Asian Journal of Chemistry; Vol. 25, No. 3 (2013), 1208-1210

ASIAN JOURNAL OF CHEMISTRY

http://dx.doi.org/10.14233/ajchem.2013.12350

Studies on the Metabolites of a Fungal Endophyte *Penicillium* sp. HS-5 from *Huperzia serrata*

YOU-MIN YING[1], WEI-GUANG SHAN[1], WEN-HONG LIU[2] and ZHA-JUN ZHAN[1,*]

[1]College of Pharmaceutical Science, Zhejiang University of Technology, Hangzhou 310014, P.R. China
[2]Zhejiang Chinese Medical University, Hangzhou 310053, P.R. China

*Corresponding author: Fax: +86 571 88871075; Tel: +86 571 88871030; E-mail: zjnpr@zjut.edu.cn

(*Received*: 4 July 2011; *Accepted*: 3 September 2012) AJC-12080

Nine compounds **1-9** were isolated from the broth of the strain *Penicillium* sp. HS-5, a fungal endophyte living in *Huperzia serrata*. Structures of the isolated compounds were identified as Sumiki's acid (**1**), acetyl Sumiki's acid (**2**), 2-furoic acid (**3**), kojic acid (**4**), 4-hydroxybenzonic acid (**5**), adenosine (**6**), adenine (**7**), thymine (**8**) and ergosterol (**9**) by NMR and MS analysis as well as by comparison of their physio-chemical and spectroscopic data with literatures. Compounds **1-4** were isolated for the first time from a fungal endophyte of *Huperzia serrata* and subjected to an antibacterial assay against *S. aureus* ATCC 29213, a MRSA strain.

Key Words: Endophyte, *Huperzia serrata*, Antibacterial, Sumiki's acid.

INTRODUCTION

Endophytes are microorganisms growing in the tissues of their host plants without causing apparent disease symptoms[1]. The host plants offer the endophytes a unique biotope where they are subjected to constant metabolic and environmental interactions, which may enhance the synthesis of secondary metabolites[2]. Hence, endophytes are currently considered as a wellspring of novel secondary metabolites offering the potential for medical, agricultural and/or industrial exploitation[3].

During our investigation of endophytes, many fungal strains were obtained from the traditional Chinese medicinal plant *Huperzia serrata* and subjected to metabolites analysis[4]. In the present paper, we reported the isolation and structural elucidation of the metabolites produced by a *Huperzia serrata*-derived fungal endophyte *Penicillium* sp. HS-5. By repeated column chromatography over silica gel, MCI gel CHP 20P, Lichroprep RP-18 and Toyopearl HW-40C, nine compounds **1-9** were isolated from the broth of the strain and their structures were determined by spectroscopic analysis and by comparison of their physiochemical and spectroscopic data with those reported. Compounds **1-4** were isolated for the first time from a fungal endophyte of *Huperzia serrata* and subjected to antibacterial assay against *S. aureus* ATCC 29213, a MRSA strain.

EXPERIMENTAL

All NMR experiments were recorded on a Bruker AV-500 spectrometer operating at 500 and 125 MHz for ^{1}H NMR and ^{13}C NMR, respectively. ESIMS spectra were recorded on an Agilent 6210 Lc/Tof mass spectrometer and EI-MS were recorded on a Finnigan LCQoacw mass spectrometer. Silica gel (200-300 mesh), MCI-CHP20P gel (75-150 m; Mitsubishi Chemimcal Industries Ltd.), Toyopearl HW-40C gel (50-100 mm; Tosoh) and Lichroprep RP-18 (40-63 mm; Merck) were used for column chromatography and a pre-coated silica gel GF254 plate (Qingdao Marine Chemical Plant, Qingdao, P.R. China) was used for TLC. All solvents used were of analytical grade (Hangzhou Gaojing Fine Chemical Plant, Hangzhou, P.R. China).

Fungal material and culture condition: The fungus HS-5 was isolated from the healthy stems of *Huperzia serrata* collected in Xishuangbanna Tropical Plant Garden, Chinese Academy of Science, Yunnan Province, P. R. China in September 2007 and identified on the morphology level as *Penicillum* sp. by Prof. Wen-Hong Liu of Zhejiang Chinese Medical University. The original culture (ZJUT HS-5070723) was deposited at Zhejiang University of Technology, P.R. China. The fungus was cultured in liquid Sabouraud's medium (peptone 10 g/L, glucose 40 g/L) in Erlenmeyer flasks to a total of 50 L (250 mL × 200) at 28 ºC. The flasks were firstly inoculated on rotary shakers for 6 days at 185 rpm and then cultivated for another 20 days without agitation.

Extraction and isolation: At the end of fermentation, the culture was filtered through cheese cloth. The broth was condensed at 40 ºC to a volume of 5 L which was then successively partitioned between ethyl acetate (5 × 3 L) and *n*-BuOH (5 × 3 L). The ethyl acetate fraction (15 g) was subjected to

silica gel column chromatography and eluted with $CHCl_3$-MeOH (20:1→5:1) to give five fractions (Fr. 1-5). Fr. 3 was subjected to column chromatography containing MCI gel CHP 20P and eluted with MeOH-H_2O (5:95-95:5) to give compound **3** (567 mg), compound **2** (17 mg), compound **5** (14 mg) and compound **10** (55 mg). Compound **9** (1.37 g) was crystallized from this fraction. Fr. 4 was applied to RP-18 silica gel column eluted with MeOH-H_2O (5:95) to afford compound **1** (14 mg). Fr. 5 was also purified on a RP-18 silica gel column eluted with MeOH-H_2O (5:95) to furnish compound **4** (18 mg). The *n*-BuOH fraction (23 g) was separated by repeated silica gel column eluted with $CHCl_3$-MeOH (8:1→2:1) and HW-40C size exclusion chromatography eluted with MeOH to give compound **6** (10 mg), compound **7** (8 mg) and compound **8** (13 mg).

Compound **1**, brown oil, EI-MS m/z 142 $[M]^+$, 125, 114, 97. ^{1}H NMR (500 MHz, MEOD) δ: 7.18 (1H, d, *J* = 3.5, H-3), 6.48 (1H, d, *J* = 3.5, H-4), 4.59 (2H, s, H-6). ^{13}C NMR (125 MHz, MEOD) δ: 162.0 (C-1), 160.7 (C-5), 145.7 (C-2), 120.2 (C-3), 110.5 (C-4), 57.6 (C-6). These data were consistent with those reported of Sumiki's acid[5].

Compound **2**, brown oil, EI-MS m/z 184 $[M]^+$, 142, 125, 97. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 7.24 (1H, d, *J* = 3.0, H-3), 6.54 (1H, d, *J* = 3.0, H-4), 5.11 (2H, s, H-6), 2.11 (3H, s, H-8). The ^{1}H NMR spectra of **2** was similar with that of **1**. The differences include the downfield shift of H-6 from 4.59 to 5.11 in the acetylated derivative, together with an extra methyl group signal at 2.11 ppm. These and the MS data established the structure of acetyl Sumiki's acid (**7**). Both the ^{1}H NMR and MS data were consistent with those of reference[5].

Compound **3**, pale yellow crystal, EI-MS m/z 112 $[M]^+$, 95, 84, 67, 55. ^{1}H NMR (500 MHz, MEOD) δ: 7.70 (1H, d, *J* = 1.0, H-5), 7.17 (1H, d, *J* = 3.0, H-3), 6.57 (1H, dd, *J* = 3.0, 1.0, H-4). ^{13}C NMR (125 MHz, MEOD) δ: 162.5 (C-1), 147.5 (C-5), 146.1 (C-2), 118.4 (C-3), 112.6 (C-4). These data were consistent with those reported of 2-furoic acid[6].

Compound **4**, colourless crystal, EI-MS m/z 142 $[M]^+$, 113, 69, 57. ^{1}H NMR (500 MHz, DMSO-d_6) δ: 9.06 (1H, brs, 5-OH), 8.03 (1H, s, H-6), 6.34 (1H, s, H-3), 5.67 (1H, brs, 7-OH), 4.29 (1H, s, H-7). ^{13}C NMR (125 MHz, DMSO-d_6) δ: 173.9 (C-4), 168.1 (C-2), 145.7 (C-5), 139.3 (C-6), 109.8 (C-3), 59.5 (C-7). These data were consistent with those reported of Kojic acid[7].

Compound **5**, yellow crystal, ESI-MS m/z 137 $[M-H]^-$. ^{1}H NMR (500 MHz, MEOD) δ: 7.86 (2H, dd, *J* = 8.5, 2.5, H-2 and H-6), 6.80 (2H, dd, *J* = 8.5, 2.5, H-3 and H-5). ^{13}C-NMR (125 MHz, MEOD) δ: 169.8 (COOH), 163.1 (C-4), 132.6 (C-3 and C-5), 122.5 (C-1), 115.7 (C-2 and C-6). These data were consistent with those reported of 4-hydroxybenzonic acid[8].

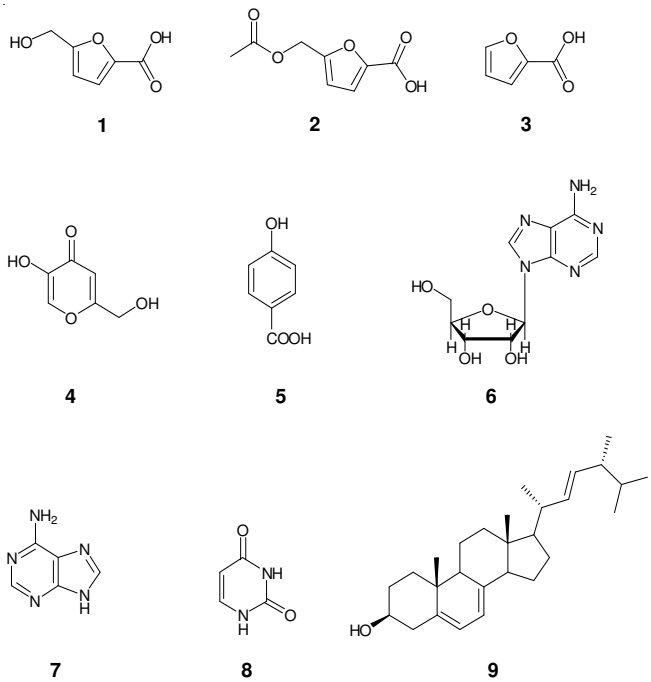
Compound **6**, white powder, ESI-MS m/z 290 $[M+Na]^+$. ^{1}H NMR (500 MHz, DMSO-d_6) δ: 8.36 (1H, s, H-2), 8.14 (1H, s, H-4), 7.36 (2H, s, 1-NH_2), 5.88 (1H, d, *J* = 6.0, H-1'), 5.41 (1H, d, *J* = 6.0, 2'-OH), 5.39 (1H, d, *J* = 6.0, 5'-OH), 5.16 (1H, d, *J* = 4.2, 3'-OH), 4.61 (1H, t, *J* = 3.0, H-2'), 4.15 (1H, dd, *J* = 4.5, 3.0, H-3'), 3.97 (1H, d, *J* = 3.0, H-4'), 3.68 (1H, dd, *J* = 12.5, 4.0, Ha-5'), 3.56 (1H, dd, *J* = 12.5, 4.0, Hb-5'). ^{13}C NMR (125 MHz, DMSO-d_6) δ: 156.6 (C-1), 152.9 (C-2), 149.5 (C-3), 140.4 (C-4), 119.8 (C-5), 88.4 (C-1'), 86.4 (C-4'), 73.9 (C-2'), 71.1 (C-3'), 62.1 (C-5'). These data were consistent with those reported of adenosine[9].

Compound **7**, white powder, EI-MS m/z 135 $[M]^+$, 108, 81, 54. ^{1}H NMR (500 MHz, DMSO-d_6) δ: 12.86 (1H, s, NH), 8.11 (2H, s, H-2, 4), 7.11 (2H, s, 1-NH_2). ^{13}C NMR (125 MHz, DMSO-d_6) δ: 155.8 (C-1), 152.3 (C-2), 150.4 (C-3), 138.9 (C-4), 118.6 (C-5). These data were consistent with those reported of adenine[10].

Compound **8**, white powder, ^{1}H-NMR (500 MHz, DMSO-d_6) δ: 11.02 (1H, s, 3-NH), 10.61 (1H, s, 1-NH), 7.25 (1H, s, H-6), 1.72 (3H, s, 5-CH_3). ^{13}C NMR (125 MHz, DMSO-d_6) δ: 164.9 (C-4), 151.5 (C-2), 137.7 (C-6), 107.7 (C-5), 11.8 (5-CH_3). These data were consistent with those reported of thymine[11].

Compound **9**, colourless crystal, EI-MS m/z 396 $[M]^+$. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 5.57 (1H, dd, *J* = 5.5, 2.0, H-6), 5.38 (1H, m, H-7), 5.15-5.25 (2H, m, H-22, 23), 3.63 (1H, m, H-3), 1.03 (3H, d, *J* = 6.5, H-21), 0.95 (3H, s, H-19), 0.92 (3H, d, *J* = 7.0, H-28), 0.84 (3H, d, *J* = 7.5, H-27), 0.83 (3H, d, *J* = 7.0, H-26), 0.63 (3H, s, H-18). ^{13}C NMR (125 MHz, $CDCl_3$) δ: 141.4 (C-8), 139.8 (C-5), 135.6 (C-22), 132.0 (C-23), 119.6 (C-6), 116.3 (C-7), 70.5 (C-3), 55.8 (C-17), 54.6 (C-14), 46.3 (C-9), 42.8 (C-13, 24), 40.8 (C-4), 40.4 (C-20), 39.1 (C-12), 38.4 (C-1), 37.1 (C-10), 33.1 (C-25), 32.0 (C-2), 28.3 (C-16), 23.0 (C-15), 21.1 (C-11, 21), 20.0 (C-26), 19.7 (C-27), 17.6 (C-28), 16.3 (C-19), 12.1 (C-18). These data were consistent with those reported of ergosterol[8].

Antibacterial assay: A disk diffusion method was employed to evaluate the antibacterial activity of the isolates. Sterilized paper disks (5.5 mm) were impregnated with 100 mg of the pure isolated compound and placed on agar plates with the test bacterium: *S. aureus* ATCC 29213. The plates were checked for inhibition zones after 24 h of incubation at 37 ºC.



Structure of compounds **1-9**

RESULTS AND DISCUSSION

Cultivation of the fungal endophyte *Penicillium* sp. HS-5 in Sabouraud's medium afforded nine compounds **1-9**, with four of them **1-4** isolated for the first time from a fungal endophyte of *Huperzia serrata*.

Sumiki's acid (**1**), acetyl Sumiki's acid (**2**) and 2-furoic acid (**3**) are carboxylic acid derivatives originate from 5-hydroxymethylfurfurl (HMF) and furfural, two intermediate generated *via* dehydration of hexose or pentose monomer units. The formation process of 5-hydroxymethylfurfurl and furfural *in vitro* has been well studied by the synthetic chemists[12,13]. It is speculated that 5-hydroxymethylfurfurl and furfural are produced by the fungus in a similar way. The sucrose used as carbon source in the medium was firstly hydrolyzed to form glucose and fructose, both of which, after isomerrization, were dehydrated *via* an open-chain or the cyclic furanose intermediate pathways to produce 5-hydroxymethylfurfurl and other additional dehydration and fragmentation products such as furfural[14]. The 5-hydroxymethylfurfurl was oxidized to give **1** and further acetylated to afford **2**. Similarly, the furfural can also be oxidized to form **3**.

Kojic acid (**4**) is a γ-pyrone derivative produced by several species of fungi, especially *Aspergillus oryzae* which has the Japanese common name koji[15]. The biosynthesis of kojic acid was studied by isotope tracer technique in 1950. Results of the studies revealed that the major biosynthetic pathway was a direct conversion of glucose to kojic acid, without breaking the pyranose ring[16]. Interest in kojic acid was mainly due to its inhibitory activity on tyrosinase and its inhibition of melaninogenesis. And the cosmetic use of kojic acid as a skin lightening agent was approved by health authorities in Japan in 1988[16].

Ergosterol (**9**) was identified on the basis of ^{1}H, ^{13}C NMR spectral data and comparison with authentic samples (co-TLC). Ergosterol is an ergostane-type sterol that is highly specific to fungi and generally absent in higher plants, which has been used as a marker to estimate fungal biomass in soil[17], leaf litter[18], seed[19] and other plant-fungal systems[20].

Antibacterial assay was employed to evaluate the antibacterial activity of **1-4** against a MRSA stain *S. aureus* ATCC 29213. To our disappointment, only weak activities were observed (Table-1), although **1** and **2** were reported[4] to be active against *S. aureus* ATCC 25923.

TABLE-1
INHIBITION ZONES (mm) OF COMPOUNDS **1–4** IN BACTERIAL CULTURE

	S. aureus ATCC 29213
Compound **1** (100 µg)	8.2
Compound **2** (100 µg)	7.0
Compound **3** (100 µg)	6.5
Compound **4** (100 µg)	7.5

ACKNOWLEDGEMENTS

Financial support of the Scientific Foundation of Zhejiang Province (Y2101290) is gratefully acknowledged.

REFERENCES

1. R.X. Tan and W.X. Zou, *Nat. Prod. Rep.*, **18**, 448(2001).
2. B. Schulz, C. Boyle, S. Draeger, A.K. Römmert and K. Krohn, *Mycol. Res.*, **106**, 996 (2002).
3. G. Strobel, B. Daisy, U. Castillo and J. Harper, *J. Nat. Prod.*, **67**, 257 (2004).
4. W.G. Shan, Y.M. Ying, H.N. Yu, W.H. Liu and Z.J. Zhan, *Helv. Chim. Acta*, **93**, 722 (2010).
5. R. Jadulco, P. Proksch, V. Wray, Sudarsono, A. Berg and U. Gräfe, *J. Nat. Prod.*, **64**, 527 (2001).
6. V. Nair, V. Varghese, R.R. Paul, A. Jose, C.R. Sinu and R.S. Menon, *Org. Lett.*, **12**, 2653 (2010).
7. X.F. Li, J.H. Jeong, K.T. Lee, J.R. Rho, H.D Choi, J.S. Kang and B.W. Son, *Arch. Pharm. Res.*, **26**, 532 (2003).
8. F. Wang and J.K. Liu, *Nat. Prod. Res. Dev.*, **16**, 204 (2004).
9. S.M. Zhou, K. Zhou and D.J. Xiao, *Chin. J. Mar. Drugs*, **24**, 31 (2005).
10. Q. Chen, L.J. Wu and L.J. Ruan, *J. Shenyang Pharm. Univ.*, **19**, 257 (2002).
11. G.C. Su, S. Zhang and S.H. Qi, *Chin. Tradit. Herbal Drugs*, **39**, 1606 (2008).
12. C. Moreau, R. Durand, S. Razigade, J. Duhamet, P. Faugeras, P. Rivalier, P. Ros and G. Avignon, *Appl. Catal. A*, **145**, 211 (1996).
13. X. Qian, M.R. Nimlos, M. Davis, D.K. Johnson and M.E. Himmel, *Carbohydr. Res.*, **340**, 2319 (2005).
14. J.N. Chheda, Y. Román-Leshkov and J.A. Dumesic, *Green Chem.*, **9**, 342 (2007).
15. T. Yabuta, *J. Chem. Soc.*, **125**, 575 (1924).
16. R. Bentley, *Nat. Prod. Rep.*, **23**, 1046 (2006).
17. A.W. West, W.D. Grant and G.P. Sparling, *Soil Biol. Biochem.*, **19**, 607 (1987).
18. S.Y. Newell, T.L. Arsuffi and R.D. Fallon, *Appl. Environ. Microbiol.*, **54**, 1876 (1988).
19. L.M. Seitz, H.E. Mohr, R. Burroughs and D.B. Sauer, *Cereal Chem.*, **54**, 1207 (1977).
20. F. Martin, C. Delaruelle and J.-L. Hilbert, *Mycol. Res.*, **94**, 1059 (1990).