

# Fumigaclavines D–H, New Ergot Alkaloids from Endophytic *Aspergillus fumigatus*

## Authors

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## Key words

- *Aspergillus fumigatus*
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- *Cynodon dactylon*
- Poaceae
- clavine-type ergot alkaloids
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## Abstract

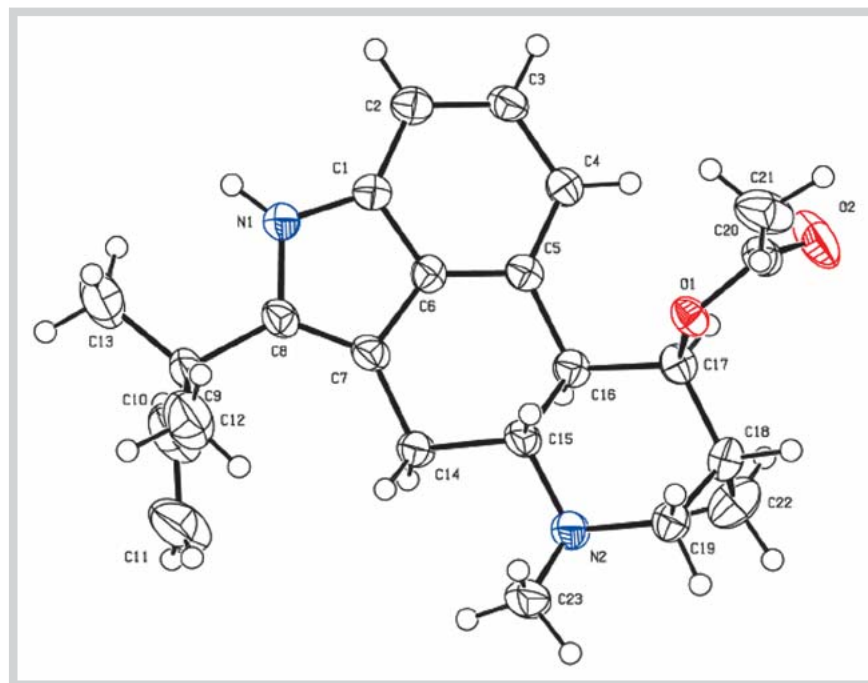
Ergot alkaloids are toxins which are produced biotechnologically on an industrial scale. The chemical investigation of endophytic *Aspergillus fumigatus* resulted in the isolation of five new ergot alkaloids named fumigaclavines D–H (2–6), along with three known analogues, fumigaclavine C (1), festuclavine (7), and fumigaclavine A (8). Their structures were unequivocally elucidated by extensive spectroscopic analyses in association with X-ray single-crystal diffraction. Fumigaclavines D–H are interesting clavine-type ergot alkaloids featuring a reverse prenyl moiety at C-2, with 1–4, 6, and 8 bearing additional substituents, e.g., an OH or OAc group at C-9. Compounds 2, 4, and 6–8 showed a broad spectrum of antimicrobial activity against a panel of anaerobic microorganisms, of which compounds 4 and 6 were the most active against *Veillonella parvula* with an MIC = 16 µg/mL compared to that (0.12 µg/mL) of tinidazole, co-assayed as a positive reference.

Supporting information available online at <http://www.thieme-connect.de/products>

## Introduction

Fungal endophytes are receiving increasing attention due to their productivity of biologically potent and structurally unforeseeable compounds, which are of significance in drug discovery [1–3]. The genus *Aspergillus* (Trichocomaceae), which contains about 180 recognized species, has been proven to be a rich source of bioactive metabolites [4]. Among the species of this genus, *Aspergillus fumigatus* Fresen. is a common airborne fungus and the most important *Aspergillus* species causing infective lung diseases. This species proved to be particularly productive regarding the accumulation of diverse natural products and resulted in the discovery of over 226 potentially bioactive secondary metabolites with 18 of them being ergot alkaloids [5]. The ergot alkaloids are a complex family of indole-derived mycotoxins that interact with several monoamine receptors of the central nervous system, negatively affecting cardiovascular, nervous, reproductive, and immune systems of exposed humans and animals [6,7]. The intensive use of antibiotics inevitably selects resistant microorganisms, which issue a continuing and cyclical need for new antibiotics. A search for new chemical structures with antimicrobial

activity traditionally requires testing of large batteries of different microbial strains [8]. During our ongoing search for chemically new and biologically potent metabolites from plant endophytes [9–13], we recently described the isolation and identification of several new ergot alkaloids from the endophytic fungus *A. fumigatus* isolated from a healthy stem of *Cynodon dactylon* (Poaceae) [14, 15]. A continued investigation of the minor constituents obtained after the fermentation of the fungus on solid medium afforded five new clavine-type metabolites named fumigaclavines D–H (2–6), along with three previously known compounds, fumigaclavine C (1), festuclavine (7), and fumigaclavine A (8). Some clinically relevant anaerobic microorganisms, *Peptostreptococcus anaerobius*, *Bacteroides diasonis*, *Veillonella parvula*, *Actinomyces israelii*, *Bacteroides vulgatus*, and *Streptococcus anaerobius*, were adopted for the antimicrobial screening. In the current paper, the isolation, structure elucidation, and antimicrobial activities of these compounds are reported herein.



**Fig. 1** Single-crystal X-ray structure of **1**. (Color figure available online only.)

## Results and Discussion

The mycelia and culture medium of the fungus *A. fumigatus* were extracted with ethyl acetate. The resulting ethyl acetate extract was dried and chromatographed on silica gel and Sephadex LH-20 followed by reversed-phase chromatography to yield eight alkaloids (**1–8**). Compounds **2**, **4**, and **6–8** showed a broad spectrum of antimicrobial activity against a panel of anaerobic microorganisms, of which compounds **4** and **6** were the most active against *V. parvula*.

Compound **1** was obtained as a colorless crystal. Its molecular formula was determined as  $C_{23}H_{30}N_2O_2$  by HR-ESI-MS ( $m/z$  367.37860, calcd. for  $[M + H]^+$  367.23855). Compound **1** was identified as fumigaclavine C [16] according to its  $^1H$  and  $^{13}C$  NMR spectra along with a set of 2D NMR experiments ( $^1H$ - $^1H$  COSY, NOESY, HSQC and HMBC). Although this compound was discovered as early as 1977, its absolute configuration has not ever been reported so far, which somewhat causes confusion about the C-8 stereochemistry with both 8S and 8R configurations being assigned in the literature [17]. In this paper, we report herein the absolute configuration of **1** by using low-temperature (100 K) single-crystal X-ray diffraction with Cu K $\alpha$  radiation analysis (● Fig. 1), and a (5R, 8R, 9S, 10R) stereochemistry of fumigaclavine C (**1**) was allowed to be established for the first time.

Fumigaclavine D (**2**), a white amorphous powder, has the molecular formula  $C_{22}H_{28}N_2O_5$ , established by HR-ESI-MS ( $m/z$  353.22165, calcd. for  $[M + H]^+$  353.22290), implying ten degrees of unsaturation. The  $^1H$  and  $^{13}C$  NMR data of **2** (● Tables 1 and 2) indicated that six of the ten units of unsaturation come from five carbon–carbon double bonds and a carbonyl. Therefore, the other four units of unsaturation come from four rings. The  $^1H$  NMR data of **2** and the information from its  $^1H$ - $^1H$  COSY spectrum revealed the presence of a three-spin system made of vicinal aromatic hydrogens (H-12 to H-14) and the partial structures of the dihydroergine moiety (H-2 to H-10) and ethylene group (H-22 to H-23). Comparison of the NMR data with those reported for fumigaclavine C (**1**) suggests that both compounds shared the same ba-

sic tetracyclic ergoline ring skeleton, except for the absence of an N-CH $_3$  signal within **2**. This conclusion was supported by the discernible HMBC interaction of H-7 with C-5 ( $\delta_C$  55.2), C-9 ( $\delta_C$  73.2), and C-18 ( $\delta_C$  15.7). In addition, the remarkable upfield shifts for C-5 ( $\delta_C$  55.2) and C-7 ( $\delta_C$  47.3) and downfield shifts for H-5 ( $\delta_H$  3.17, m) and H-7 [ $\delta_H$  3.19, dd, 13.3, 4.4 Hz, H-7 $\alpha$ ;  $\delta_H$  2.71, br d, 13.3 Hz, H-7 $\beta$ ] were observed. The NOESY correlations between H-10 and H-9, H-18, and between H-5 and H-25 were found in both compounds **1** and **2** (● Fig. 2) and corroborated that fumigaclavines C and D have the same relative configuration. Hence, **2** was finally identified as (5R, 8R, 9S, 10R)-6-demethylated fumigaclavine C.

Fumigaclavine E (**3**) was found to have the molecular formula  $C_{21}H_{28}N_2O$ , established by HR-ESI-MS ( $m/z$  325.33131, calcd. for  $[M + H]^+$  325.22799), which differed from that of **1** by 43 amu, suggesting the lack of an acetyl group. Comparison of NMR data indicated the compound featured the same core structure as **1** and possessed a hydroxyl group at C-9 rather than an acetoxy group as in 9-deacetylfumigaclavine C, previously isolated in our laboratory [14]. The position of the substituent was confirmed by the significant downfield shift of H-9 ( $\delta_H$  4.52, br s) and COSY correlations from H-9 to H-8 ( $\delta_H$  2.09, m) and H-10 ( $\delta_H$  3.15, d, 10.6 Hz). The relative stereochemistry of **3** was the same as **1**, as evidenced from the similar NMR data and NOESY relationships (● Fig. 2). Especially the NOESY correlations between H-10 and H-9, H-18 but not with H-5 are of importance as they directly proved the relative configuration of C-10. The similar Cotton effect in comparison with those of **1** thus supposed the assignment of the same stereochemistry of **3** as (5R, 8R, 9S, 10R)-9-deacetylfumigaclavine C.

Fumigaclavine F (**4**) shared the same molecular formula as **3**, as determined by the HR-ESI-MS ( $[M + H]^+$   $m/z$  325.32954, calcd. for  $C_{21}H_{28}N_2O$ , 325.22799) and NMR data. The  $^1H$  and  $^{13}C$  NMR data of **4** (● Tables 1 and 2) and HMBC correlations, which closely resemble those of **3**, showed that these two compounds had the same gross structure. The only difference is that the H-5 resonance of **4** shifted upfield to  $\delta_H$  2.58 (br d, 11.0 Hz) compared to

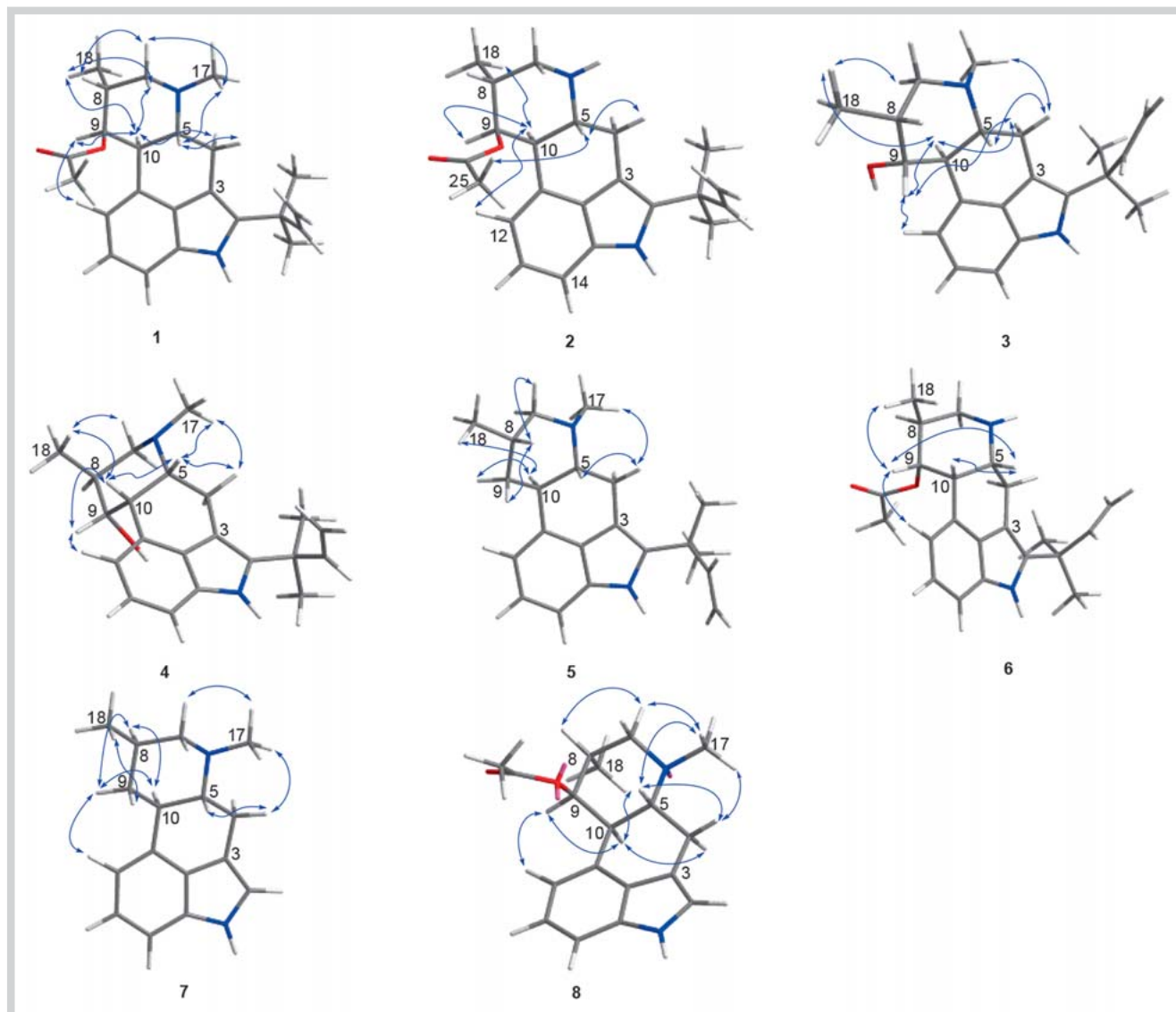
**Table 1** <sup>1</sup>H NMR (500 MHz) data (*J* in Hz) for fumigaclavines C–H (**1–6**) and festuclavine (**7**) and <sup>1</sup>H NMR (400 MHz) data (*J* in Hz) for fumigaclavine A (**8**).

No	1 <sup>b</sup>	2 <sup>a</sup>	3 <sup>a</sup>	4 <sup>a</sup>	5 <sup>a</sup>	6 <sup>a</sup>	7 <sup>a</sup>	8 <sup>a</sup>
2								6.90, s
4	3.51 (br d, 10.5)	3.24 (dd, 14.5, 4.5)	3.54 (dd, 14.4, 4.6)	3.50 (dd, 13.5, 3.5)	3.51 (dd, 14.5, 4.5)	3.32 (over- lapped)	3.43 (dd, 14.4, 4.2)	3.43 (dd, 20.4, 10.3)
	2.66 (t, 11.0)	2.71 (dd, 14.5, 10.7)	2.59 (dd, 14.4, 11.3)	2.58 (dd, 13.5, 11.0)	2.59 (dd, 14.5, 11.0)	2.79 (dd, 12.0, 3.3)	2.58 (dd, 14.4, 11.0)	2.66 (over- lapped)
5	2.61 (br d, 11.0)	3.17 (m)	2.74 (td, 11.3, 4.6)	2.54 (br d, 11.0)	2.03 (td, 10.5, 4.5)	3.31 (m)	2.54 (td, 11.0, 4.2)	2.66 (br d, 10.6)
7	2.71 (dd, 11.5, 3.5)	3.19 (dd, 13.3, 4.4)	2.98 (dd, 11.3, 3.7)	2.86 (dd, 11.5, 3.5)	2.85 (br d, 11.6)	3.30 (over- lapped)	2.99 (dt, 10.0, 1.9)	2.75 (br d, 11.9)
	2.64 (br d, 11.5)	2.71 (br d, 13.3)	2.71 (br d, 11.3)	2.60 (br d, 11.5)	2.47 (dd, 11.6, 3.5)	2.91 (br d, 13.2)	1.97 (t, 10.0)	1.97 (br d 11.9)
8	2.09 (m)	2.03 (m)	2.09 (m)	2.04 (m)	2.15 (m)	2.12 (m)	2.01 (m)	2.09 (m)
9	5.66 (br s)	5.68 (br s)	4.52 (br s)	4.49 (br s)	2.44 (m)	5.70 (br s)	2.67 (br d, 12.0)	5.64 (br s)
					1.63 (td, 13.0, 5.0)		1.09 (t, 12.0)	
10	3.30 (br d, 8.5)	3.13 (br d, 13.3)	3.15 (d, 10.6)	3.10 (dt, 9.0, 1.0)	3.00 (td, 11.0, 3.5)	3.24 (br d, 13.0)	2.92 (td, 13.0, 3.6)	3.32 (m)
12	6.71 (d, 7.0)	6.58 (d, 8.0)	6.89 (d, 7.1)	6.87 (d, 7.5)	6.75 (d, 8.0)	6.60 (d, 7.3)	6.83 (d, 7.1)	6.62 (d, 7.5)
13	7.03 (dd, 8.0, 7.0)	6.94 (t, 8.0)	7.03 (dd, 8.0, 7.1)	7.01 (dd, 8.0, 7.5)	6.96 (t, 8.0)	6.96 (dd, 8.0, 7.3)	7.04 (dd, 8.0, 7.1)	7.01 (dd, 8.1, 7.4)
14	7.07 (d, 8.0)	7.07 (d, 8.0)	7.09 (d, 8.0)	7.07 (d, 8.0)	7.07 (d, 8.0)	7.09 (d, 8.0)	7.12 (d, 8.0)	7.11 (d, 8.1)
17	2.43 (s)		2.48 (s)	2.49 (s)	2.39 (s)		2.49 (s)	2.45 (s)
18	1.31 (d, 7.0)	1.27 (d, 7.5)	1.28 (d, 7.5)	1.26 (d, 7.5)	1.30 (d, 7.0)	1.29 (d, 7.1)	1.03 (d, 6.3)	1.32 (d, 7.4)
20	1.52 (s)	1.52 (s)	1.52 (s)	1.50 (s)	1.52 (s)	1.52 (s)		
21	1.52 (s)	1.52 (s)	1.52 (s)	1.50 (s)	1.52 (s)	1.52 (s)		
22	6.09 (dd, 17.5, 10.5)	6.13 (dd, 17.5, 10.5)	6.13 (dd, 17.3, 10.6)	6.12 (dd, 17.0, 10.5)	6.13 (dd, 17.0, 10.5)	6.14 (dd, 17.6, 10.3)		
23	5.13 (d, 17.5)	5.04 (dd, 10.0, 1.2)	5.05 (dd, 10.6, 1.2)	5.03 (dd, 10.5, 1.5)	5.04 (dd, 10.5, 1.2)	5.05 (dd, 10.3, 1.2)		
	5.12 (d, 10.5)	5.03 (dd, 13.0, 1.2)	5.03 (dd, 17.3, 1.2)	5.01 (dd, 17.5, 1.5)	5.03 (dd, 17.0, 1.2)	5.05 (dd, 17.6, 1.2)		
25	1.88 (s)	1.85 (s)				1.86 (s)		1.88 (s)

<sup>a</sup> In methanol-*d*<sub>4</sub>; <sup>b</sup> in CDCl<sub>3</sub>**Table 2** <sup>13</sup>C NMR (125 MHz) data for fumigaclavines C–H (**1–6**) and festuclavine (**7**), and <sup>13</sup>C NMR (100 MHz) data for fumigaclavine A (**8**).

Position	1 <sup>b</sup>	2 <sup>a</sup>	3 <sup>a</sup>	4 <sup>a</sup>	5 <sup>a</sup>	6 <sup>a</sup>	7 <sup>a</sup>	8 <sup>a</sup>
2	136.7 (s)	138.3 (s)	138.4 (s)	138.7 (s)	138.2 (s)	130.4 (s)	119.2 (d)	119.5 (d)
3	106.1 (s)	106.5 (s)	105.9 (s)	106.8 (s)	106.6 (s)	106.5 (s)	111.3 (s)	111.0 (s)
4	28.1 (t)	31.6 (t)	28.5 (t)	29.3 (t)	29.0 (t)	30.7 (t)	27.6 (t)	27.4 (t)
5	61.6 (d)	55.2 (d)	62.3 (d)	62.7 (d)	70.1 (d)	55.2 (d)	68.7 (d)	63.7 (d)
7	57.8 (t)	47.3 (t)	58.7 (t)	58.9 (t)	64.4 (t)	47.1 (t)	66.1 (t)	59.0 (t)
8	33.1 (d)	34.3 (d)	36.8 (d)	37.4 (d)	29.5 (d)	33.9 (d)	31.4 (d)	34.6 (d)
9	71.4 (d)	73.2 (d)	69.4 (d)	70.3 (d)	34.7 (t)	72.5 (d)	37.6 (t)	73.0 (d)
10	39.4 (d)	41.2 (d)	41.5 (d)	42.2 (d)	36.2 (d)	40.6 (d)	41.7 (d)	40.9 (d)
11	129.2 (s)	129.5 (s)	129.6 (s)	130.5 (s)	133.6 (s)	128.9 (s)	133.5 (s)	130.1 (s)
12	112.8 (d)	112.4 (d)	113.8 (d)	114.3 (d)	113.2 (d)	112.9 (d)	113.4 (d)	113.1 (d)
13	122.2 (d)	122.4 (d)	122.7 (d)	123.1 (d)	122.6 (d)	122.7 (d)	123.5 (d)	123.5 (d)
14	107.7 (d)	109.2 (d)	109.1 (d)	109.5 (d)	109.0 (d)	109.5 (d)	109.7 (d)	109.8 (d)
15	134.5 (s)	132.6 (s)	134.5 (s)	135.0 (s)	134.1 (s)	134.8 (s)	135.1 (s)	135.5 (s)
16	128.0 (s)	128.4 (s)	129.4 (s)	130.0 (s)	128.5 (s)	129.3 (s)	127.4 (s)	128.2 (s)
17	43.5 (q)		43.7 (q)	43.4 (q)	44.2 (q)		43.3 (q)	44.0 (q)
18	16.6 (q)	15.7 (q)	17.7 (q)	17.7 (q)	18.8 (q)	15.6 (q)	19.9 (q)	17.0 (q)
19	39.0 (s)	40.1 (s)	40.8 (s)	40.8 (s)	40.3 (s)	40.3 (s)		
20	27.2 (q)	28.0 (q)	28.0 (q)	28.5 (q)	28.0 (q)	28.0 (q)		
21	27.4 (q)	28.1 (q)	28.1 (q)	28.7 (q)	28.2 (q)	28.1 (q)		
22	145.7 (d)	147.6 (d)	147.6 (d)	148.1 (d)	147.6 (d)	147.5 (d)		
23	111.8 (t)	111.6 (t)	111.5 (t)	111.9 (t)	111.4 (t)	111.7 (t)		
24	170.9 (s)	172.7 (s)				172.5 (s)		172.7 (s)
25	21.2 (q)	21.0 (q)				20.9 (q)		21.1 (q)

<sup>a</sup> In methanol-*d*<sub>4</sub>; <sup>b</sup> in CDCl<sub>3</sub>



**Fig. 2** Key NOE correlations of fumigaclavines 1–8. (Color figure available online only.)

$\delta_{\text{H}}$  2.74 (td, 11.3, 4.6) of **3**. Also, there were strong NOE correlations between H-10 ( $\delta_{\text{H}}$  3.17, d, 10.6 Hz) and H-5, H-9 ( $\delta_{\text{H}}$  4.52, br s), H-18 ( $\delta_{\text{H}}$  1.28, d, 7.5 Hz), as observed in **4**, suggesting that fumigaclavines E (**3**) and F (**4**) were closely similar (diastereomeric) with the opposite configuration of C-8, C-9, and C-10 in **4**. Therefore, **4** was an epimer of **3**, (5*R*, 8*S*, 9*R*, 10*S*)-9-deacetylfumigaclavine C.

The HR-ESI-MS and NMR data gave the molecular formula of fumigaclavine G (**5**) as  $\text{C}_{21}\text{H}_{28}\text{N}_2$ , 16 amu less than compound **3**, indicating the lack of a hydroxyl group. The NMR spectroscopic data (Tables 1 and 2) of **5** were very compatible with those of fumigaclavine E (**3**) except for the replacement of the 9-methine group [ $\delta_{\text{H}}$  4.52, brs;  $\delta_{\text{C}}$  69.4] in **3** by a methylene group [ $\delta_{\text{H}}$  2.44, m; 1.63, td, 13.0, 5.0 Hz;  $\delta_{\text{C}}$  34.7] in **5**. Confirming evidence was obtained from the  $^1\text{H}$ - $^1\text{H}$  COSY correlation from H-8 ( $\delta_{\text{H}}$  2.15, m) through H-10 ( $\delta_{\text{H}}$  3.00, td, 11.0, 3.5 Hz) and key HMBC correlations from H-9 ( $\delta_{\text{H}}$  2.15, m) to C-10 ( $\delta_{\text{C}}$  36.2), C-11 ( $\delta_{\text{C}}$  133.6), and C-18 ( $\delta_{\text{C}}$  18.8). Similar NOE correlations observed between H-10 with H-4 $\beta$  ( $\delta_{\text{H}}$  2.59, dd, 14.5, 11.0 Hz) and H<sub>3</sub>-18 ( $\delta_{\text{H}}$  1.30, d, 7.0 Hz), H-5 ( $\delta_{\text{H}}$  2.03, td, 10.5, 4.5 Hz) with H-4 $\alpha$  ( $\delta_{\text{H}}$  3.51, dd,

14.5, 4.5 Hz) revealed that **5** shared the same relative stereochemistry as **3**. Hence, **5** was (5*R*, 8*R*, 10*R*)-9-deacetylfumigaclavine C.

Fumigaclavine H (**6**) had the same molecular formula as **2** and the HR-ESI-MS ( $[\text{M} + \text{H}]^+$   $m/z$  353.24883, calcd. for  $\text{C}_{22}\text{H}_{28}\text{N}_2\text{O}_5$ , 353.22290) indicated that **6** was a diastereoisomer of **2**. The  $^1\text{H}$  and  $^{13}\text{C}$  NMR data of **5** (Tables 1 and 2) were very similar to those of **2**, which was featured by the presence of an acetoxy group bound to C-9 ( $\delta_{\text{C}}$  72.5), the  $^3J$  HMBC correlation of H<sub>3</sub>-25 ( $\delta_{\text{H}}$  1.86, s) with C-24 ( $\delta_{\text{C}}$  172.5), and  $^1\text{H}$ - $^1\text{H}$  COSY correlations from H-8 ( $\delta_{\text{H}}$  2.12, m) through H-10 ( $\delta_{\text{H}}$  3.24, br d, 13.0). The distinction was ascribed to the multiplet signal of an oxymethine group at  $\delta_{\text{H}}$  3.17 in **2** that shifted downfield to  $\delta_{\text{H}}$  3.32 in **6**, suggesting **6** was an epimer of **2**. This was confirmed by the NOE correlations between H-10 and H-5, and between H-9 and H-18, as can be seen in Fig. 2. Thus, the  $\alpha$ -orientation of H-5 was established and **6** was assigned to (5*R*, 8*S*, 9*R*, 10*S*)-6-demethylated fumigaclavine C.

All isolated compounds were evaluated for their activities against a panel of anaerobic microorganisms that included *P. anaerobius*,

**Table 3** Activity (minimum inhibitory concentration) of compounds **1–8** against different anaerobic microorganisms.

Compound	MIC [ $\mu\text{g/mL}$ ]					
	<i>P. anaerobius</i>	<i>B. diatasonis</i>	<i>V. parvula</i>	<i>A. israelii</i>	<i>B. vulgatus</i>	<i>S. anaerobius</i>
<b>1</b>	> 128	> 128	> 128	> 128	> 128	> 128
<b>2</b>	64	64	32	64	128	128
<b>3</b>	> 128	> 128	> 128	> 128	> 128	> 128
<b>4</b>	32	32	16	32	64	32
<b>5</b>	> 128	> 128	> 128	> 128	> 128	> 128
<b>6</b>	32	32	16	32	> 128	32
<b>7</b>	64	32	32	32	64	32
<b>8</b>	128	128	64	128	128	128
Tinidazole <sup>a</sup>	0.5	0.5	0.12	8.0	0.5	0.25

<sup>a</sup> A prescribed drug used as a positive control in the study

*B. diatasonis*, *V. parvula*, *A. israelii*, *B. vulgatus*, and *S. anaerobius*. The results summarized in **Table 3** indicate that the compounds behaved very differently. Compounds **2**, **4**, and **6–8** exhibited a broad spectrum of antimicrobial activities towards all tested microorganisms with MIC values ranging from 16–128  $\mu\text{g/mL}$ , and compounds **4** and **6** displayed antimicrobial activity against *V. parvula* (MIC = 16  $\mu\text{g/mL}$ ). However, the other investigated compounds were proved to be devoid of significant activity at any reasonable concentration (MIC > 128  $\mu\text{g/mL}$ ).

Prior to the discovery of fumigaclavines D–H (**2–6**), the only examples of the clavinet-type ergot alkaloids with a reverse prenyl moiety at C-2 were fumigaclavine C (**1**), 9-deacetylfumigaclavine C, and 9-deacetoxyfumigaclavine C, which were only found in *Aspergillus* [5]. Fumigaclavines D–H (**2–6**) are new members featuring a unique reverse prenyl moiety at C-2, **1–4**, and **6**, bearing additional substituents, e.g., an OH or OAc group at C-9. Precursors of ergot alkaloids with a tricyclic structure carry a 6,7-*seco*-D-ring [17]. With the functional proof of biosynthetic enzymes, it should be possible for the naturally occurring substance chanoclavine-I as well as its two isomers, chanoclavine-II and isochanoclavine-I, to produce the corresponding derivatives. Clearly they are closely structurally related to the previously reported fumigaclavine C (**1**), festuclavine (**7**), and fumigaclavine A (**8**). Their putative biogenetic pathway A to C originating from naturally occurring secoergolones is depicted in **Fig. 3**.

## Materials and Methods



### General experimental procedures

Optical rotations were recorded on a Perkin-Elmer 341 digital polarimeter. UV spectra were recorded on a Hitachi U-3000 spectrophotometer. The IR spectra were measured on a Nexus 870 FT-IR spectrometer. HRESIMS spectra were recorded on an Agilent 6210 mass spectrometer. NMR data were acquired on Bruker AV500 and AV300 NMR spectrometers using TMS as the internal standard, and chemical shifts were recorded as  $\delta$  values. Single-crystal X-ray diffraction data were collected on an Agilent Technologies SuperNova Dual diffractometer with an Atlas detector (Cu K $\alpha$  radiation,  $\lambda$  = 1.54184 Å). Optical rotations were recorded on a Rudolph Autopol III automatic polarimeter. UV spectra were recorded on a Hitachi U-3000 spectrophotometer. Silica gel (200–300 mesh) for column chromatography was purchased from Qingdao Marine Chemical Factory. Sephadex LH-20 was purchased from Pharmacia Biotech. All other chemicals were of analytical grade. All anaerobes isolated from clinic specimens were

supplied by Jiangsu Provincial Center for Disease Prevention and Control (Nanjing, China). Tinidazole (99% purity) was purchased from Arden Chemical Company

### Isolation and cultivation of the fungus

The title strain of *A. fumigatus* (strain No. CY018) was isolated from the healthy stem of *C. dactylon* collected in November 2001 from Yancheng Biosphere Reserve, Jiangsu Province. The collected plants were authenticated by Prof. L.X. Zhang (Nanjing University), with a voucher specimen preserved under the number YC01–11–34 in the herbarium of Nanjing University. The isolate was identified as *A. fumigatus* by one of the authors (Dr. Y.C. Song) using a molecular biological protocol by DNA amplification and sequencing of the ITS region (GenBank accession no. KJ809565). A voucher specimen is deposited in our laboratory at  $-80^{\circ}\text{C}$ . The working strain was preserved on potato dextrose agar slants and stored at  $4^{\circ}\text{C}$ .

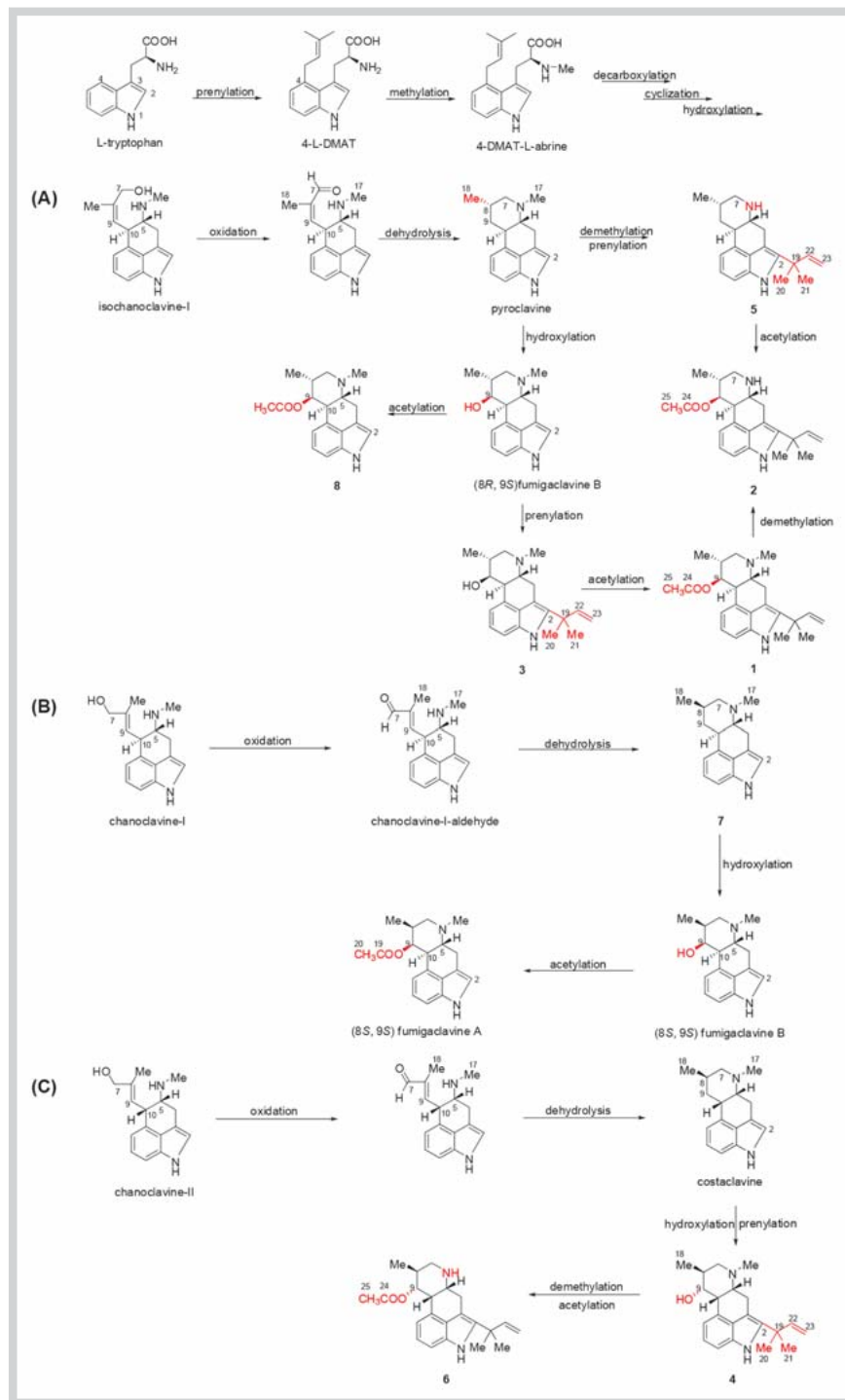
### Fermentation and extraction

The fungal strain was cultured on petri dishes of potato dextrose agar (PDA) at  $28^{\circ}\text{C}$  for five days. The agar patches with the title fungus were inoculated into Erlenmeyer flasks (1000 mL) each containing 300 mL of PDA medium. After four days, the seed culture was transferred into 250 mL flasks, each preloaded with the evenly mingled medium composed of 7.5 g of grain, 7.5 g of bran, 0.5 g of yeast extract, 0.1 g of sodium tartrate, 0.01 g of FeSO<sub>4</sub>·7H<sub>2</sub>O, 0.1 g of sodium glutamate, 0.1 mL of pure corn oil, and 30 mL of H<sub>2</sub>O, and grown for 40 days at  $28 \pm 1^{\circ}\text{C}$  with the relative humidity in the range of 60–70%.

The harvested culture (1000 g dry weight) was extracted at room temperature with alcohol ( $3 \times 2\text{ L}$ ). Evaporation of the solvent under reduced pressure yielded a black oil (60 g), which was diluted with H<sub>2</sub>O to give an aqueous suspension. The suspension was successively extracted with petroleum ether and EtOAc. The EtOAc fraction was shown to be cytotoxic, and it was concentrated *in vacuo* to give a residue (42 g) for further separation.

### Isolation and purification

The EtOAc extract (42 g) was subjected to automatic medium pressure liquid chromatography employing a step gradient of dichloromethane-methanol. Si 60 F<sub>254</sub> for TLC and H-Sil gel (200–300 mesh) for column chromatography (CC) were purchased from Qingdao Marine Chemical Company. Forty-one fractions were collected using a dichloromethane-methanol-based solvent system to yield eight fractions, F1–F8. Promising fraction F2 (3.2 g) was subjected to further medium pressure liquid chroma-



**Fig. 3** A putative biogenetic pathway of fumigaclavines 1–8. (Color figure available online only.)

tography with petroleum ether-acetone as the solvent. Subfraction F2–6 (376 mg) was further purified by RP C-18 column (2.5 × 60 cm, MeOH/H<sub>2</sub>O = 6 : 4) to afford compounds **1** (77.4 mg) and **3** (1.5 mg). F3 (460 mg) was processed in the same way as F2 on medium pressure liquid chromatography using petroleum ether-acetone as the eluent. Subfraction F3–5 was eluted with Sephadex LH-20 using dichloromethane-methanol 1 : 1. F3–5–1, with petroleum ether-dichloromethane-acetone 1 : 1 : 2 as the eluent, was purified by silica gel CC (2 × 80 cm) to afford compounds **2** (1.5 mg), **5** (3.4 mg), and **6** (2.6 mg). F4 (2.1 g) was subjected to medium pressure liquid chromatography using petroleum ether-acetone as the eluent. Subfraction F4–3 was passed

through Sephadex LH-20 using a methanol/H<sub>2</sub>O mixture, 8 : 2. Final purification was achieved by using an RP C-18 column (2.5 × 60 cm, MeOH/H<sub>2</sub>O = 7 : 3) of F4–3–3 to yield compounds **4** (505 mg), **7** (6.0 mg), and **8** (6.95 mg).

#### Antimicrobial assays

Antimicrobial assays using *P. anaerobius*, *B. diatonis*, *V. parvula*, *A. israelii*, *B. vulgatus*, and *S. anaerobius* were conducted as previously described [18, 19]. The MICs were determined after incubating the clinical anaerobic bacteria for 48 h at 35 °C in an atmosphere of 80% N<sub>2</sub>, 10% CO<sub>2</sub>, and 10% H<sub>2</sub>. The microtiter plates were read visually, and the minimum concentration of the ex-

tracts that produced no turbidity was recorded as the MIC. All assays were performed at the Department of Clinical Laboratory of the First Affiliated Hospital of Nanjing Medical University and repeated three times to maximize reliability and reproducibility. The purity of all compounds tested was greater than 95% as determined by TLC and NMR. Tinidazole (purity  $\geq 99\%$ , Sigma-Aldrich) was co-assayed as a positive control in all of the antimicrobial assays.

### Isolates

**Fumigaclavine C (1):** colorless crystal (MeOH);  $[\alpha]_D^{25} - 92.5$  (c 0.20, CHCl<sub>3</sub>); UV (MeOH)  $\lambda_{\max}$  (log  $\epsilon$ ) 228 (8.24), 281 (2.12) nm; <sup>1</sup>H and <sup>13</sup>C NMR data, see **Tables 1** and **2**, respectively; HR-ESI-MS  $m/z$  367.3786 [M + H]<sup>+</sup> (calcd. for C<sub>23</sub>H<sub>31</sub>N<sub>2</sub>O<sub>2</sub>, 367.2386).

**Fumigaclavine D (2):** white amorphous powder (MeOH);  $[\alpha]_D^{25} + 46.2$  (c 0.20, CHCl<sub>3</sub>); UV (MeOH) (log  $\epsilon$ )  $\lambda_{\max}$  229 (8.13), 282 (2.09) nm; <sup>1</sup>H and <sup>13</sup>C NMR data, see **Table 1**; HR-ESI-MS  $m/z$  353.2216 [M + H]<sup>+</sup> (calcd. for C<sub>22</sub>H<sub>29</sub>N<sub>2</sub>O, 353.2229).

**Fumigaclavine E (3):** white amorphous powder (MeOH);  $[\alpha]_D^{25} + 12.9$  (c 0.20, CHCl<sub>3</sub>); UV (MeOH) (log  $\epsilon$ )  $\lambda_{\max}$  229 (7.89), 282 (2.01) nm; <sup>1</sup>H and <sup>13</sup>C NMR data, see **Tables 1** and **2**, respectively; HR-ESI-MS  $m/z$  325.3313 [M + H]<sup>+</sup> (calcd. for C<sub>21</sub>H<sub>29</sub>N<sub>2</sub>O, 325.3315).

**Fumigaclavine F (4):** white amorphous powder (MeOH);  $[\alpha]_D^{25} - 41.7$  (c 0.20, CHCl<sub>3</sub>); UV (MeOH)  $\lambda_{\max}$  (log  $\epsilon$ ) 229 (8.29), 281 (2.13) nm; <sup>1</sup>H and <sup>13</sup>C NMR data, see **Tables 1** and **2**, respectively; HR-ESI-MS  $m/z$  325.3295 [M + H]<sup>+</sup> (calcd. for C<sub>21</sub>H<sub>29</sub>N<sub>2</sub>O, 325.2280).

**Fumigaclavine G (5):** white amorphous powder (MeOH);  $[\alpha]_D^{25} - 50.3$  (c 0.20, CHCl<sub>3</sub>); UV (MeOH) (log  $\epsilon$ )  $\lambda_{\max}$  230 (8.37), 282 (2.20) nm; <sup>1</sup>H NMR data, see **Table 1**; HR-ESI-MS  $m/z$  309.2341 [M + H]<sup>+</sup> (calcd. for C<sub>21</sub>H<sub>29</sub>N<sub>2</sub>, 309.2331).

**Fumigaclavine H (6):** white amorphous powder (MeOH);  $[\alpha]_D^{25} - 90.6$  (c 0.20, CHCl<sub>3</sub>); UV (MeOH) (log  $\epsilon$ )  $\lambda_{\max}$  229 (7.24), 281 (1.88) nm; <sup>1</sup>H and <sup>13</sup>C NMR data, see **Tables 1** and **2**, respectively; HR-ESI-MS  $m/z$  353.2488 [M + H]<sup>+</sup> (calcd. for C<sub>22</sub>H<sub>29</sub>N<sub>2</sub>O<sub>5</sub>, 353.2229).

**Festoclavine (7) [20]:** white amorphous powder (MeOH);  $[\alpha]_D^{25} + 13.4$  (c 0.20, CHCl<sub>3</sub>); UV (MeOH) (log  $\epsilon$ )  $\lambda_{\max}$  221 (6.97), 280 (1.83) nm; <sup>1</sup>H and <sup>13</sup>C NMR data, see **Table 1**; HR-ESI-MS  $m/z$  241.1843 [M + H]<sup>+</sup> (calcd. for C<sub>16</sub>H<sub>21</sub>N<sub>2</sub>, 241.1705).

**Fumigaclavine A (8):** white amorphous powder (MeOH);  $[\alpha]_D^{25} - 91.3$  (c 0.20, CHCl<sub>3</sub>); UV (MeOH) (log  $\epsilon$ )  $\lambda_{\max}$  223 (5.94), 281 (1.71) nm; <sup>1</sup>H and <sup>13</sup>C NMR data, see **Tables 1** and **2**, respectively; HR-ESI-MS  $m/z$  299.2420 [M + H]<sup>+</sup> (calcd. for C<sub>18</sub>H<sub>23</sub>N<sub>2</sub>O<sub>2</sub>, 299.1756).

### Supporting information

<sup>1</sup>H NMR, <sup>13</sup>C NMR, <sup>1</sup>H-<sup>1</sup>H COSY, HMQC, HMBC, ROSEY, HR-ESI-MS and CD spectra for compounds **1–8** are available as Supporting Information.

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### Conflict of Interest



There is no conflict of interest among all authors.

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