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Marinamide, a novel alkaloid and its methyl ester produced by the application of mixed fermentation technique to two mangrove endophytic fungi from the South China Sea

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Abstract A novel 1-isoquinolone analog designated as marinamide (**A**) and its methyl ester (**B**), were produced by the application of mixed fermentation technique to two mangrove endophytic fungi (strains Nos. 1924 and 3893) from the South China Sea. Their structures were elucidated by comprehensive spectra methods (mainly by 2D NMR) as 4-(2-pyrrolyl)-1-isoquinolone-3-carboxylic acid (**A**) and methyl 4-(2-pyrrolyl)-1-isoquinolone-3-carboxylate (**B**), respectively. Compounds **A** and **B** were not obtained when either strain was cultured individually under the same conditions. The results showed that the application of mixed fermentation technique maybe represents a potentially important approach to discover novel metabolites.

Keywords: endophytic fungus, mixed fermentation, metabolite, alkaloid.

For a long time, terrestrial microorganisms are abundant natural pharmaceutical resource for human, but it becomes more and more difficult to find new species and microorganisms with special functions from land as the research goes on. Consequently, the speed of finding new metabolites from terrestrial microorganisms becomes slow. At the other hand, many drug-resistant pathogens have emerged recently because of the antibiotics misuse. Especially some new pathogens and new virus emerge unceasingly. It is clear that new classes of antibiotics are urgently needed. Thus, marine microorganisms became a new resource for screening for novel precursors of drug[1]. Marine microorganisms have developed unique metabolic pathways because of the special marine environment and its special functions in the ecosystem, and provided a lot of metabolites not produced by terrestrial microorganisms. Recently, a lot of bioactive metabolites with novel structure were isolated continuously from marine fungi[2−5], marine actinomycetes[6−10] and marine bacteria[11−14]. From the 1990s, we began to screen for novel bioactive metabolites from marine-derived mangrove endophytic fungi from the South China Sea, and found many significant novel structures[15−25].

However, although marine microorganisms were proved to be an abundant resource for new species and new compounds, but like terrestrial microorganisms, a lot of marine microorganisms produced special chemical components only under special conditions, such as existing special nutritional components, or competition for nutrients and limited space, or guarding against natural enemies attacking or maintaining the messages transmitting between species. Thus, in order to find more novel structures from marine microorganisms and enhance metabolites production, new methods must be developed and applied. During the research on microorganisms, it was found continuously that many important biochemical pathways could not be finished or could be finished weakly by an individual strain, and it needs two or more strains working together to finish the biochemical pathways, which inspired human to coculture with two or more strains to obtain the effects that a single strain could not make. Accordingly, we have begun exploring the use of mixed fermentation for the discovery of novel natural products from marine-derived mangrove endophytic fungi from the South China Sea. In this paper, a novel 1-isoquinolone analog designated as marinamide (**A**) and its methyl ester (**B**), are produced by the application of mixed fermentation technique to two mangrove endophytic fungi (strains Nos. 1924 and 3893) from the South China Sea. Their structures were elucidated by comprehensive spectra methods as 4-(2-pyrrolyl)-1-isoquinolone-3-carboxylic acid (A) and methyl 4-(2-pyrrolyl)-1-isoquinolone-3-carbo- xylate (B), respectively. Compounds A and B were not obtained when either strain was cultured individually under the same conditions[26,27],1). The results showed that the application of

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mixed fermentation technique maybe represents a potentially important approach to discover novel metabolites.

1 Materials and methods

1.1 Instruments and reagents

The following instruments were used: Bruker VECTOR 22 FT-IR spectrometer, Varian Inova 500NB NMR spectrometer, VG-ZAB-HS mass spectrometer, Elementar Vario EL CHNS-O elemental analyzer, Varian Cary-100 UV spectrophotometer, and Peking X4 microscope melting determination instrument.

All reagents were C. P., A. R. or B. R., respectively

1.2 Fungal strain culture

Fungal strain: Marine fungal strains (Nos. 1924 and 3893) were isolated from a plant from an estuarine mangrove in Hong Kong, no spora formed and species unidentified. The fungal strains were provided by Prof. L L P Vrijmoed and Prof. E B G Jones in the City University of Hong Kong and preserved at the Zhongshan University and the City University of Hong Kong, respectively.

Culture media: glucose 10 g, peptone 2 g, yeast extracts 1 g, crude marine salt 3.5 g, running water 1 L, pH 7.0.

Mixed fermentation of fungal strains: Total 60 L liquid media were filled in 500 mL Erlenmeyer flasks, with every flask containing 200 mL liquid media, then sterilized for 15 min under 1.25×10^5 Pa. After cooling to room temperature, the flasks were incubated with the fungal strains (Nos. 1924 and 3893). And the mixed culture fermentation was allowed to proceed for 12 d at 25℃.

1.3 Extraction and separation

The total cultures (60 L) were filtered through cheesecloth to give mycelia. The mycelia were air-dried and weight was about 220 g, then extracted with ethanol (5 L×5). The combined ethanol were condensed below 50℃ to give crude extracts. The combined extracts (about 20 g) were chromatographed on silical gel by using a gradient elution from petroleum to ethyl acetate, then from ethyl acetate to methanol. The components eluted by ethyl acetate and ethyl acetate-methanol (9∶1) were chromatographed on silical gel repeatedly and crystallized from ethanol to obtain compounds **A** (20 mg) and **B** (40 mg), respectively.

1.4 Experimental data

Compound **A**: yellow solid, m.p.>300℃, FABMS (*m/z*): 255 $[M+H]^+$, 237; NMR data: see Table 1; IR (KBr) ν: 3430, 3333, 3128, 1643, 1614, 1452, 1401, 1133, 942, 872, 819, 779, 753 cm^{-1}; UV (MeOH) λ_{max}: 216, 316, 348, 356 nm.

Compound **B**: pale-yellow prism, m.p.>300 ℃, FABMS (*m/z*): 269 $[M+H]^+$, 237; NMR data: see Table 2; IR (KBr) ν: 3431, 3332, 3195, 1642, 1614, 1560, 1454, 1401, 1354, 1297, 1234, 1138, 1003, 905, 881, 807, 738 cm^{-1}; UV (MeOH) λ_{max}: 216, 284, 332 nm; Found (calculated) %: C 67.06 (67.16), H 4.612 (4.508), N 10.324 (10.442).

2 Results and discussion

Compound **B** was analyzed for the molecular formula of $C_{15}H_{12}N_2O_3$ by the FABMS (obsd $[M+H]^+$ at *m/z* 269) and elemental analysis. The number of carbon atoms and protons were in agreement with that in NMR spectra.

Analysis of ^{1}H NMR, ^{13}C NMR and DEPT spectra for compound **B** showed the presence of 2 carbonyls groups (δ_C 173.5 and 167.5), 5 unsaturated quaternary carbon atoms, 7 unsaturated CH and one methoxy group. The fact that it did not react with ferric chloride indicated that compound **B** was not a phenol and further proved that the signals at δ_H 11.61 and 11.56 were not the resonance of the active protons of the hydroxyls in phenol. The presence of 1,2-disubstituted benzene substructure was deduced by the connectivity of H-8, H-7, H-6 and H-5 in the ^{1}H-^{1}H COSY NMR spectra in combination with the key HMBC correlations between C-1 and H-8, C-9 and H-8, C-8 and H-7, C-7 and H-6, C-6 and H-5, C-5 and H-6, C-10 and H-5. The α-pyrrolyl group was established by the connectivity of H-11, H-13, H-14 and H-15 in the ^{1}H-^{1}H COSY NMR spectra in combination with the key HMBC correlations between C-12 and H-13 and H-11, C-13 and H-14, C-14 and H-15, C-15 and H-11 and H-14. The key HMBC correlation between C-16 and H-17 indicated the presence of methyl carboxylate residue. There were some correlations between C-4 and H-2 and H-11, which indicated that the α-pyrrolyl group was linked to C-4. And there was no any HMBC correlation for C-3, which indicated that methyl carboxylate residue was linked to C-3. Finally, the structure of compound **B** was established as methyl 4-(2-pyrrolyl)-1-isoquinolone-3-carboxylate by the comprehensive analysis of spectra (see Fig. 1).

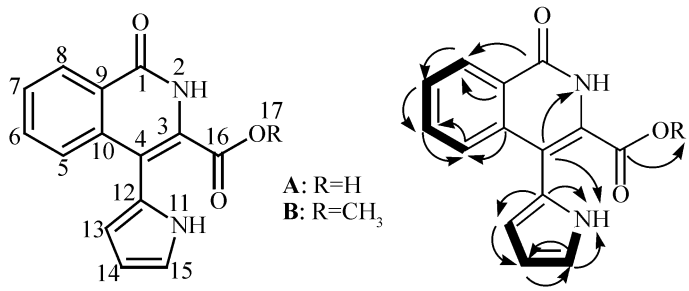

Fig. 1. Structures of compounds **A** and **B** and their key correlations of HMBC and COSY.

Table 1 NMR data for compound **A** (500 MHz, DMSO-d_6)

Position No.	δ_C	δ_H (J)*	HMBC	^{1}H-^{1}H COSY
1	178.4(s)		H-8	
2		12.34(br s, NH)		
3	147.5(s)			
4	106.2(s)			
5	119.1(d)	7.88(d, 8.0Hz, 1H)	H-6, 7, 8	H-6, 7, 8
6	125.3(d)	7.52(t, 8.0Hz, 1H)	H-5, 7, 8	H-5, 7, 8
7	133.6(d)	7.83(t, 8.0Hz, 1H)	H-5, 6, 8	H-5, 6, 8
8	124.9(d)	8.24(d, 8.0Hz, 1H)	H-5, 6, 7	H-5, 6, 7
9	138.7(s)		H-5, 6, 7, 8	
10	122.8(s)		H-5, 6	
11		11.81(br s, NH)		H-13, 14, 15
12	123.5(s)		H-13, 14, 15	
13	113.6(d)	6.84(m, 1H)	H-14, 15;11-NH	H-14, 15;11-NH
14	108.7(d)	6.28(m, 1H)	H-13, 15	H-13, 15;11-NH
15	123.0(d)	7.14(m, 1H)	H-13, 14	H-13, 14;11-NH
16	166.1(s)		OH	
17		16.13(br s, OH)		

*Assigned by HMQC.

Table 2 NMR data for compound **B** (500 MHz, DMSO-d_6)

Position No.	δ_C	δ_H (J)*	HMBC	^{1}H-^{1}H COSY
1	173.5(s)		H-8	
2		11.61 (br s, 1H, NH)		
3	140.6(s)			
4	113.5(s)		2-NH, 11-NH	
5	118.4(d)	7.66 (d, 8.0Hz, 1H)	H-6	H-6, 7, 8
6	123.5(d)	7.35 (t, 8.0 Hz, 1H)	H-5, 7	H-5, 7, 8
7	132.2(d)	7.69 (t,8.0 Hz,1H)	H-6, 8	H-5, 6, 8
8	124.7(d)	8.07 (d,8.0 Hz,1H)	H-5, 6, 7	H-5, 6, 7
9	139.4(s)		H-5, 7, 8	
10	124.2(s)		H-5, 6; 2-NH	
11		11.56 (br s,1H,NH)		H-13, 14, 15
12	122.9(s)		H-13, 14, 15; 11-NH	
13	111.8(d)	6.47 (m,1H)	H-14, 15	H-14, 15; 11-NH
14	109.6(d)	6.25 (m,1H)	H-13, 15	H-13, 15; 11-NH
15	122.4(d)	7.12 (m,1H)	H-13, 14; 11-NH	H-13, 14; 11-NH
16	167.5(s)		H-17	
17	51.7(q)	3.67 (s,3H)		

*Assigned by HMQC.

The NMR spectra for compound **A** were very like those for compound **B**. The difference between them was that there were no signals of methoxy group, but showing the signals of active proton in carboxy group at δ_H 16.13 (br s). Apparently, compound **A** was the product that the methoxy group in compound **B** was replaced by carboxy group. Finally, compound **A** was elucidated by the comprehensive analysis of spectra (mainly by HMQC, COSY and HMBC spectra) as 4-(2-pyrrolyl)-1-isoquinolone-3-carboxylic acid (see Fig. 1).

In order to find compounds having selective central nervous system actions, various thyrotropin releasing hormone (TRH) analogs in which the pyroglutamic acid residue is replaced by (3*S*)-1-oxo-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid and related derivatives were prepared by Maeda *et al.*[28] in 1988, and 1-isoquinolone-3-carboxylic acid, the mother structure in marinamides (**A** and **B**) containing no pyrrolyl group was also prepared by them. In 2001, Ukita *et al.*[29] reported a novel class of potent and selective phosphodiesterase 5 (PDE5) inhibitors, 4-aryl-1-isoquinolinone derivatives. Among them, compound T-1032, the sulfate form of methyl 2-(4-aminophenyl)-1,2-dihydro-1-oxo-7-(2-pyridinylmethoxy)-4-(3,4,5-trimethoxyphenyl)-3-isoquinoline carboxylate was selected for further biological and pharmacological evaluation of erectile dysfunction. Different from the above compounds, new compounds marinamide (**A**) and its methyl ester (**B**) belong to a new class of 1-isoquinolone alkaloids in which C-4 was substituted by an α-pyrrolyl group. In the primary bioassay, marinamide (**A**) and its methyl ester (**B**) exhibited significant antibacterial activities against *Escherichia coli* (diameter of bacteriostatics ring/cm: 1.4 (**A**); 2.0 (**B**)), *Pseudomonas pyocyanea* (0.9 (**A**); 1.7 (**B**)) and *Staphylococcus auereu* (1.0 (**A**); 1.3 (**B**)) at the concentration of 1 mg/mL.

Mixed fermentation has been used in the food industry[30] and to enhance enzyme production[31]; however, it is not clear that this method has been used extensively by the pharmaceutical industry for new metabolite discovery. Given that antibiotics may be produced in nature to provide a competition advantage[32], it is possible for the pathways responsible for the biosynthesis of certain compounds to be regulated by factors elicited by one microbe and detected by another. This proposal is supported by the following evidence: Burgess *et al.*[33] found that antibiotic production can be induced in response to microbial antagonism and Sonnenbichler *et al.*[34] found that the production of specific secondary metabolites can be increased up to 400-fold when strains grow in the presence of an antagonist. Cueto *et al.*[35] obtained a new antibiotic designated as pestalone from the mixed fermentation of a marine fungus *Pestalotia* sp. CNL-365 and a drug-resistant marine bacterium CNJ-328. Pestalone was not detected when either strain was cultured individually. Recently, Oh *et al.*[36] found that the marine bacterium CNJ-328 induced another marine fungus *Libertella* sp. to produce 4 new cytotoxic metabolites designated as libertellenones in mixed fermentation. These new compounds were not observed in pure cultures of the fungus or the bacterium. In this paper, a new alkaloid designated as marinamide (**A**) and its methyl ester (**B**) are obtained from the mixed fermentation of two marine-derived mangrove endophytic fungi (strains Nos. 1924 and 3893) from the South China Sea. The results further demonstrated that the application of mixed fermentation technique maybe represents a potentially important approach to discover novel metabolites.

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References

1 Blunt J W, Copp B R, Munro M H G, et al. Marine natural products. Nat Prod Rep, 2005, 22: 15－61
2 Wei H, Itoh T, Kinoshita M, et al. Shimalactone A, a novel polyketide, from marine derived fungus Emericella variecolor GF10. Tetrahedron, 2005, 61(33): 8054－8058
3 Wei H, Itoh T, Kinoshita M, et al. Cytotoxic sesterterpenes, 6-epi-ophiobolin G and 6-epi-ophiobolin N, from marine derived fungus Emericella variecolor GF10. Tetrahedron, 2004, 60(28): 6015－6019
4 Klemke C, Kehraus S, Wright A D, et al. New secondary metabolites from the marine endophytic fungus Apiospora montagnei. J Nat Prod, 2004, 67(6): 1058－1063
5 Tsukamote S, Miura S, Yamashita Y, et al. Aspermytin A: A new neurotrophic polyketide isolated from a marine-derived fungus of the genus Aspergillus. Bioorg & Med Chem Lett, 2004, 14(2): 417－420
6 Koch I, Maskey R P, Biabani M A F, et al. 1-hydroxy-1-norresistomycin and resistoflavinmethyl ether: New antibiotics from ma-

rine-derived streptomycetes. J Antibiot, 2005, 58(8): 530－534
7 Li F C, Maskey R P, Qin S, et al. Chinikomycins A and B: Isolation, structure elucidation, and biological activity of novel antibiotics from a marine Streptomyces sp. Isolate M045. J Nat Prod, 2005, 68(3): 349－353
8 Maskey R P, Helmke E, Kayser O, et al. Anti-cancer and antibacterial trioxacarcins with high anti-malaria activity from a marine streptomycete and their absolute stereochemistry. J Antibiot, 2004, 57(12): 771－779
9 Stritzke K, Schulz S, Laatsch H, et al. Novel caprolactones from a marine streptomycete. J Nat Prod, 2004, 67(3): 395－401
10 Maskey R P, Sevvana M, Uson I, et al. Gutingimycin: A highly complex metabolite from a marine streptomycete. Angew Chem Int Ed, 2004, 43(10): 1281－1283
11 Liu H W, Fujiwara T, Nishikawa T, et al. Lissoclibadins 1-3, three new polysulfur alkoloids, from the ascidian Lissoclinum cf. badium. Tetrahedron, 2005, 61(36): 8611－8615
12 Silipo A, Leone S, Molinaro A, et al. Complete structural elucidation of a novel lipooligosaccharide from the outer menbrane of the marine bacterium Shewanella pacifica. Eur J Org Chem, 2005, (11): 2281－2291
13 Matsuo Y, Imagawa H, Nishizawa M, et al. Isolation of an algal morphogenesis inducer from a marine bacterium. Science, 2005, 307(5715): 1598－1598
14 Hickford S J H, Kupper F C, Zhang G P, et al. Petrobactin sulfonate, a new siderophore produced by the marine bacterium marinobacter hydrocarbonoclasticus. J Nat Prod, 2004, 67(11): 1897－1899
15 Wu X Y, Liu X H, Jiang G C, et al. Xyloketal G, a novel metabolite from the mangrove fungus Xylaria sp. 2508. Chem Nat Comp, 2005, 41(1): 27－29
16 Yin W Q, Zou J M, She Z G, et al. Two cyclic peptides produced by the endophytic fungus #2221 from Castaniopsis fissa on the South China Sea coast. Chin Chem Lett, 2005, 16(2): 219－222
17 Li H J, Lin Y C, Yao J H, et al. Two new metabolites from the mangrove endophytic fungus no. 2524. J Asian Nat Prod Res, 2004, 6(3): 185－191
18 Li H J, Lin Y C, Vrijmoed L L P, et al. A new cytotoxic sterol produced by an endophytic fungus from Castaniopsis fissa at the South China Sea coast. Chin Chem Lett, 2004, 15(4): 419－422
19 Zhu F, Lin Y C, Zhou S N. Anthroquinone derivatives isolated from marine fungus #2526 from the South China Sea. Chin J Org Chem, 2004, 24(9): 1114－1117
20 Chen G Y, Lin Y C, Wen L, et al. Two new metabolites of a marine endophytic fungus (No. 1893) from an estuarine mangrove on the South China Sea coast. Tetrahedron, 2003, 59(26): 4907－4909
21 Lin Y C, Li H J, Jiang G C, et al. A novel γ-lactone, eutypoid-A and other metabolites from marine fungus Eutypa sp. (#424) from the South China Sea. India J Chem Sec B, 2002, 41B: 1542－1544
22 Lin Y C, Wang J, Wu X Y, et al. A novel compound enniatin G from the mangrove fungus Halosarpheia sp. (strain 732) from the South China Sea. Aust J Chem, 2002, 55: 225－227
23 Lin Y C, Wang J, Zhou S N, et al. New isocoumarins from the mangrove endophytic fungus #2533. Chem J Internet, 2001, 3(7): 30－33
24 Lin Y C, Wu X Y, Feng S, et al. A novel N-cinnamoylcyclopeptide containing an allenic ether from the fungus Xylaria sp. (strain #2508) from the South China Sea. Tetrahedron Lett, 2001, 42: 449－451
25 Lin Y C, Wu X Y, Feng S, et al. Five unique compounds: Xyloketals from mangrove fungus Xylaria sp. From the South China Sea coast. J Org Chem, 2001, 66: 6252－6256
26 Zhu F, Liu X H, Wen L, et al. Metabolites of marine fungus #1924 from the South China Sea. Acta Scientia Natur Univ Sunyatseni, 2005, 44(4): 66－69
27 Zhu F, Lin Y C. Two sphingolipids isolated from a mangrove endophytic fungus. Chin J Org Chem, 2005, 25(S2): 584
28 Maeda H, Suzuki M, Sugano H, et al. Synthesis and central nervous system actions of thyrotropin-releasing hormone analogs containing a 1-oxo-1,2,3,4-tetrahydroisoquinoline moiety. Chem Pharm Bull, 1988, 36(1): 190－201
29 Ukita T, Nakamura Y, Kubo A, et al. Novel, potent, and selective phosphodiesterase 5 inhibitors: Synthsesis and biological activities of a series of 4-aryl-1-isoquinolinone derivatives. J Med Chem, 2001, 44: 2204－2218
30 Wiesel I, Rehm H J, Bisping B. Improvement of tempe fermentations by application of mixed cultures consisting of Rhizopus sp. and bacterial strains. Appl Microbiol Biotechnol, 1997, 47: 218－225
31 Gutierrez-Corres M, Tengerdy R P. Xylanase production by fungal mixed culture solid substrate fermentation on sugar canebagasse. Biotechnol Lett, 1998, 20: 45－47
32 Strongman D B, Miller J D, Calhoun L, et al. The biochemical basis for interference competition among some lignicolous marine fungi. Bot Mar, 1987, 30(1): 21－26
33 Burgess J G, Jordan E M, Bregu M, et al. Microbial antagonism: A neglected avenue of natural products research. J Biotechnol, 1999, 70: 27－32
34 Sonnenbichler J, Dietrich J, Peipp H. Secondary fungal metabolites and their biological activities, 5. Inverstigations concerning the induction of the biosynthesis toxic secondary metabolites in basidiomycetes. Biol Chem Hoppe-Seyler, 1994, 375(1): 71－79
35 Cueto M, Jensen P R, Kauffman C, et al. Pestalone, a new antibiotic produced by a marine fungus in response to bacterial challenge. J Nat Prod, 2001, 64: 1444－1446
36 Oh D C, Jensen P R, Kauffman C A, et al. Libertellenones A-D: Induction of cytotoxic diterpenoid biosynthesis by marine microbial competition. Bioorg & Med Chem, 2005, 13(17): 5267－5273