Structural and Biological Properties of Vermistatin and Two New Vermistatin Derivatives Isolated from the Marine-Mangrove Endophytic Fungus *Guignardia* sp. No. 4382

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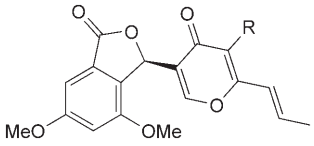
Vermistatin (=(3*R*)-4,6-dimethoxy-3-{4-oxo-6-[(1*E*)-prop-1-en-1-yl]-4*H*-pyran-3-yl}-2-benzofuran-1(3*H*)-one; **1**) and two new vermistatin derivatives, compounds **2** and **3**, were isolated from the fungal strain *Guignardia* sp. No. 4382 obtained from the South-China Sea. Their structures were elucidated by various methods, including circular dichroism (CD), 1D- and 2D-NMR, and HR-EI-MS. The structures of **1** and **2** were unequivocally corroborated by X-ray crystallography, and their absolute configurations were derived by CD spectroscopy based on a literature comparison. The *in vitro* cytotoxic and antifungal activities of **1** and **2** were tested.

Introduction. – Endophytic fungi are a well-established source for structurally diverse and biologically active secondary metabolites [1]. In continuation of our ongoing search for potential antitumor and antifugal natural products from marine-mangrove fungi [2], the metabolites of the *Guignardia* fungal strain No. 4382, a marine endophytic fungus collected from the South-China Sea, were studied. Together with the known vermistatin (**1**), two new compounds were isolated: methoxyvermistatin (**2**) and hydroxyvermistatin (**3**). Their structures and absolute configurations were elucidated by circular-dichroism (CD) and 1D- and 2D-NMR experiments, as well as by HR-EI-MS and X-ray diffraction analyses. The cytotoxic activities of **1** and **2** *in vitro* and their antifugal activities were tested.



1 R = H
2 R = OMe
3 R = OH

Results and Discussion. – 1. *Isolation and Structure Elucidation*. The AcOEt extract derived from the fermentation material of the entophytic fungal strain *Guignardia* sp.

No. 4382 was repeatedly purified chromatographically to yield compounds **1**–**3**. Related 'phthalidopyranone' derivatives have been reported before as secondary metabolites of fungi belonging to the genera *Penicillium* [3–6].

Compound **2** was obtained as colorless crystals. Its IR spectrum showed absorption bands for a 1,2,3,5-tetra-substituted benzene (850 cm^{-1}) and a five-membered lactone ring (1765 cm^{-1}), and the absorptions at 1665 and 1649 cm^{-1}, respectively, indicated the presence of C=O and C=C moieties (4*H*-pyranone). The molecular formula $C_{19}H_{18}O_7$ was determined by HR-EI-MS (*m*/*z* 358.1048 (M^+)).

In the ^{1}H-NMR spectrum of **2** (*Table 1*), a Me group at δ(H) 1.92 (*dd*, *J* = 2.8, 2.0 Hz), three MeO groups at δ(H) 3.83 (*s*), 3.86 (*s*), and 3.91 (*s*), a methine at δ(H) 6.34 (*s*), two olefinic resonances at δ(H) 6.58–6.61, and two aromatic signals at δ(H) 6.84 (*d*, *J* = 2.0) and 6.94 (*d*, *J* = 2.0 Hz) were observed. The ^{13}C-NMR spectrum of **2** showed 19 signals: four Me and six CH groups, and nine quaternary C-atoms (including two C=O groups), as determined by DEPT experiments.

Table 1. *^{1}H- and ^{13}C-NMR Data of* **2** *and* **3**. Recorded in (D_6)acetone at 400/100 MHz, resp.; δ in ppm, *J* in Hz. Arbitrary atom numbering.

Position	**2**		**3**		HMBC[a])
	δ(C)	δ(H)	δ(C)	δ(H)	
1	170.3		171.4		3, 7
3	74.8	6.34 (*s*)	75.7	6.33 (*s*)	2′
4	155.0		155.6		5, 4-MeO
5	105.5	6.84 (*d*, *J* = 2.0)	106.5	6.78 (*d*, *J* = 2.0)	7
6	163.6		166.6		5, 7, 6-MeO
7	99.8	6.94 (*d*, *J* = 2.0)	103.8	6.84 (*d*, *J* = 2.0)	5
8	130.2		131.4		
9	128.1		127.8		3, 5, 7
2′	155.4	7.97 (*s*)	156.3	7.88 (*s*)	3
3′	124.4		125.6		3, 2′
4′	173.9		174.7		3, 2′
5′	143.3		144.4		
6′	156.0		157.3		2′, 1″, 3″
1″	119.1	6.58–6.61 (*m*)[b])	120.5	6.58–6.61 (*m*)[c])	3″
2″	135.0	6.58–6.61 (*m*)[d])	136.2	6.58–6.61 (*m*)[e])	3″
3″	18.9	1.92 (*dd*, *J* = 2.8, 2.0)	19.8	1.94 (*dd*, *J* = 4.0, 1.6)	1″, 2″
4-MeO	56.3	3.91 (*s*)	61.5	3.83 (*s*)	
6-MeO	56.3	3.86 (*s*)	57.3	3.81 (*s*)	
5′-MeO or 5′-OH	60.6	3.83 (*s*)		9.28–9.39 (*m*)	

[a]) Identical C → H correlations for **2** and **3**. [b]) In CD_3OD: δ(H) 6.71 (*dq*, *J* = 15.6, 0.9). [c]) In CD_3OD: δ(H) 6.70 (*dq*, *J* = 15.6, 2.0). [d]) In CD_3OD: δ(H) 6.60 (*dq*, *J* = 15.6, 6.6). [e]) In CD_3OD: δ(H) 6.60 (*dq*, *J* = 15.6, 5.6).

The complete assignment of the ^{1}H- and ^{13}C-NMR data[1]) was accomplished by 2D-NMR (HMQC, HMBC, COSY) experiments. The ^{1}H,^{1}H-COSY spectrum revealed a contiguous sequence of coupled signals from H–C(1″) to H–C(3″), and the HMBC

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

data helped us to assemble the overall structure of **2**. The multiple HMBC correlations (*Table 1*) from H–C(3) and H–C(7) to C(1), from H–C(5), H–C(7), and 6-MeO to C(6), from H–C(5) and 4-MeO to C(4), and from H–C(3) to both C(1) and C(9) established the partial structure of **2** from C(1) to O(2), and located the position of the MeO groups. The correlations from H–C(1″) and H–C(2′) to C(6′) revealed the partial structure from C(2′) to C(3″). The pyran ring was attached to a furan at C(3) based on the HMBC correlation from H–C(3) to C(1), C(9), C(2′), C(3′), and C(4′). The signals for H–C(1″) and H–C(2″) could not be assigned by ^{1}H-NMR in (D_6)acetone, but in CD_3OD they appeared at δ(H) 6.71 (*dq*, J = 15.6, 0.9 Hz) and 6.60 (*dq*, J = 15.6, 6.6 Hz). The proposed structure of **2** was further confirmed by X-ray crystallography (*Fig. 1*).

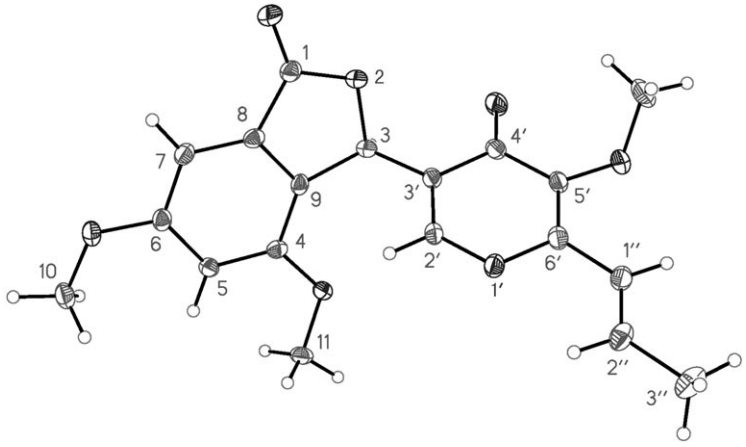


Fig. 1. *X-Ray crystal structure of* **2**

Compound **3** was obtained as a colorless powder. Its IR spectrum showed bands at 3243, 1770, 1658, 1640, 1601, and 857 cm^{-1}, which indicated the presence of OH, C=O, C=C, and 1,2,3,5-tetrasubstituted benzene moieties. The molecular formula was assigned as $C_{18}H_{16}O_7$ by HR-EI-MS (*m*/*z* 344.0887 (M^+)). In the ^{13}C-NMR (DEPT) spectrum of **3** (*Table 1*), 18 signals were discerned: three Me and six CH groups, and nine quaternary C-atoms. The ^{1}H- and ^{13}C-NMR data of **3** were similar to those of **1** (*Table 1*), except that one MeO signal was missing, as confirmed by EI-MS (M^+ at *m*/*z* 358 *vs.* 344), and a new, broad signal instead appeared at δ(H) 9.28–9.39 in the spectrum of **3**. The ^{1}H-NMR spectrum of **3** revealed a Me signal at δ(H) 1.94 (*dd*), two MeO groups at δ(H) 3.83 (*s*) and 3.81 (*s*), two aromatic signals at δ(H) 6.84 and 6.78 (2*d*), and three methines at δ(H) 6.33 (*s*, 1 H) and 6.58–6.61 (*m*, 2 H). In the ^{1}H,^{1}H-COSY spectrum, a correlation from H–C(3″) to H–C(1″) was observed. Full analysis of the 2D-NMR data then allowed us to assemble the overall structure of **3**.

The structure of vermistatin proper (**1**), which was isolated from the same fungus, was elucidated spectroscopically, by comparison with literature data [4], and by X-ray crystallography (*Fig. 2*). The absolute configuration of **1** had been determined previously by means of circular-dichroism (CD) experiments relative to a congener with known configuration [4]. We now unequivocally confirmed the structure and

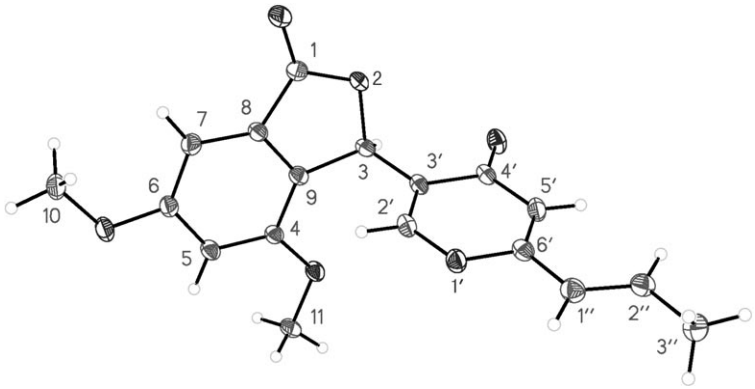


Fig. 2. *X-Ray crystal structure of* **1**

absolute configuration of **1** by single-crystal X-ray diffraction (*Oxford Diffraction Xcalibur Nova* X-ray single-crystal diffractometer with CuK_α radiation; *Flack* parameter $-0.02(14)$; see *Table 2*).

The absolute configurations of **2** and **3** were deduced by comparison of their CD data with that of **1** (*Fig. 3*). The absolute configuration at the stereogenic center at C(3) is (*R*) in all cases. The CD spectrum of **2** (in MeOH; λ in nm (θ in mdeg)) showed bands at 313 (+1.229) and 319 (sh, +1.247). The CD spectrum of **3** showed bands at 309 (+1.910), 320 (sh, +3.481), and 328.5 (+3.236). And that of **1** showed bands at 301 (+1.657) and 323 (+3.236).

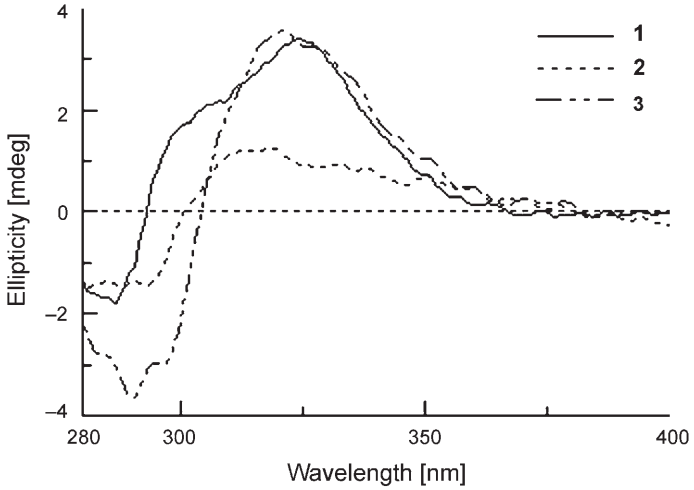


Fig. 3. *CD Spectra of* **1**–**3**. Recorded in MeOH at ambient temperature.

2. *Biological Properties.* Vermistatin (**1**) was originally isolated from *Penicillium vermiculatum* as a cytotoxic compound against tumor cells. It was reported to inhibit

the RNA synthesis in EAC and P-388 leukemia cells [3], as an elastase inhibitor [7], and as a phytotoxin on various banana cultivars [7][8]. Vermistatin (**1**) was reported to have no antifungal activity [9], except towards *Candida albicans* [10].

We found that methoxyvermistatin (**2**) exhibits modest *in vitro* cytotoxic activity against KB and KBv200 cells, with IC_{50} values of 20.0 and 15.1 μg/ml, respectively. For comparison, vermistatin proper (**1**) inhibited the KB tumor cell line with an IC_{50} value of 90.2 μg/ml, cisplatin being used as positive control, the latter giving rise to IC_{50} values of 0.56 and 0.78 μg/ml against the above two cell lines, respectively. Notably, the MeO congener **2** is a stronger antitumor agent that the parent compound **1** lacking a 5′-MeO group. Finally, we found that neither **1** nor **2** exhibited any antifungal activity.

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Experiment Part

General. Column chromatography (CC): silica gel (200–300 mesh; *Qingdao Haiyang Chemical Co.*, China). Melting point (m.p.): *Fisher-Johns* hot-stage apparatus; uncorrected. CD Spectra: *Jasco J-810* circular-dichroism spectrometer; λ in nm (θ in mdeg). IR Spectra: *Nicolet 5DX* FT-IR spectrophotometer; in cm^{-1}. ^{1}H- and ^{13}C-NMR Spectra: *Bruker AVANCE-400* spectrometer, in (D_6)acetone or CD_3OD; δ in ppm, *J* in Hz. HR-EI-MS: *VG-ZAB* mass spectrometer; in *m*/*z*.

Biological Material. A strain of the fungus *Guignardia* sp. No. 4382 was isolated from the bark of *Kandelia candel* (endophyte) from the Mai Po Marshes, Hong Kong, China. Voucher samples of the fungus are stored in the Department of Biology and Chemistry, City University of Hong Kong, Hong Kong, and the Department of Applied Chemistry, Zhongshan University, Guangzhou, P. R. China. Starter cultures (obtained from Prof. *E. B. G. Jones* and Prof. *L. L. P. Vrijmoed*, Department of Biology and Chemistry, City University of Hong Kong, Hong Kong) were maintained on cornmeal seawater agar. Plugs of agar supporting mycelial growth were cut and transferred aseptically to a 250-ml *Erlenmeyer* flask containing 100 ml of liquid medium made from glucose (10 g/l), peptone (2 g/l), yeast extract (1 g/l), and NaCl (30 g/l). The flask was incubated at 30° on a rotary shaker for 5–7 d. The mycelium was aseptically transferred to 200 1-l *Erlenmeyer* flasks containing a total of 120 l of liquid medium, and then incubated at r.t. for 30 d.

Extraction and Purification of Meatabolites. The cultures (150 l in total) were filtered through cheesecloth. The filtrate was concentrated below 50° to a final volume of *ca*. 5 l, and extracted by shaking with an equal volume of AcOEt (5×). The combined org. extracts were subjected to CC (SiO_2; 120 × 6 cm column; petroleum ether (PE) → AcOEt gradient) to yield six fractions (*Fr. 1–Fr. 6*), containing 3.0, 1.0, 1.5, 2.0, 2.1, and 2.0 g of material, resp. *Fr. 3* and *Fr. 4* were further purified by CC (SiO_2; 50 × 2.5 cm column; PE/AcOEt 60:40) to afford **2** (7.8 mg). Further purification by prep. TLC (SiO_2; PE/AcOEt 50:50) of the residual material of *Fr. 3* and *Fr. 4* yielded **3** (10.0 mg) and **1** (2.0 mg).

Methoxyvermistatin (=*(3*R*)-4,6-Dimethoxy-3-{5-methoxy-4-oxo-6-[(1*E*)-prop-1-en-1-yl]-4*H*-pyran-3-yl}-2-benzofuran-1(3*H*)-one*; **2**). Colorless crystals. M.p. 198–199°. $[\alpha]_D^{20} = -30$ ($c = 0.1$, acetone). CD (MeOH): 313 (+1.229), 319 (sh, +1.247). IR (KBr): 3084, 2941, 2848, 1765, 1665, 1649, 1621, 1507, 1445, 1381, 1299, 1226, 1206, 1172, 1122, 850. ^{1}H- and ^{13}C-NMR: see *Table 1*. HR-EI-MS: 358.1048 (M^+, $C_{19}H_{18}O_7^+$; calc. 358.1053).

Hydroxyvermistatin (=*(3*R*)-4,6-Dimethoxy-3-{5-hydroxy-4-oxo-6-[(1*E*)-prop-1-en-1-yl]-4*H*-pyran-3-yl}-2-benzofuran-1(3*H*)-one*; **3**). Colorless powder. M.p. 226–227°. $[\alpha]_D^{20} = -15.4$ ($c = 0.1$, acetone). CD (MeOH): 309 (+1.910), 320 (sh, +3.481), 328.5 (+3.236). IR (KBr): 3243, 2961, 2925, 2854, 1770, 1741, 1658, 1640, 1601, 1523, 1459, 1381, 1347, 1286, 1261, 1223, 1171, 1117, 1039, 857. ^{1}H- and ^{13}C-NMR: see *Table 1*. HR-EI-MS: 344.0887 (M^+, $C_{18}H_{16}O_7^+$; calc. 344.0896).

X-Ray Crystallography[2]). The X-ray diffraction data for compound **1** were measured on an *Oxford Diffraction Xcalibur-Nova* single-crystal diffractometer using CuK_α radiation at 150 K (*Table 2*, *Fig. 2*). The data were processed with the software CrysAlis [11]. The structure was solved by direct methods and refined using full-matrix least-squares based on F^2 with the SHELXL program [12]. H-Atoms were added in ideal positions and refined as riding models. As anticipated, the two benzene rings of vermistatin (**1**) were situated at a dihedral angle of 86° (nearly perpendicular to each other) to minimize steric hindrance.

The X-ray diffraction data for compound **2** were measured in the $2\theta/\omega$ scan mode on a *Bruker Smart-1000 CCD* diffractometer using MoK_α radiation (*Table 2*, *Fig. 1*). The data were processes as described above.

Table 2. *X-Ray Crystal and Refinement Data of* **1** *and* **2**

	1	**2**
Crystallized from	petroleum ether/AcOEt	petroleum ether/AcOEt
Empirical formula	$C_{18}H_{16}O_6$	$C_{19}H_{18}O_7$
M_r [Da]	328.31	358.33
Crystal size	0.40 × 0.10 × 0.05 mm	0.48 × 0.24 × 0.12 mm
Unit-cell dimensions:		
a [Å]	4.8168 (9)	4.1217(8)
b [Å]	8.457(4)	8.0574(16)
c [Å]	18.953(6)	13.001(3)
α [°]	85.97	100.073
β [°]	89.13	91.457
γ [°]	87.67	94.589
V [Å^3]	769.4(5)	423.42(14)
Crystal system	triclinic	triclinic
Space group	$P1$	$P1$
Z	2	1
λ [Å]	1.54178	0.71073
T [°C]	293(2)K	173(2) K
Calculated density [Mg/m^3]	1.417	1.405
Absorption coefficient [mm^{-1}]	0.898	0.108
θ-Range [°] for data collection	2.34 – 63.30	2.58 – 25.99
θ_{max}	60.89	25.52
F(000)	344	188
Refinement method	Full-matrix least-squares on F^2	Full-matrix least-squares on F^2
Data, restraints, parameters	4534, 3, 434	2938, 3, 239
Reflections collected	22815	3286
Independent reflections	4534	2938
Goodness-of-fit on F^2	1.082	1.074
Final R_1 ($I > 2\sigma(I)$)	0.0386	0.0387
wR_2	0.1087	0.0770
R Indices (all data)	$R_1 = 0.0404$, $wR_2 = 0.1106$	$R_1 = 0.0310$, $wR_2 = 0.0723$
Abs. structure parameter	− 0.02(14)	− 0.2(8)
Largest diff. peak and hole	0.0368, − 0.232 e Å^{-3}	0.173, − 0.153 e Å^{-3}

[2]) The crystallographic data of **1** and **2** have been deposited with the *Cambridge Crystallographic Data Centre* as supplementary publication numbers CCDC-634972 and CCDC-634973, resp. Copies of the data can be obtained, free of charge, at http://www.ccdc.cam.ac.uk/data_request/cif.

Cytotoxicity Assay. KB or KBv200 Cells were harvested and seeded in 96-well plates at 3.0×10^3 cells/well in a final volume of 190 µl. After 24 h of incubation, 10 µl of cytotoxic agent or compound vehicles were added to each well. After 68 h, 10 µl of MTT (= 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide) soln. was added to each well. After 4 h, DMSO (100 µl) was added. The concentration (IC_{50}) required to inhibit cell growth by 50% were calculated from the cytotoxicity curves using *Bliss*'s software.

Antifungal Assay. The antifungal activities of **1** and **2** were tested by using the agar-impregnated disk method. 9-mm Disks were impregnated with 40 µg of test substance and then exposed to three different fungi, *Saccharomyces cerevisiae*, *Aspergillus niger*, and *Staphylococcus aureus*, but none gave rise to an inhibition zone.

REFERENCES

[1] D. J. Newman, G. M. Cragg, *J. Nat. Prod.* **2007**, *70*, 461.
[2] R. Y. Yang, C. Y. Li, Y. C. Lin, G. T. Peng, Z. G. She, S. N. Zhou, *Bioorg. Med. Chem. Lett.* **2006**, *16*, 4205; Y. C. Lin, X. Y. Wu, S. Feng, G. C. Jiang, J. H. Luo, S. N. Zhou, L. L. P. Vrijmoed, E. B. Gareth Jones, K. Krohn, K. Steingrover, F. Zsila, *J. Org. Chem.* **2001**, *66*, 6252.
[3] J. Fuska, A. Fuskova, P. Nemec, *Biologia (Bratislava, Slovakia)* **1979**, *34*(9), 735.
[4] J. Fuska, D. Uhrin, B. Proksa, Z. Votick, J. Ruppeldt, *J. Antibiot.* **1986**, *39*, 1605.
[5] S. I. Komai, T. Hosoe, T. Itabashi, K. Nozawa, T. Yaguchi, K. Fukushima, K. I. Kawai, *Heterocycles* **2005**, *65*, 2771.
[6] L. Merlini, G. Nasini, A. Selva, *Tetrahedron* **1970**, *26*, 2739.
[7] M. Sturdikova, B. Proksa, J. Fuska, M. Stancikova, *Biologia (Bratislava)* **1995**, *50*(3), 233.
[8] R. K. Upadhyay, G. A. Strobel, S. J. Coval, J. Clardy, *Experientia* **1990**, *46*, 982.
[9] B. Proksa, S. Uhrinova, J. Adamcova, J. Fuska, *Monatsh. Chem.* **1992**, *123*, 251.
[10] M. Arai, H. Tomoda, T. Okuda, H. Wang, N. Tabata, R. Masuma, Y. Yamaguchi, S. Omura, *J. Antibiot.* **2002**, *55*, 172.
[11] CrysAlis, Version 1.171.31.8, Oxford Diffraction Ltd., 2007.
[12] G. M. Sheldrick, 'SHELXL-97: Program for Crystal Structure Solution and Refinements', University of Göttingen, Germany, 1997.

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