Diketopiperazine Alkaloids from *Penicillium* spp. HS-3, an Endophytic Fungus in *Huperzia serrata*

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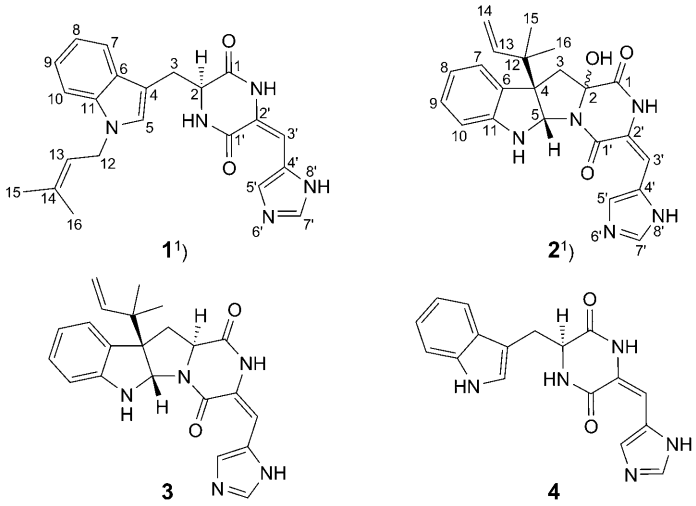
Cultivation of the fungus *Penicillium* spp. HS-3, an endophytic fungus from the stems of *Huperzia serrata*, led to the isolation of a new diketopiperazine alkaloid, named as tryhistatin (**1**), together with three known ones, **2**–**4**, all of which share a similar backbone. The structure of **1** was established by detailed interpretation of the 1D- and 2D-NMR spectra, and HR-ESI-MS data. Furthermore, 1D- and 2D-NMR experiments were employed to elucidate the structure of **2** which was deduced previously only on the basis of the LC/MS method. In this article, we confirmed the correctness of the previously reported structure and made a complete assignment of the NMR signals of **2** for the first time.

Introduction. – Endophytic fungi are fungal microorganisms which spend the whole or part of their life cycle colonizing inter- and/or intracellularly inside healthy tissues of their host plants, typically causing no apparent symptoms of disease [1]. This population was a hotspot of intense research over the past decade and proved to be a good producer of structurally diverse and biologically active metabolites [2–7]. Furthermore, results from many researches indicated that endophytic fungi could metabolize the same substance with their own host plants [8–10], which offered us a hint that endophytic fungi may be an alternate source of secondary plant metabolites.

Huperzia serrata, a traditional Chinese herb medicine rich in lycopodium alkaloids, has become famous worldwide for the discovery of hupzine A, a potent acetylcholinesterase inhibitor [11]. In the process of our research on the endophytic fungi from *Huperzia serrata*, the strain *Penicillium* spp. HS-3 was isolated from stems of the plant and found to produce several alkaloids in the preliminary experiments. Systematic experiments aiming at investigating of the alkaloid constituents of this fungus led to the isolation of a new diketopiperazine alkaloid, along with three known ones. Its structure was assigned by mass-spectrometric and spectroscopic analyses, especially 2D-NMR techniques (HMQC, HMBC, and NOESY).

Results and Discussion. – Tryhistatin (**1**) was obtained as a white amorphous powder. The HR-ESI-MS provided the molecular formula $C_{22}H_{23}N_5O_2$ (*m/z* 390.1917, $[M+1]^+$), implying the presence of 14 degrees of unsaturation, in accordance with the ^{1}H- and ^{13}C-NMR spectral data (*Table*). The IR spectrum showed an absorption band for the amide group (1669 cm^{-1}), which was confirmed by ^{13}C-NMR signals at δ(C) 169.9 and 164.2. Twenty-two C-atom signals corresponding to seven quaternary C-atoms, eleven CH, two CH_2, and two Me groups were observed in the ^{13}C-NMR and

DEPT spectra. The ^{1}H-NMR spectrum (*Table*) of **1** showed the presence of two Me groups at δ(H) 1.65 (Me(15)[1]) and 1.71 (Me(16)), two aliphatic CH_2 groups at δ(H) 3.06 (*dd*, $J = 14.5, 4.4$, 1 H of CH_2(3)), 3.45 (*dd*, $J = 14.5, 3.0$, 1 H of CH_2(3)), and 4.27–4.29 (CH_2(12)), one aliphatic CH group at δ(H) 4.26 (*dd*, $J = 4.4, 3.0$), five olefinic H-atoms at δ(H) 5.06 (H–C(13)), 5.31 (H–C(3′)), 6.87 (H–C(5)), 6.93 (H–C(5′)), and 7.63 (H–C(7′)), and four *AA′BB′* system H-atoms at δ(H) 7.00 (*d*, $J = 7.7$), 7.02 (*d*, $J = 7.5$), 7.06 (*d*, $J = 7.5$), and 7.51 (*d*, $J = 7.7$) ascribed to a 1,2-disubstituted phenyl group. Interpretation of NMR data of **1** indicated the presence of an indole group and an imidazole group, which was confirmed by the UV-spectroscopic data. The data mentioned above indicated that **1** was a diketopiperazine alkaloid derivative containing an indole, an imidazole, and an isopentenyl moiety.



Analysis of the ^{1}H- and ^{13}C-NMR, HSQC, and HMQC spectra of **1** enabled us to assign all the H-atoms to their bonding C-atoms. The NMR spectra of **1** and **4** exhibited similarity, by intensive study of which conclusion was finally made that **1** contained an extra isopentenyl unit compared with **4** [12]. The linkage of the three main structure fragments was established by an HMBC experiment (*Fig.*). The linkage of C(11), C(12), and C(5) each *via* the N-atom was suggested by the correlation of H–C(12)/C(5), H–C(5)/C(11), and H–C(12)/C(11). The HMBCs H–C(3)/C(1) and H–C(2)/C(3) indicated the linkage of C(1) with C(3) *via* C(2); the linkage of C(3) with C(5) *via* C(4) could be deduced by the HMBC cross-peaks H–C(3)/C(5), H–C(5)/C(4), and H–C(3)/C(4); in the same way, the HMBCs H–C(3′)/C(1′) and H–C(3′)/C(2′) indicated the connectivity of C(1′) and C(3′) *via* C(2′). The absolute configuration of **1** was determined to be the same as that of **4** [12] by comparison of their optical rotation ($[\alpha]_D = +274.5$ for **1** *vs.* $+164.5$ for **4**). The structure of **1** was thus established and named as tryhistatin.

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[1]) Arbitrary atom numbering. For the systematic name, see *Exper. Part.*

Table. *^{1}H- and ^{13}C-NMR Data* (in CD_3OD, at 500 and 125 MHz, resp.) *of* **1** *and* **2**. δ in ppm, J in Hz.

	1[1])		**2**[1])	
	δ(H)	δ(C)	δ(H)	δ(C)
H–C(1)	–	169.9	–	168.8
H–C(2)	4.26 (*dd*, *J* = 4.4, 3.0)	58.4	–	88.7
CH_2(3)	3.45 (*dd*, *J* = 14.5, 3.0), 3.06 (*dd*, *J* = 14.5, 4.4)	31.9	2.61 (*d*, *J* = 13.8), 2.56 (*d*, *J* = 13.8)	45.4
H–C(4)	–	108.0	–	61.6
H–C(5)	6.87 (*s*)	129.8	5.76 (*s*)	80.9
H–C(6)	–	130.1	–	133.2
H–C(7)	7.50 (*d*, *J* = 7.7)	120.2	7.20 (*d*, *J* = 7.7)	126.3
H–C(8)	7.02 (*t*, *J* = 7.5)	121.6	6.68 (*t*, *J* = 7.4)	119.2
H–C(9)	7.06 (*t*, *J* = 7.5)	122.6	7.02 (*t*, *J* = 7.4)	129.6
H–C(10)	7.00 (*d*, *J* = 7.7)	110.5	6.57 (*d*, *J* = 7.8)	110.3
C(11)	–	137.9	–	151.5
CH_2(12)	4.27–4.29 (*m*)	45.0	–	42.8
H–C(13)	5.06 (*t*, *J* = 6.9)	121.6	6.03 (*dd*, *J* = 17.4, 10.9)	145.6
H–C(14)	–	137.2	5.13 (*dd*, *J* = 10.9, 1.1), 5.10 (*dd*, *J* = 17.4, 1.1)	115.1
Me(15)	1.65 (*s*)	26.0	0.97 (*s*)	23.5
Me(16)	1.71 (*s*)	18.3	1.12 (*s*)	23.1
C(1′)	–	164.2	–	162.2
C(2′)	–	123.6	–	126.0
H–C(3′)	5.31 (*s*)	109.1	6.29 (*s*)	112.5
C(4′)	–	128.2	–	128.8
H–C(5′)	6.93 (*s*)	132.6	7.33 (*s*)	133.2
H–C(7′)	7.63 (*s*)	137.4	7.52 (*s*)	137.9

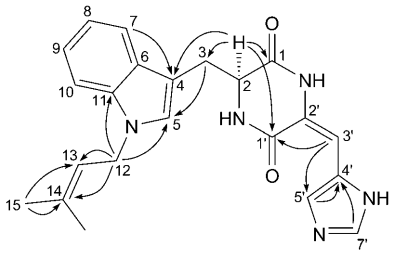

Figure. *Key HMBCs* (H → C) *of* **1**[1])

Compound **2**, with the molecular formula $C_{22}H_{23}N_5O_3$, deduced by HR-ESI-MS at *m*/*z* 406.1876 ($[M+1]^+$), was obtained as a white amorphous powder. High similarity was observed between the NMR data of **2** and those of **3**, implying a close structural resemblance of the two compounds. The major differences were the loss of an aliphatic H-atom signal and the downfield-shifted signal of C(2) in the NMR data of **2** by comparison with those of **3**, which indicated that there was an additional heteroatom linked to C(2) in **2**. A OH group was preferentially and tentatively considered as the substituent judging from the chemical shifts [13], which was supported by the molecular formula. Thus, the structure of **2** was elucidated as 16-hydroxyroquefortine C, whose

structure was reported only by LC/MS method [14]. Hereby, the complete assignment of the NMR signals of **2** was also reported by high-quality 1D- and 2D-NMR experiments in this study (*Table*).

The structures of the other two known compounds were identified as roquefortine C (**3**) [15][16] and cyclo(dehydrohistidyl-L-tryptophyl) (**4**) [17] by comparison of their spectroscopic data with literature values.

All of the four compounds isolated belong to diketopiperazine alkaloids, an important class of fungal metabolites biosynthesized by condensation of two amino acids, such as tryptophane, proline, histidine, and phenylalanine [18]. Compounds of this class have drawn wide attention due to their bioactive profiles in various pharmacological assay systems [19–25] and shown great potential of becoming therapeutic drugs.

Experimental Part

General. All solvents used were of anal. grade (*Shanghai Chemical Plant*). TLC: pre-coated silica gel GF_{254} plates (*Qingdao Haiyang Chemical Plant*). Column chromatography (CC): silica gel (SiO_2; 230–400 mesh), *Lichroprep RP-18* (40–63 μ), and *MCI-CHP20P* gel (75–150 μ; *Mitsubishi Chemical Industries Ltd.*). Optical rotations: *Perkin-Elmer 341* polarimeter. UV Spectra: *Shimadzu UV-2450* spectrometer; λ_{max} (log ε). IR Spectra: *Thermo Nicolet-6700* spectrophotometer; in cm^{-1}. NMR Spectra: *Bruker AM-500* apparatus; δ in ppm rel. to Me_4Si, J in Hz. ESI-MS: *Agilent 6210-Lc/Tof* mass spectrometer; in *m/z*.

Microbial Material. The fungal strain HS-3 of *Penicillium* sp. was isolated from the healthy stems of *Huperzia serrata* collected from Xishuangbanna Tropical Plant Garden, Chinese Academy of Science, Yunnan Province, P. R. China. Plant samples were designed to undergo a process described by *Schulz* as surface sterilization [26]. The surface-sterilized samples were cut into 0.5-cm fragments and placed onto the surface of potato-dextrose agar (PDA) as medium in *Petri* dishes with six fragments in each, which was then incubated at 28° for 4–7 d. During cultivation, the hyphal-tip method [27] was adopted for the purification of the endophytic fungi. The growing fungi were successively removed onto fresh PDA medium and cultivated at 28° for 7 d. After purification, the pure strain HS-3 was transferred into 500-ml *Erlenmeyer* flasks containing 300 ml of potato-dextrose liquid medium for fermentation. The flasks were firstly inoculated on rotary shakers at 28° for 6 d at 185 r.p.m. and then cultivated for another 20 d at 28° without agitation.

Extraction and Isolation. The culture was filtered through cheesecloth. The mycelium was air-dried (dried weight 1.65 kg) and extracted three times in 95% EtOH by refluxing. The combined extracts were evaporated to dryness under reduced pressure to afford the residue (456 g). The residue was dissolved in H_2O (2 l) to form a suspension, which was then adjusted with 0.5M HCl to pH *ca.* 4. The acidic mixture was extracted with AcOEt (6 × 0.5 l) to remove the non-alkaloid components. The aq. phase was brought to pH *ca.* 10 by addition of 1M Na_2CO_3 and extracted with $CHCl_3$ (6 × 0.5 l) to give the crude alkaloids (2.07 g), which were then subjected to CC (*MCI-CHP20P* gel; MeOH/H_2O 2:3 → 9:1) to afford three major fractions *A–C*. *Fr. A* was applied to CC (*RP-18*; MeOH/H_2O 1:1) to yield **4** (27.1 mg). *Fr. B* was separated by CC (SiO_2; $CHCl_3$/MeOH/Et_2NH 25:1:0.05) to give **1** (9.9 mg) and **2** (3.0 mg). *Fr. C* was also purified by CC (*RP-18*; MeOH/H_2O 7:3) to afford **3** (30.0 mg).

Tryhistatin (=(*3*E,*6*S)*-3-(1*H*-Imidazol-5-ylmethylidene)-6-{[1-(3-methylbut-2-en-1-yl)-1*H*-indol-3-yl]methyl}piperazine-2,5-dione*; **1**). White amorphous powder. $[\alpha]_D^{20} = +274.5$ ($c = 1.1$, MeOH). UV (MeOH): 222 (4.65), 296 (4.24), 322 (4.31). IR (KBr): 3185, 2922, 2858, 1669, 1616, 1425, 1203, 1092, 1018, 734. ^{1}H- and ^{13}C-NMR: *Table.* ESI-MS (pos.): 390 ($[M+H]^+$). HR-ESI-MS: 390.1917 ($[M+H]^+$, $C_{22}H_{24}N_5O_2^+$; calc. 390.1925).

16-Hydroxyroquefortine C (=(*3*E,*5a*S,*10b*R*)-6,10b,11,11a-Tetrahydro-11a-hydroxy-3-(1*H*-imidazol-5-ylmethylidene)-10b-(2-methylbut-3-en-2-yl)-2*H*-pyrazino[1′,2′:1,5]pyrrolo[2,3-*b*]indole-1,4(3*H*,5*a*H)-

dione; **2**). White amorphous powder. $[\alpha]_D^{20} = -626.7$ ($c = 0.3$, MeOH). IR (KBr): 3254, 2969, 1669, 1603, 1403, 1251, 1169, 1093, 926, 825, 750. UV (MeOH): 233 (4.20), 332 (4.40). ^{1}H- and ^{13}C-NMR: *Table*. ESI-MS (pos.): 406 ($[M+H]^+$). HR-ESI-MS: 406.1876 ($[M+H]^+$, $C_{22}H_{24}N_5O_3^+$; calc. 406.1874).

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