Secondary Metabolites from *Aspergillus fumigatus*, an Endophytic Fungus from the Liverwort *Heteroscyphus tener* (STEPH.) SCHIFFN.

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Three new metabolites, asperfumigatin (**1**), isochaetominine (**10**), and 8′-*O*-methylasterric acid (**21**), together with nineteen known compounds, were obtained from the culture of *Aspergillus fumigatus*, an endophytic fungus from the Chinese liverwort *Heteroscyphus tener* (STEPH.) SCHIFFN. Their structures were established by extensive analysis of the spectroscopic data. The absolute configurations of **1** and **10** were determined by analysis of their respective CD spectra. Cytotoxicity of these isolates against four human cancer cell lines was also determined.

Introduction. – Endophytic fungi are microorganisms that live in the host tissues without causing any disease symptoms, and most of them are reported to have a beneficial effect on their host organisms [1][2]. Due to the microorganisms living in the host plants, various physiological functions are being realized [3]. In the process of co-evolution with the host, endophytic fungi produce the same or similar metabolites as the host [4]. In addition, endophytic fungi have become an important source for the discovery of novel structures displaying a variety of biological activities [5][6].

Species in *Aspergillus* have been sources of lifesaving drugs and are renowned for their medical and commercial importance [7]. Recent studies have demonstrated that the metabolites with a broad range of biological activities from *Aspergillus* can be found in the ocean, soil, and plants. This provides a new potential source of novel medicinal compounds [8–10]. In the course of our ongoing efforts to discover potential anticancer agents from liverwort and the endophytes within it, the extract of an endophytic strain of *Aspergillus fumigatus* isolated from the liverwort *Heteroscyphus tener* (STEPH.) SCHIFFN. showed strong cytotoxicity against the PC3 human prostate cancer cell line with an IC_{50} value of 16.72 μg/ml. Bioassay-guided separation afforded 22 compounds (*Fig. 1*) including two new alkaloids and one new diphenyl ether. Cytotoxicity evaluation against four human cancer cell lines revealed that most of them have cytotoxic activities with the IC_{50} value of 19.9–39.9 μM. Herein, we report the isolation, structure elucidation, and bioassay of the compounds.

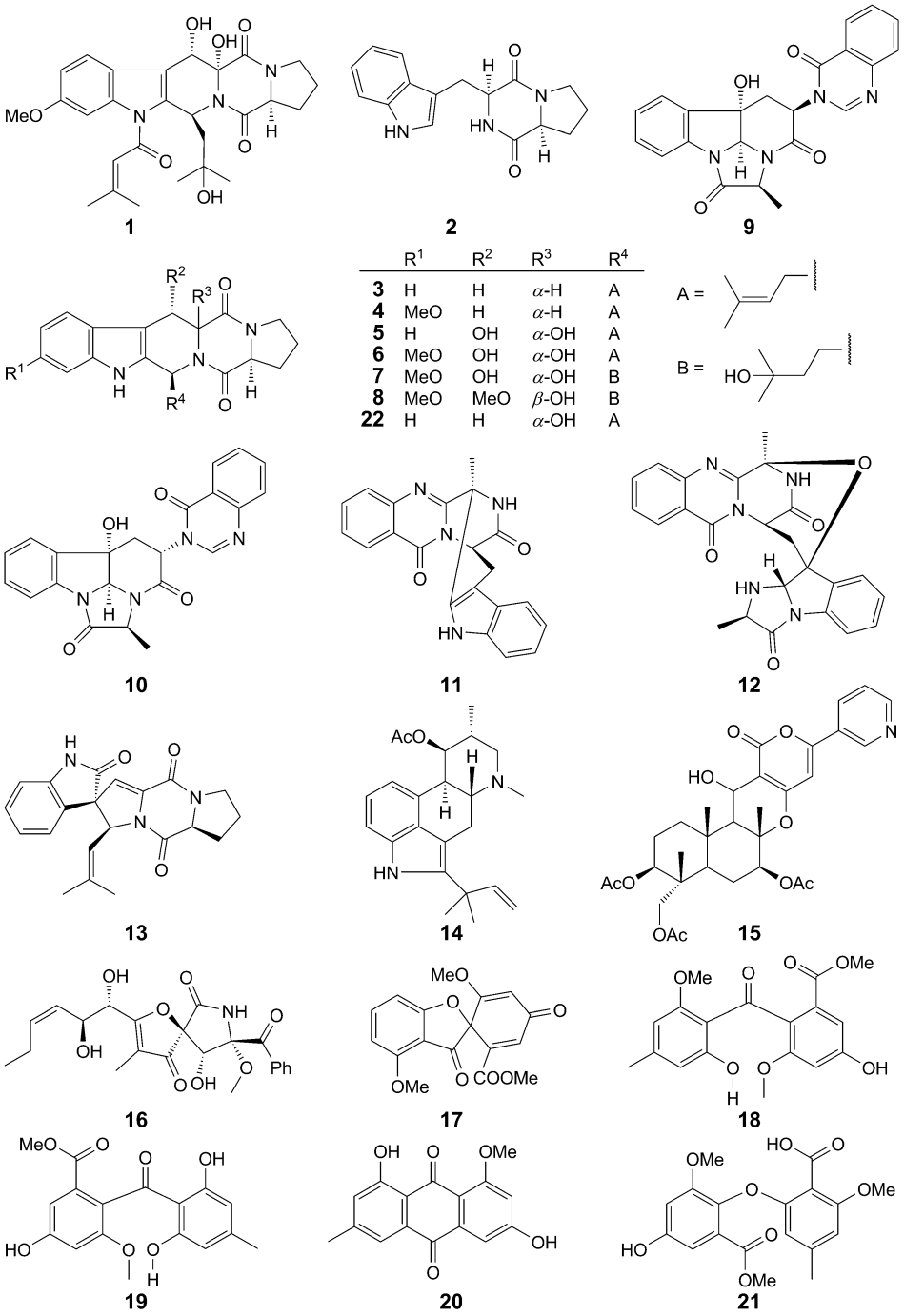

Fig. 1. *Structures of compounds* **1**–**22**

Results and Discussion. – The AcOEt extract of *A. fumigatus* afforded compounds **1**–**22** after a combination of size-exclusion, normal phase, and reversed-phase chromatography.

Structure Elucidation. Compound **1** was obtained as yellow amorphous solid. The molecular formula was determined as $C_{27}H_{33}N_3O_7$ by HR-ESI-MS (*m*/*z* 512.2388 ($[M+H]^+$; calc. 512.2397)). The ^{1}H-NMR spectrum of **1** (*Table 1*) displayed four Me signals (δ(H) 1.14, 1.28, 2.09, and 2.20, all (*s*)), one MeO signal (δ(H) 3.85), four olefinic/aromatic H-atom signals (δ(H) 6.33, 6.89, 7.39, and 7.89), and three exchangeable H-atom signals (δ(H) 3.48, 3.92, and 4.63). The ^{13}C-NMR spectrum (*Table 1*) exhibited 27 signals accounted for the functional groups described above and three amide C=O signals (δ(C) 165.7, 166.1, and 171.9). The ^{1}H- and ^{13}C-NMR data were similar to those of verruculogen TR-2 (**7**) [10], except that NH(1) was substituted by an amide side chain in **1**. This additional group was determined to be 3-methylbut-2-enoate by the HMBCs from H–C(26) to C(25), C(27), C(28), and C(29) and those from Me(28) and Me(29) to C(26) (*Fig. 2*). Furthermore, an upfield shift of C(25) (δ(C) 165.7) confirmed the presence of a conjugated amide group. Thus, the structure of **1** was established as an indole-diketopiperazine bearing an additional 3-methylbut-2-enoate at N(1) (*Fig. 2*). By comparison of its NOESY correlations, CD spectra (*Fig. 3,a*), and the specific optical rotation value ($[\alpha]_D^{25} = -45.5$ ($c = 0.2$, MeOH)) [11] with those of compound **7**, the absolute configuration of **1** was determined to be (3*S*,6*S*,12*R*,13*S*). The trivial name asperfumigatin was given to this new compound.

Compound **10** was obtained as colorless crystals. It possessed the same molecular formula as chaetominine (**9**) [6], $C_{22}H_{18}O_4N_4$, established by HR-ESI-MS (*m*/*z* 403.1398 ($[M+H]^+$; calc. 403.1406)). The similar ^{1}H- and ^{13}C-NMR spectral data of

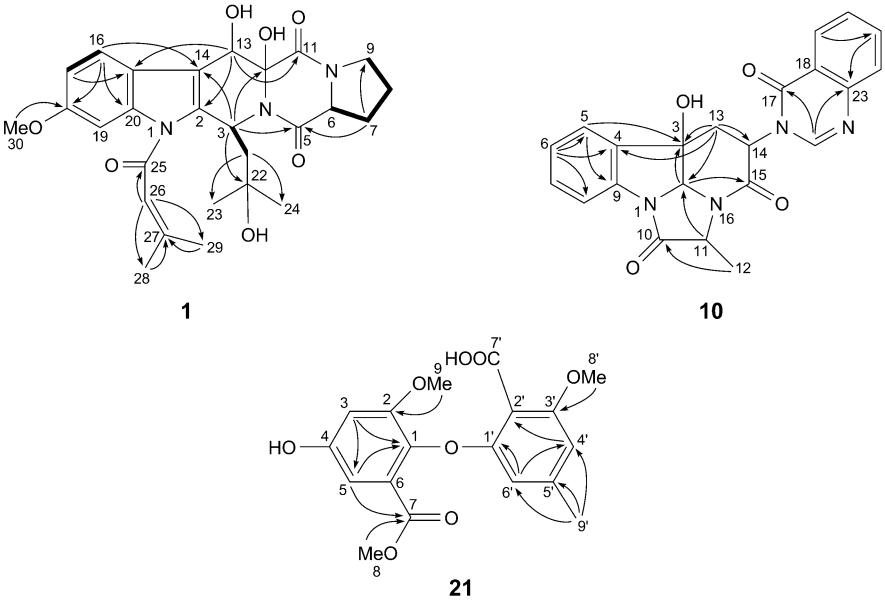

Fig. 2. *Key HMBC* (H→C) *and* ^{1}*H,*^{1}*H-COSY* (—) *correlations of* **1**, **10**, *and* **21**

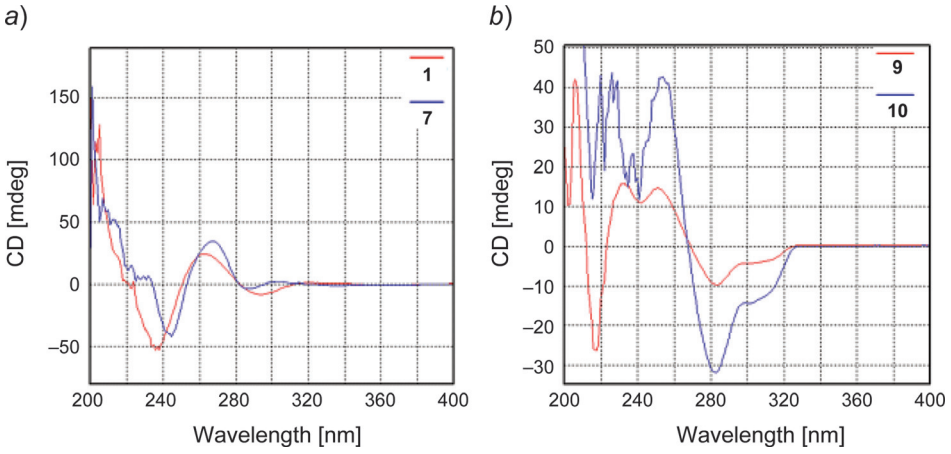

Fig. 3. a) *CD Spectra of compounds* **1** *and* **7**, b) *CD Spectra of compounds* **9** *and* **10**

Table 1. *^{1}H- and ^{13}C-NMR Data*[a]) ($CDCl_3$) *of Compound* **1**. δ in ppm, J in Hz.

Position	δ(H)	δ(C)	Position	δ(H)	δ(C)
2		133.4	17	6.89 (*d*, *J* = 9.0)	111.7
3	6.28–6.30 (*m*)	48.1	18		158.8
5		166.1	19	7.39 (*s*)	99.9
6	4.49–4.52 (*m*)	59.5	20		137.2
7	2.46–2.50 (*m*, H_a), 2.06–2.11 (*m*, H_b)	29.0	21	1.80–1.85 (*m*, H_a), 1.67–1.71 (*m*, H_b)	50.8
8	2.04–2.10 (*m*, H_a), 1.94–1.99 (*m*, H_b)	23.0	22		69.5
			23	1.28 (*s*, 3 H)	30.0
9	3.60–3.64 (*m*, 2 H)	45.3	24	1.14 (*s*, 3 H)	29.8
11		171.9	25		165.7
12		83.7	26	6.33 (*s*)	120.1
13	5.73 (*s*)	68.5	27		158.8
14		113.7	28	2.09 (*s*, 3 H)	27.5
15		121.9	29	2.20 (*s*, 3 H)	21.3
16	7.89 (*d*, *J* = 9.0)	122.0	30	3.85 (*s*, 3 H)	55.8

[a]) Recorded at 600 and 150 MHz, respectively. Data assignments were based on ^{1}H,^{1}H-COSY, HMQC, HMBC, and NOESY experiments.

compounds **9** and **10** (*Table 2*) demonstrated that they have the same gross structure, which was confirmed by HMBCs (*Fig. 2*). Based on a comparison of the NOESY and CD spectra of compound **9** with those reported previously [6], the absolute configuration of **9** was elucidated as (2*S*,3*S*,11*S*,14*R*), as in the case of compound **9** [6]. In the NOESY spectrum of **10**, the correlations of H–C(2)/HO–C(3) and H–C(2)/H–C(11) implied that the relative configurations of C(2), C(3), and C(11) were the same as those in compound **9**. However, lack of a NOESY correlation between H–C(14) and H–C(2) indicated different configuration at C(14) in **10**. Another

Table 2. *^{1}H- and ^{13}C-NMR Data*[a])[b]) ((D$_6$)DMSO) *of Compound* **10**. δ in ppm, J in Hz.

Position	δ(H)	δ(C)	Position	δ(H)	δ(C)
2	5.85 (*s*)	82.9	14	5.91 (br. *s*)	49.0
3		76.6	15		160.0
4		137.6	17		166.3
5	7.47	124.7	18		121.0
6	7.24 (*t*, *J*=7.2)	125.6	19	8.19 (*d*, *J*=7.2)	126.5
7	7.42 (*t*, *J*=7.2)	129.8	20	7.59 (*t*, *J*=7.2)	127.3
8	7.48	114.5	21	7.87 (*t*, *J*=7.2)	134.8
9		137.7	22	7.70 (*d*, *J*=7.2)	127.2
10		170.9	23		146.7
11	4.64 (*q*, *J*=6.6)	59.7	25	8.23 (br. *s*)	147.4
12	1.49 (*d*, *J*=6.6)	15.1	3-OH	6.78 (*s*)	
13	2.47 (H$_a$), 2.93 (*t*, *J*=12.2, H$_b$)	38.4			

[a]) Recorded at 600 and 150 MHz, respectively. Signal assignments are based on ^{1}H,^{1}H-COSY, HMQC, HMBC, and NOESY experiments. [b]) Some coupling constants could not be determined due to signal overlap.

evidence can be seen in the ^{13}C-NMR spectrum of **10**, in which C(14) was shifted upfield from δ(C) 50.1 in **9** to δ(C) 49.0 in **10**. From a biosynthetic standpoint, together with comparison of the CD spectra of compounds **9** and **10** (*Fig. 3,b*), the absolute configuration of **10** was established as (2*S*,3*S*,11*S*,14*S*). Finally, the structure of compound **10**, named isochaetominine, was unambiguously determined as depicted in *Fig. 1*.

Compound **21** was obtained as yellow amorphous solid. The molecular formula was determined as $C_{18}H_{18}O_8$ by HR-ESI-MS (*m*/*z* 363.1078 ($[M+H]^+$; calc. 363.1080)). In the ^{1}H-NMR spectrum of **21**, one Me signal at δ(H) 2.14, three MeO signals at δ(H) 3.61, 3.67, and 3.76, four aromatic H-atom signals at δ(H) 5.75, 6.47, 6.74, and 6.76, two OH signals at δ(H) 9.95 and 12.56 (br.) were observed. The NMR data of **21** were similar to those of asterric acid [12], with the noticeable difference of additional resonances (δ(H) 3.76; δ(C) 55.7) due to a MeO group at C(3′). This finding was supported by the HMBCs of **21** (*Fig. 2*). Accordingly, the structure of compound **21** was determined as 8′-*O*-methylasterric acid.

The known compounds were identified as brevianamide F (**2**) [13], demethoxyfumitremorgin C (**3**) [14], fumitremorgin C (**4**) [15], cyclotryprostatin C (**5**) [16], 12,13-dihydroxyfumitremorgin C (**6**) [17], verruculogen TR-2 (**7**) [10], 20-hydroxycyclotryprostatin B (**8**) [18], chaetominine (**9**) [6], fumiquinazoline J (**11**) [19], fumiquinazoline C (**12**) [20], spirotryprostatin B (**13**) [21], fumigaclavine C (**14**) [22], pyripyropene A (**15**) [23], pseurotin A (**16**) [24], trypacidin (**17**), 1,2-*seco*-trypacidin (**18**) [25], sulochrin (**19**) [26], questin (**20**) [27], and 13-dehydroxycyclotryprostatin C (**22**) [28] by comparing their spectroscopic data with previously reported data.

Biological Activity. All of the isolated compounds were evaluated to determine their cytotoxic activity against the human prostate cancers PC3, multiple drug resistance PC3D cells, the human lung adenocarcinoma epithelial cell line A549, and the human lung cancer cell line NCI-H460 using the MTT (= 3-(4,5-dimethylthiazol-2-

Table 3. *Cytotoxicity of Compounds* **1**–**22** *against Four Cell Lines*[a])

Compound	PC3	PC3D	A549	NCI-H460
1	30.6 ± 0.2	> 40	> 40	> 40
2	> 40	> 40	> 40	> 40
3	32.0 ± 0.5	> 40	> 40	> 40
4	28.9 ± 0.2	39.4 ± 2.5	> 40	> 40
5	33.9 ± 0.2	> 40	> 40	> 40
6	36.2 ± 0.4	39.6 ± 1.0	> 40	> 40
7	38.9 ± 0.5	> 40	> 40	> 40
8	32.5 ± 0.8	> 40	> 40	> 40
9	30.1 ± 0.7	> 40	> 40	> 40
10	32.2 ± 0.5	> 40	> 40	> 40
11	> 40	> 40	> 40	26.9 ± 0.6
12	27.8 ± 0.4	> 40	> 40	33.4 ± 0.7
13	35.2 ± 0.5	> 40	> 40	> 40
14	26.6 ± 0.7	> 40	> 40	> 40
15	23.4 ± 0.8	27.7 ± 0.7	38.3 ± 0.8	> 40
16	> 40	> 40	> 40	> 40
17	19.9 ± 0.5	39.6 ± 1.1	33.8 ± 0.8	31.0 ± 0.5
18	> 40	> 40	> 40	> 40
19	> 40	> 40	> 40	> 40
20	> 40	> 40	> 40	> 40
21	> 40	> 40	> 40	> 40
22	35.9 ± 0.6	39.9 ± 1.3	> 40	> 40
Cisplatin[b])	9.1 ± 0.5	8.23 ± 0.8	13.5 ± 1.3	10.7 ± 0.8

[a]) Results are listed as the mean values of $IC_{50} \pm$ S. D. in μM. [b]) Cisplatin was used as positive control. The experiments were repeated three times.

yl)-2,5-diphenyl-2*H*-tetrazolium bromide) assay [29]. As shown in *Table 3*, most of these compounds showed weak cytotoxic activity against the PC3 cell lines.

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Experimental Part

General. TLC: Pre-coated silica gel GF_{254} plates (*Qingdao Marine Chemical Industry*); spots of compounds were visualized under UV (254 nm) light or by spraying with 10% H_2SO_4/EtOH followed by heating. Column chromatography (CC): silica gel (SiO_2; 200–300 mesh; *Qingdao Haiyang Chemical Co. Ltd.*), *Sephadex LH-20* gel (*Pharmacia Biotek*). Semi-prep. HPLC: *Agilent 1100-G1310A* isopump equipped with a *G1322A* degasser, a *G1314A VWD* detector (210 nm) and a *ZORBAX SB-*C_{18} column (9.4 × 250 mm, 5 μm). Optical rotations: *Perkin-Elmer 241 MC* polarimeter. UV Spectra: *Shimadzu UV-2450* spectrophotometer; λ_{max} (log ε) in nm. CD Spectra: *Chirascan* spectropolarimeter; λ ($\Delta\varepsilon$) in nm. IR Spectra: *Thermo-Nicolet 670* spectrometer; KBr disks; $\tilde{\nu}$ in cm^{-1}. ^1H- and ^{13}C-NMR spectra: *Bruker Avance-DRX-600* spectrometer; at 600 (^1H) or 150 MHz (^{13}C) ; δ in ppm rel. to Me_4Si as internal standard, *J* in Hz. 2D spectra were recorded with standard pulse programs and acquisition parameters. ESI-MS: *API-4000* triple-stage quadrupole instrument; in *m*/*z*. HR-ESI-MS: *Finnigan LC-*Q^{DECA} mass spectrometer; in *m*/*z*.

Fungal Material. The endophytic fungus *Aspergillus fumigatus* identified based on the nuclear 18S rDNA sequences was isolated from Chinese liverwort *H. tener*, collected from Maoer Mountain, Guangxi Zhuang Autonomous Region, P. R. China. The fungus assigned the accession No. 2011041507–5 was deposited with the Key Laboratory of Chemical Biology of Ministry of Education, School of Pharmaceutical Science, Shandong University, Jinan. The fungus was cultured in three flasks (300 ml) each containing 100 ml of PDB at 25° on a rotary shaker (120 rpm) for 5 d to obtain the seed culture. Then, the seed broth was added to 20 flasks (500 ml) each containing sterile culture medium made of 80 g rice and 120 ml water. Finally, these flasks were maintained at 25° for 50 d.

Extraction and Isolation. The mycelium obtained the culture medium was cut into small pieces and extracted with AcOEt. The org. solvent was concentrated under reduced pressure at 38° using a rotary evaporator. The crude extract (69.3 g) was separated by column chromatography (CC) over SiO_2 and eluted with a gradient of CH_2Cl_2/MeOH from 100 : 0 to 0 : 100 to give 14 fractions (*Frs. A–N*). *Frs. B–H* were found to show the cytotoxic activity against the human PC3 cell line with the IC_{50} values of 20.8, 26.2, 25.6, 49.0, 35.5, 26.8, and 39.1 μg/ml, resp. *Fr. E* (1.1 g) was subjected to a *Sephadex LH-20* CC and eluted with CH_2Cl_2/MeOH (1 : 1) to obtain five subfractions *Frs. E1–E4*. *Fr. E2* (67.1 mg) was separated by semi-prep. HPLC (73% MeOH/H_2O) to afford **15** (t_R 14.0 min; 7.2 mg). *Fr. E3* (336.5 mg) was subjected to MPLC (MeOH/H_2O from 2 : 3 to 100 : 0) to give twelve subfractions, *Frs. E3.1 – E3.12*. Then, **1** (t_R 32.1 min; 6.4 mg) was isolated from *Fr. E3.8* (13.5 mg) by semi-prep. HPLC (63% MeOH/H_2O). *Fr. E3.3* (177.1 mg) gave **2** (semi-prep. HPLC, (50% MeOH/H_2O): t_R 13.5 min; 12.6 mg). *Fr. E3.10* (42.9 mg) was purified by semi-prep. HPLC (70% MeOH/H_2O) to yield **11** (t_R 27.0 min; 10.2 mg). Purification of *Fr. E3.5* (79.2 mg) with semi-prep. HPLC (50% MeOH/H_2O) afforded **7** (t_R 31.0 min; 8.2 mg), **8** (t_R 40.0 min; 6.4 mg), **10** (t_R 35.5 min; 4.9 mg), and **19** (t_R 26.7 min; 2.3 mg). *Fr. E3.9* (12.5 mg) was purified by semi-prep. HPLC (60% MeOH/H_2O) to afford **22** (t_R 21.3 min; 5.7 mg). *Fr. F* (781.0 mg) was fractioned by a *Sephadex LH-20* column (CH_2Cl_2/MeOH 1 : 1) to give four subfractions (*Frs. F1 – F4*). By semi-prep. HPLC (51% MeOH/H_2O) **16** (t_R 20.0 min; 1.7 mg) was afforded from *Fr. F2*. *Fr. G* (607.7 mg) was also subjected to a *Sephadex LH-20* column (CH_2Cl_2/MeOH 1 : 1) to afford subfractions *Frs. G1 – G5*. *Fr. G3* (131.8 mg) was separated to subfractions *Frs. G3.1 – G3.6* by MPLC (MeOH/H_2O from 2 : 3 to 100 : 0). *Fr. G3.4* (13.7 mg) was purified to give **21** (t_R 14.0 min; 4.1 mg) by using semi-prep. HPLC (57% MeOH/H_2O, 0.2% AcOH). Further purification of *Fr. G3.5* (39.1 mg) with semi-prep. HPLC (40% MeCN/H_2O) yielded **9** (t_R 18.0 min; 17.4 mg) and **13** (t_R 14.0 min; 1.9 mg). Separation of *Fr. D* (2.0 g) following a procedure similar to that used for *Fr. E*, which was purified by semi-prep. HPLC gave **3** (t_R 18.0 min; 4.7 mg), **12** (t_R 12.0 min; 4.2 mg), **17** (t_R 20.0 min; 48.8 mg), and **18** (t_R 22.3 min; 4.2 mg) (50% MeCN/H_2O), **4** (t_R 24.0 min; 2.8 mg), **5** (t_R 27.0 min; 2.4 mg), and **6** (t_R 30.0 min; 2.1 mg) (32% MeCN/H_2O), and **20** (50% MeCN/H_2O, 0.2% AcOH; t_R 23.0 min, 4.5 mg). *Fr. H* (1.5 g) was purified by HPLC (40% MeOH/H_2O, 0.2% AcOH) to afford **14** (t_R 12.0 min; 148.0 mg).

Asperfumigatin (=*(5a*R,*6*S,*12*S,*14a*S*)-1,2,3,5a,6,11,12,14a-Octahydro-5a,6-dihydroxy-12-(2-hydroxy-2-methylpropyl)-9-methoxy-11-(3-methylbut-2-enoyl)-5*H,*14*H-*pyrrolo[1″,2″:4′,5′]pyrazino-[1′,2′:1,6]pyrido[3,4-*b*]indole-5,14-dione*; **1**). Yellow solid. $[\alpha]_D^{25} = -45.5$ ($c = 0.2$, MeOH). UV (MeOH): 212 (4.70), 222 (4.68), 266 (2.77). CD (MeOH): 236 (−8.3), 263 (−3.7), 294 (−1.3). IR: 3428, 2969, 2931, 1667, 1379, 1155. ^{1}H- and ^{13}C-NMR: see *Table 1*. ESI-MS (pos.): 512.6 ($[M+H]^+$), 534.4 ($[M+Na]^+$). HR-ESI-MS: 512.2388 ($[M+H]^+$, $C_{27}H_{34}N_3O_7^+$; calc. 512.2397).

Isochaetominine (=*(2*S,*4*S,*5a*S,*9c*S*)-4,5,5a,9c-Tetrahydro-5a-hydroxy-2-methyl-4-(4-oxoquinazolin-3(4*H*)-yl)-3*H*-2a,9b-diazacyclopenta[*jk*]fluorene-1,3(2*H*)-dione*; **10**). Colorless crystals. $[\alpha]_D^{25} = -110.9$ ($c = 0.1$, MeOH). UV (MeOH): 212 (3.97), 233 (4.21). CD (MeOH): 205 (+17.1), 220 (+5.2), 226 (+5.3), 241 (+1.4), 253 (+5.17), 283 (−3.9). IR: 3505, 3409, 3250, 1732, 1660, 1615, 1481, 1462, 1377, 1280, 755. ^{1}H- and ^{13}C-NMR: see *Table 2*. ESI-MS (pos.): 403.5 ($[M+H]^+$), 425.4 ($[M+Na]^+$). HR-ESI-MS: 403.1398 ($[M+H]^+$, $C_{22}H_{19}N_4O_4^+$; calc. 403.1406).

8′-O-*Methylasterric Acid* (=*2-[4-Hydroxy-2-methoxy-6-(methoxycarbonyl)phenoxy]-6-methoxy-4-methylbenzoic Acid*; **21**). Yellow solid. UV (MeOH): 212 (2.62), 240 (3.25). IR: 3355, 2976, 1701, 1612, 1091, 1050. ^{1}H-NMR ((D$_6$)DMSO, 600 MHz): 12.56 (*s*, COOH); 9.95 (*s*, OH); 6.76 (*s*, H–C(3)); 6.74 (*s*, H–C(5)); 6.47 (*s*, H–C(6′)); 5.75 (*s*, H–C(4′)); 3.76 (*s*, Me(8′)); 3.67 (*s*, Me(9)); 3.61 (*s*, Me(8)); 2.14 (*s*, Me(9′)). ^{13}C-NMR (150 MHz, (D$_6$)DMSO): 166.6 (C(7′)); 165.5 (C(7)); 156.3 (C(3′)); 155.5 (C(1′)); 155.2 (C(4)); 153.5 (C(2)); 139.6 (C(5′)); 133.6 (C(1)); 125.9 (C(6)); 107.4 (C(5)); 105.7 (C(6′)); 105.2

(C(4′)); 104.8 (C(3)); 56.1 (C(9)); 55.7 (C(8′)); 52.2 (C(8)); 21.6 (C(9′)). ESI-MS (pos.): 363.4 ($[M+H]^+$), 385.3 ($[M+Na]^+$). HR-ESI-MS: 363.1078 ($[M+H]^+$, $C_{18}H_{19}O_8^+$; calc. 363.1080).

Cytotoxicity Assay. The human lung adenocarcinoma epithelial cell line A549 and the human lung cancer cell line NCI-H460 were cultured in *Dulbecco*'s modified *Eagle*'s (DMEM) medium containing 10% fetal bovine serum (FBS, *Hyclone*). The human prostate cancers PC3 and multiple drug resistance PC3D cells were grown in Roswell Park Memorial Institute (RPMI) 1640 medium (*Hyclone*) with 10% FBS (*Hyclone*). All of these cells were maintained in 5% CO_2 at 37°. The cytotoxic activity *in vitro* of the compounds **1**–**22** was measured using MTT assay. 2×10^5 Cells/ml were seeded in a 96-well plate and respectively treated with vehicle or four different concentration of compounds (20, 10, 5, and 2.5 μg/ml) for further 48 h, then the MTT colorimetric assay was used as described previously. Simply, cells were incubated with MTT for 4 h at 37°. After finishing the incubation, the supernatants were removed and DMSO was added (100 μl/well). The absorbance was measured at 570 nm on a plate reader (*BioRad*, USA). Each concentration was tested in triplicate and each assay was performed three times.

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