For example, *F. solani* has the ability to produce cyclooxygenase-2 (COX-2) inhibitor fusopoltide A [41] and anti-tumor naphthoquinone compound solaninaphthoquione [42]. Kakeya et al. reported the isolation and structure elucidation of lucilactaene with unique structure, a cell cycle inhibitor derived from *Fusarium* sp. RK 97-94 [43]. Renner et al. focused on marine *Fusarium* species to mine novel bioactive secondary metabolites. Neomangicols, halogenated sesterterpenoids with cytotoxic activity, and mangicols A−G, sesterterpene polyols with remarkable anti-inflammatory activity, were identified from *F. heterosporum* [44]. In order to obtain alternatives of biohazardous nematocides, Bogner et al. got three secondary metabolites, gibepyrone D, indole-3-acetic acid, and 4-hydroxybenzoic acid, with prominent nematode-antagonistic activity from strain *F. oxysporum* 162 [45]. These results provided clues for the finding of novel biohazardous nematocides. Parnafungin A and B were initially isolated from *F. larvarum*, as antifungal agents. In addition, antiviral medicine podophyllotoxin [46], anti-malaria drug quinine and cinchonidine [47], as well as cardiovascular agent ginkgolide B [48], were reported to be prepared from *Fusarium* species. Furthermore, *F. proliferatum* is known to produce a variety of mycotoxins [49,50]. These mycotoxins can increase health risks when fungal contaminated agricultural products are consumed. However, there are many secondary metabolites from these species which have not been investigated.

This study on the pathogen *F. proliferatum* isolate FP13294 expands the knowledge about the chemical production and biological capabilities of the genus *Fusarium*. Compounds **1**–**2** were reported here for the first time and belongs to the alkenol family. Compound **3** is a sesquiterpenoid, identified from *Trichoderma lignorum* in 2004 [22] but have not yet been reported in the genus *Fusarium*. Our results showed

compounds **1** and **2** exhibited weak antibacterial activity against *S. epidermidi* (MIC = 100 μM).

Alkenols are widely distributed in nature and are reported to be utilized as antibacterial, anti-inflammatory and anti-tumor agents, and even as pheromones. Avocadene was reported to exhibit anti-bacterial as well as anti-inflammatory activities [51]. As a component of many essential oils, linalool showed anti-tumor effect [52] and was used in leukemia therapy [53]. In addition, 11-eicosen-1-ol was found to be the major ingredient of alarm pheromones of worker honeybee [54]. Therefore, potentiality of alkenol no matter in bioactive drugs development than in interaction progress between pathogen and host plants are cause for concern.

Declaration of competing interest

The authors declare that they have no conflicts of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.synbio.2021.10.001.

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