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ASPERGICIN, A NEW ANTIBACTERIAL ALKALOID PRODUCED BY MIXED FERMENTATION OF TWO MARINE-DERIVED MANGROVE EPIPHYTIC FUNGI

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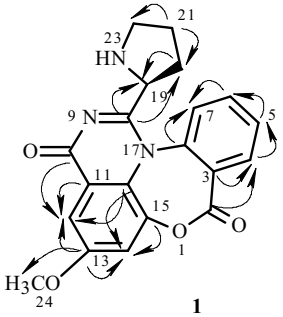
*A new alkaloid designated as aspergicin (**1**), together with a previous secondary metabolite, neoaspergillic acid (**2**), and a common compound, ergosterol (**3**), were isolated from the mixed cultured mycelia of two marine-derived mangrove epiphytic* Aspergillus *sp. fungi. Extensive application of 1D and 2D NMR techniques was made to characterize the structure and to establish the ^{1}H and ^{13}C assignments of compound **1**. In the antibacterial assays, both compounds **1** and **2** showed significant antibacterial activity against some selected Gram bacteria.*

Keywords: aspergicin, mangrove epiphytic fungus, mixed fermentation, NMR assignment, antibacterial activity.

Application of mixed fermentation was proved to contribute to the discovery of new natural products from marine-derived microorganisms. To date, at least nine new biologically active compounds have been identified by mixed fermentation of marine-derived microorganisms, pestalone [1], libertellenones A–D [2], marinamides A and B [3], and emericellamides A and B [4]. Results of mixed fermentation include increased antibiotic activity in the crude extract, increased yields of previously described metabolites, increased yields of previously undetected metabolites, analogues of known metabolites resulting from combined pathways, and induction of previously unexpected pathways for bioactive constituents [5].

Marine-derived mangrove fungi were proved to be an abundant resource for novel natural compounds [6, 7]. As part of our ongoing search for new bioactive metabolites from mixed fermentation of marine-derived mangrove fungi from the South China Sea [3, 8], two antibacterial compounds, neoaspergillic acid and kojic acid, were obtained as major secondary metabolites from the mixed fermentation liquid of two marine-derived mangrove epiphytic *Aspergillus* fungi, which were isolated from a rotten fruit of mangrove *Avicennia marina* in the South China Sea [9]. Further chemical investigations of the mixed fermentation mycelia resulted in the isolation of a new alkaloid designated as aspergicin (**1**), together with a previous secondary metabolite, neoaspergillic acid (**2**), and a commom compound, ergosterol (**3**). We report herein their isolation, structural elucidation, and antibacterial activities.



1

Fig. 1. The structure and key HMBC correlations (from C to H) of compound **1**.

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TABLE 1. NMR Data for Compound **1*** (DMSO-d_6, δ, ppm, TMS, J/Hz)

C atom	δ_C	δ_H	HMBC	C atom	δ_C	δ_H	HMBC
2	164.0 (s)		H-4	14	108.3 (d)	6.88 (d, J = 2.76)	H-12
3	133.8 (s)		H-4, 5, 7	15	154.8 (s)		H-14
4	129.5 (d)	7.82 (dd, J = 7.90, 1.54)	H-5, 6	16	130.3 (s)		H-12, 14
5	131.0 (d)	7.64 (m)[b]	H-4, 6, 7	18	151.3 (s)		H-19, 20a
6	128.9 (d)	7.58 (m)[b]	H-4, 5, 7	19	59.1 (d)	4.59 (dd, J = 6.80, 1.76)	H-22b (weak)
7	129.5 (d)	7.56 (m)[b]	H-5, 6	20	26.7 (t)	a: 2.11 (m); b: 3.24 (m)	H-19, 21a (weak), 22b
8	132.8 (s)		H-6, 7	21	23.9 (t)	a: 1.95 (m); b: 2.11 (m)	H-19, 20a, 22a, 22b
10	161.6 (s)		H-12	22	46.6 (t)	a: 3.44 (m); b: 3.59 (m)	H-19 (weak)
11	122.9 (s)		H-12	23		9.83 (s, NH)	
12	98.5 (d)	7.04 (d, J = 2.76)	H-14	24	56.0 (q)	3.83 (s)	
13	159.5 (s)		H-12, 14, 24				

*δ_H were recorded at 400 MHz, and δ_C were recorded at 100 MHz. Assignments were made by DEPT, HSQC, and HMBC experiments. [b]Strongly coupled, overlapping resonances.

TABLE 2. Activity of Compounds **1** and **2** against Gram-positive and Gram-negative Bacteria. Gatifloxacin and Norfloxacin were Selected for Comparison

Compound	MICs, μg/mL					
	S. aureus	*S. epidermidis*	*B. subtilis*	*B. dysenteriae*	*B. proteus*	*E. coli*
1	62.50	31.25	15.62	15.62	62.50	31.25
2	0.98	0.49	1.95	7.80	7.80	15.62
Gatifloxacin	7.80	0.98	Not tested	0.49	3.90	15.62
Norfloxacin	125	15.62	1.95	3.90	31.25	> 125

The total mixed cultures of epiphytic fungi (isolates FSY-01 and FSW-02) were filtered through cheesecloth to give mycelia. The mycelia were dried by air, then extracted with methanol, to give crude extracts. The crude extracts were chromatographed on silical gel by gradient elution from petroleum to ethyl acetate and then from ethyl acetate to methanol, to afford compounds **3**, **2**, and **1** in sequence.

The molecular formula of **1**, $C_{20}H_{17}N_3O_4$, was established by elemental analysis and NMR analysis in combination with EI-MS (M^+, 363). The ^1H, ^{13}C NMR, DEPT, HSQC, and HMBC experiments revealed **1** (Table 1) to contain six aromatic protons attached to 1,2-disubstituted and 1,2,3,5-tetrasubstituted benzene moities, respectively, a methoxy group, an imino group, three saturated methylene, and nine quarternary sp^2 carbon atoms. HMBC correlations (see Fig. 1) established the substructures C-18 through C-22, C-2 through C-8, and C-10 through C-16, where the low field δ_C value of C-18 indicated a C-N double bond in position 18. The HMBC correlations (from C-13 to H-24) confirmed that the methoxy substituent is in position 13. Thus, a benzodiazepine structure derived from L-proline and anthranilic acid was elucidated. Finally, the structure of compound **1** was established as in Fig. 1 and designated as aspergicin.

With regards to the antimicrobial assays, the antibacterial effects of compounds **1** and **2** against three Gram-positive bacteria, *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Bacillus subtilis*, and three Gram-negative bacteria, *Bacillus dysenteriae*, *Bacillus proteus*, and *Escherichia coli*, were determined. Both compounds **1** and **2** showed significant inhibitory effect on the selected Gram-positive bacteria and Gram-negative bacteria (Table 2).

EXPERIMENTAL

General Experimental Procedures. Mp: uncorrected. ^1H, ^{13}C, DEPT, HSQC, HMBC NMR: Bruker AVANCE AV400 NMR spectrometer, δ in ppm, J/Hz, TMS. EI-MS: Thermo DSQ low-resolution mass spectrometer. Elemental analysis: Elementar Vario EL CHNS-O elemental analyzer.

Fungal Material. The two isolates of *Aspergillus* sp. employed in this study were assigned the accession numbers FSY-01 and FSW-02, which were deposited in Foshan University and were isolated from a rotten fruit of mangrove *Avicennia marina* in Zhanjiang, Guangdong Province, P. R. China.

Fermentation and Co-culture Experiment. Two 1 L Erlenmeyer flasks, each containing 400 mL of the medium GYP (glucose 10 g/L, yeast extract 1 g/L, peptone 2 g/L, crude seasalt 3.5 g/L, natural pH value), were each inoculated with two 0.5 cm^2 agar plugs taken from stock cultures of the isolate FSY-01. The flasks were incubated at 30°C on a rotatory shaker for 5–7 days. The isolate FSW-02 was cultivated in the same manner as the isolate FSY-01. Mixed fermentation was carried out by a known technique in the literature [3, 9]. The co-culture production fermentation was inoculated with the mycelium of the isolate FSY-01, then inoculated with the mycelium of the isolate FSW-02 immediately. The co-cultures were incubated at room temperature for 30 days.

Extraction and Isolation. The total mixed cultures (50 L) were filtered through cheesecloth to give mycelia. The mycelia were dried by air, then extracted with methanol, to give crude extracts. The crude extracts were chromatographed on silical gel by gradient elution from petroleum to ethyl acetate and then from ethyl acetate to methanol, to give compound **3** from the petroleum–ethyl acetate (v/v 7:3) fraction and compound **2** from the petroleum–ethyl acetate (v/v 2:3) fraction, and compound **1** from the ethyl acetate–methanol (v/v 9:1) fraction. Compound **1** was further purified by recrystalization from ethyl acetate to give pure **1** (25 mg) as a white solid.

Aspergicin (1): white solid; mp 248–251°C ($CH_3COOC_2H_5$); $[\alpha]_D$ –96° (*c* 0.12, MeOH). $C_{20}H_{17}N_3O_4 \cdot 0.4H_2O$. UV spectrum (MeOH, λ_{max}, nm): 338, 242. ^1H and ^{13}C NMR data, see Table 1. Mass spectrum (EI, 70 eV), *m*/*z* (I_{rel}, %): 363 (M^+, 100), 295 (55).

Neoaspergillic acid (2): yellow pellet; mp 125–128°C ($CHCl_3$). Mass spectrum (EI, 70 eV), *m*/*z* (I_{rel}, %): 224 (M^+, 16), 207 (76), 193 (10), 182 (100), 166 (40), 153 (43), 123 (45). ^1H and ^{13}C NMR spectra were in agreement with those in the literature [9, 10].

Ergosterol (3): colorless needle-shaped crystal; mp 184–186°C (MeOH). ^1H and ^{13}C NMR spectra were identical with those previously reported [11, 12].

Determination of Minimum Inhibitory Concentrations (MICs) of Compounds 1 and 2. Antibacterial susceptibility testing was performed by the broth dilution method according to the Clinical and Laboratory Standard Institute (CLSI) (formerly National Committee for Clinical Laboratory Standards, NCCLS) National Committee for Clinical Laboratory Standards, Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard. NCCLS Document M7-A4 (fourth ed.), National Committee for Clinical Laboratory Standards, Villanova, PA (1997). Compounds **1** and **2** were dissolved in DMSO. Minimum inhibitory concentrations (MICs, μg/mL) were determined on Mueller–Hinton (MH) broth (pH 7.2–7.4) with medium containing dilutions of **1** or **2** ranging from 0.245 to 125 μg/mL. The Gram-positive bacterial strains utilized in this study consisted of *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Bacillus subtilis*. The Gram-negative bacterial strains tested included *Bacillus dysenteriae*, *Bacillus proteus*, and *Escherichia coli*. The final bacterial concentration for inocula was $1–2 \times 10^8$ CFU/mL, which was incubated at 35°C for 18 h. The MIC was defined as the lowest drug concentration that completely inhibited growth of the bacteria by comparison with a positive control group.

REFERENCES

1. M. Cueto, P. R. Jensen, C. Kauffman, W. Fenical, E. Lobkovsky, and J. Clardy, *J. Nat. Prod.*, **64**, 1444 (2001).
2. D. C. Oh, P. R. Jensen, C. A. Kauffman, and W. Fenical, *Bioorg. Med. Chem.*, **13**, 5267 (2005).
3. F. Zhu and Y. Lin, *Chin. Sci. Bull.*, **51**, 1426 (2006).
4. D. C. Oh, P. R. Jensen, C. A. Kauffman, and W. Fenical, *J. Nat. Prod.*, **70**, 515 (2007).
5. R. K. Pettit, *Appl. Microbiol. Biotechnol.*, **83**, 19 (2009).
6. C. Li, W. Ding, Z. She, and Y. Lin, *Chem. Nat. Comp.*, **44**, 163 (2008).
7. N. Tan, Y. Tao, J. Pan, S. Wang, F. Xu, Z. She, Y. Lin, and E. B. G. Jones, *Chem. Nat. Comp.*, **44**, 296 (2008).
8. F. Zhu, Y. Lin, J. Ding, X. Wang, and L. Huang, *Chem. Ind. Forest Prod.*, **27** (1), 8 (2007).
9. F. Zhu, G. Hu, X. Chen, Y. Yuan, M. Huang, H. Sun, W. Xiang, and Y. Lin, *Acta Scientiarum Naturalium Universitatis Sunyatseni*, **48** (6), 79 (2009).
10. M. Masaki, Y. Chigira, and M. Ohta, *J. Org. Chem.*, **31** (12), 4143 (1966).
11. F. Zhu, X. H. Liu, L. Wen, Y. C. Lin, S. N. Zhou, and L. L. P. Vrijmoed, *Acta Scientiarum Naturalium Universitatis Sunyatseni*, **44** (4), 66 (2005).
12. D. A. Dias and S. Urban, *Nat. Prod. Commun.*, **4** (4), 489 (2009).