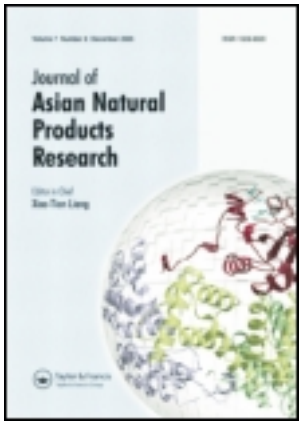

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Three new glucosides from a cold-adapted fungal strain *Mucor* sp.

Chun-Chi Feng[a†], Guo-Dong Chen[a†], Xiao-Xia Li[a], Jin-Shan Tang[a]*, Xing-Zhong Liu[b], Yan Li[c], Xin-Sheng Yao[a] and Hao Gao[a]*

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Two new naphthaline glucosides, 2-hydroxy-3-methoxy-8-methyl-1-*O*-β-D-glucopyranosylnaphthaline (**1**) and 1-hydroxy-3-methoxy-8-methyl-2-*O*-β-D-glucopyranosylnaphthaline (**2**), together with one new isocoumarin glucoside, 3-(3,3-dichloro-2-hydroxylpropyl)-6-methoxy-8-*O*-β-D-glucopyranosyl-1*H*-isochromen-1-one (**3**), were isolated from a cold-adapted fungal strain *Mucor* sp. (No. XJ07027-5). Their structures were characterized by detailed analyses of IR, MS, 1D- and 2D-NMR spectra. Among them, **2** showed moderate cytotoxic activity against five tumor cells (A-549, HL-60, MCF-7, SMMC-7721, and SW480).

Keywords: cold-adapted fungi; *Mucor* sp; naphthaline; isocoumarin; glucoside; cytotoxic activity

1. Introduction

Fungi are paid more and more attention in recent years, especially for those living in specialized environment such as alpine glacier, deep sea, saltern, and alkali soil [1–4]. Cold-adapted fungi are defined as that living in the cold habitats such as high mountain, alpine glacier, deep sea, Antarctic, and Arctic [1]. The cold-adapted fungi might produce structurally novel and biologically active compounds, but little studies on the secondary metabolites of them were implemented [5–11].

In our ongoing searching for the bioactive fungal secondary metabolites [12–17], two new naphthaline glucosides, 2-hydroxy-3-methoxy-8-methyl-1-*O*-β-D-glucopyranosylnaphthaline (**1**) and 1-hydroxy-3-methoxy-8-methyl-2-*O*-β-D-glucopyranosylnaphthaline (**2**), together with one new isocoumarin glucoside, 3-(3,3-dichloro-2-hydroxylpropyl)-6-methoxy-8-*O*-β-D-glucopyranosyl-1*H*-isochromen-1-one (**3**) (Figure 1), were obtained from a cold-adapted fungal strain *Mucor* sp. (No. XJ07027-5) derived from the soil of Xinjiang Province in China. In this contribution, we describe the isolation and structure elucidation of **1**–**3**, together with their cytotoxic activity against five tumor cells (A-549, HL-60, MCF-7, SMMC-7721, and SW480).

2. Results and discussion

The fermented rice substrate of the fungus (No. XJ07027-5) was extracted repeatedly with EtOAc, and the organic solvent was removed under reduced pressure to yield crude extract. The crude extract was suspended in 90% MeOH–H_2O and simultaneously extracted with cyclohexane to remove lipids. The residue of the 90% MeOH–H_2O layer was subjected to

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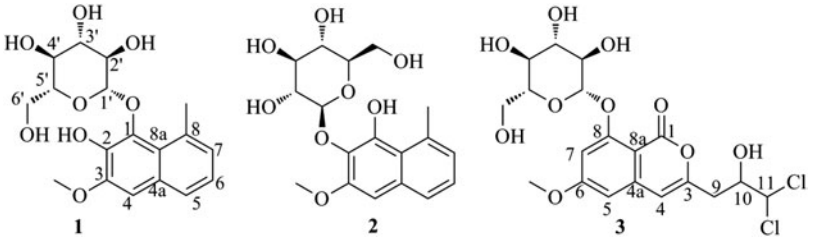

Figure 1. Structures of compounds **1**–**3**.

various chromatographic methods to yield three new compounds **1**–**3** (Figure 1).

Compound **1** was obtained as faint yellow powder. It had the molecular formula of $C_{18}H_{22}O_8$ with eight degrees of unsaturation based on the high-resolution electrospray ionization MS (HR-ESI-MS) at *m/z* 389.1215 $[M + Na]^+$. The IR spectrum exhibited absorption bands assignable to hydroxyl groups (3361 cm^{-1}) and aromatic ring (1624, 1513, and 1478 cm^{-1}). The ^1H NMR spectrum (Table 1) showed three aromatic proton signals at δ_H 7.51 (1H, d, $J = 8.0$ Hz), 7.10 (1H, dd, $J = 8.0$ and 6.8 Hz) and 7.00 (1H, d, $J = 6.8$ Hz) forming an AMX coupling system (1,2,3-trisubstituted benzene ring structure), one aromatic proton signal at δ_H 7.12 (1H, s), an anomeric proton at δ_H 4.86 (1H, d, $J = 8.0$ Hz), and two methyl protons at δ_H 3.88 (3H, s) and 2.84 (3H, s). The ^{13}C NMR (Table 1) spectrum exhibited 10 aromatic carbon signals (δ_C 104.0, 123.1, 123.6, 124.9, 126.7, 128.3, 132.8, 138.5, 139.9, and 148.7), one methyl carbon signal (δ_C 24.3), one methoxyl carbon signal (δ_C 55.5), and one set of glucopyranose carbon signals (δ_C 60.7, 69.7, 74.2, 76.3, 77.3, and 105.0) [18]. The relative configuration of the glucopyranose was determined as β according to the large *J* value of anomeric proton [δ_H 4.86 (1H, $J = 8.0$ Hz, H-1′)]. The absolute configuration of the glucopyranose was determined as D configuration according to a procedure described previously [19]. The ^{13}C NMR data, along with the degrees of unsaturation, revealed that **1** has a naphthaline skeleton [18], which was confirmed by HMBC data (Figure 2). The methoxyl group was attached to C-3 by HMBC correlation of OCH_3-3 (δ_H 3.88) with C-3 (δ_C 148.7) and the methyl group was attached to C-8 by the HMBC correlation of CH_3-8 (δ_H 2.84) with C-8 (δ_C 132.8), C-8a (δ_C 123.6), and C-7 (δ_C 126.7). The HMBC correlation of H-1′ (δ_H

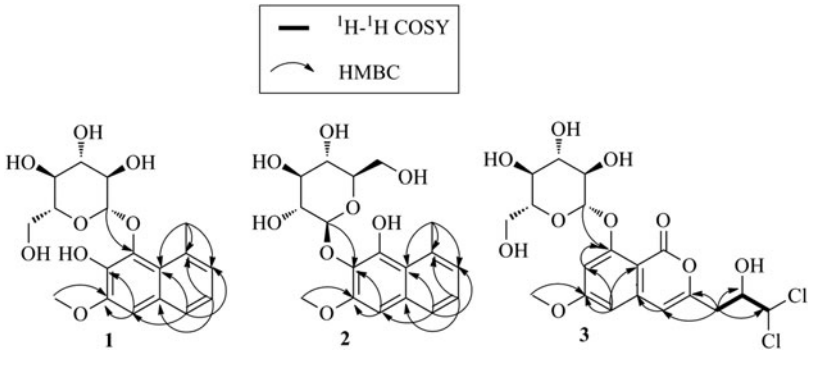

Figure 2. Key ^1H–^1H COSY and HMBC correlations of compounds **1**–**3**.

Table 1. ^{1}H (400 MHz) and ^{13}C NMR (100 MHz) spectral data for compounds **1**–**3** (in DMSO-d_6).

	1		**2**		**3**	
Position	^{1}H (J in Hz)	^{13}C	^{1}H (J in Hz)	^{13}C	^{1}H (J in Hz)	^{13}C
1		139.9		–		158.3
2		138.5		132.4		
3		148.7		151.4		154.3
4	7.12 (s)	104.0	6.84 (s)	99.1	6.51 (s)	105.2
4[a]		128.3		132.6		141.1
5	7.51 (d, 8.0)	124.9	7.50 (d, 8.4)	125.0	6.74 (d, 2.4)	102.7
6	7.10 (dd, 8.0, 6.8)	123.1	7.19 (dd, 8.4, 7.2)	125.3		165.0
7	7.00 (d, 6.8)	126.7	6.99 (d, 7.2)	125.5	6.86 (d, 2.4)	102.8
8		132.8		134.3		160.7
8[a]		123.6		119.7		102.9
9					2.85 (dd, 14.4, 2.8, Ha) 2.59 (dd, 14.4, 9.4, Hb)	36.3
10					4.17 (m)	72.2
11					6.28 (d, 3.2)	77.0
1′	4.86 (d, 8.0)	105.0	4.54 (d, 7.6)	106.2	4.95 (d, 7.6)	101.7
2′	3.40[a]	74.2	3.36[a]	73.9	3.37[a]	73.3
3′	3.27[a]	76.3	3.25[a]	76.1	3.29[a]	76.3
4′	3.17[a]	69.7	3.21[a]	69.6	3.17 (m)	69.8
5′	3.08 (m)	77.3	3.17 (m)	77.3	3.41[a]	77.4
6′	3.57 (dd, 11.0, 1.2, Ha) 3.43[a] (Hb)	60.7	3.66 (dd, 11.6, 2.0, Ha) 3.52 (dd, 11.6, 4.0, Hb)	60.8	3.73 (br.d, 11.6, Ha) 3.47 (dd, 11.6, 5.6, Hb)	60.8
3-OCH_3	3.88 (s)	55.5	3.84 (s)	55.6		
6-OCH_3					3.87 (s)	55.8
8-CH_3	2.84 (s)	24.3	2.81 (s)	24.2		

Note: –, The signal was not detected.
[a] The signals were overlapped with the signal of water peak.

Table 2. Cytotoxic activities of compounds **1**–**3**.

Compounds	Cytotoxic activity (IC_{50}, μM)				
	A-549	HL-60	MCF-7	SMMC-7721	SW480
1	>40	>40	>40	>40	>40
2	37.73	23.92	26.07	28.84	22.59
3	>40	>40	>40	>40	>40
Cisplatin	11.83	1.32	15.17	6.24	12.95
Paclitaxel	<0.008	<0.008	<0.008	<0.008	<0.008

4.86) with C-1 (δ_C 139.9) revealed that the β-D-glucopyranose was attached to C-1. Thus, the structure of **1** was determined as 2-hydroxy-3-methoxy-8-methyl-1-*O*-β-D-glucopyranosylnaphthaline, which was a new compound.

Compound **2** was isolated as faint yellow powder. Its molecular formula was $C_{18}H_{22}O_8$ with eight degrees of unsaturation, the same to that of **1**, which was established by the HR-ESI-MS at *m/z* 389.1213 $[M + Na]^+$. Comparative analyses of the ^1H and ^{13}C NMR data (Table 1) with those of **1** indicated that **2** had the similar structure to that of **1**, a naphthaline skeleton linked with a glucopyranose unit. The relative configuration of the glucopyranose was determined as β according to the large *J* value of anomeric proton [δ_H 4.54 (1H, $J = 7.6$ Hz, H-1′)]. The absolute configuration of the glucopyranose was determined as D configuration according to a procedure described previously [19]. The methyl was also attached to C-8 by HMBC correlation of CH_3-8 (δ_H 2.81) with C-8a (δ_C 119.7), C-8 (δ_C 134.3), and C-7 (δ_C 125.5). The methoxyl was attached to C-3 by HMBC correlation of OCH_3-3 (δ_H 3.84) with C-3 (δ_C 151.4). The HMBC correlation of H-1′ (δ_H 4.54) with C-2 (δ_C 132.4) revealed that the β-D-glucopyranose was attached to C-2. So, the structure of **2** could be determined as 1-hydroxy-3-methoxy-8-methyl-2-*O*-β-D-glucopyranosylnaphthaline, which was a new compound.

Compound **3** was isolated as white powder. It has the molecular formula of $C_{19}H_{22}Cl_2O_{10}$ based on the HR-ESI-MS at *m/z* 503.0474 $[M + Na]^+$ and the ratio (3:2) of the intensities of isotope peaks ($[M + Na]^+/[M + Na + 2]^+$), with eight degrees of unsaturation. Comparative analyses of the ^1H and ^{13}C NMR data (Table 1) with those of dichlorodiaportin [20] indicated that **3** was a glycosylated derivative of dichlorodiaportin, which was further confirmed by ^1H–^1H correlation spectroscopy (COSY) and HMBC correlations (Figure 2). Except for the carbon signals of the dichlorodiaportin, ^{13}C NMR spectrum exhibited one set of glucopyranose carbon signals (δ_C 60.8, 69.8, 73.3, 76.3, 77.4, and 101.7) [18]. The relative configuration of the glucopyranose was determined as β according to the large *J* value of anomeric proton [δ_H 4.95 (1H, $J = 7.6$ Hz, H-1′)]. The absolute configuration of the glucopyranose was determined as D configuration according to a procedure described previously [19]. The β-D-glucopyranose was attached to C-8 by HMBC correlation of H-1′ (δ_H 4.95) with C-8 (δ_C 160.7). Therefore, the structure of **3** was determined as 3-(3,3-dichloro-2-hydroxylpropyl)-6-methoxy-8-*O*-β-D-glucopyranosyl-1*H*-isochromen-1-one (**3**), which was a new compound.

Compounds **1**–**3** were tested for their ability to prevent the growth of five tumor cells (A-549, HL-60, MCF-7, SMMC-7721, and SW480) by MTT (3-(4,5)-dimethylthiazoyl)-3,5-diphenyltetrazolium bromide) assay [21]. Among them, **2** showed inhibitory effect on the proliferation of the tumor cells ($IC_{50} = 22.59$–37.73 μM)

(Table 2). The positive control groups were cisplatin ($IC_{50} = 1.32–15.17\ \mu M$) and paclitaxel ($IC_{50} < 0.008\ \mu M$).

3. Experimental

3.1 General experimental procedures

UV data were recorded on a JASCO V-550 UV/vis spectrometer (JASCO Corporation, Tokyo, Japan). JASCO FT/IR-480 plus Fourier transform infrared spectrometer was used for scanning IR spectra (KBr) (JASCO Corporation). ^{1}H (400 MHz), ^{13}C (100 MHz) and 2D- ($^{1}H–^{1}H$ COSY, heteronuclear single-quantum correlation, and HMBC) NMR data were acquired with Bruker AV 400 using solvent signals (DMSO-d_6: δ_H 2.50/δ_C 39.5) as internal standards (Bruker Corporation, Fallanden, Switzerland). ESI-IT-MS spectra were performed on a Finnigan LCQ Advantage MAX mass spectrometer and HR-ESI-MS spectra were obtained on Waters Synapt G2 TOF mass spectrometer (Waters Corporation, Manchester, UK). Column chromatography (CC) was carried out on silica gel (200–300 mesh) (Qingdao Haiyang Chemical Group Corporation, Qingdao, China), Sephadex LH-20 (Pharmacia, Uppsala, Sweden), and octadecylsilane (ODS) (60–80 μm, YMC, Inc., Kyoto, Japan), respectively. Analytical thin layer chromatography was performed on precoated silica gel plate (SGF_{254}, 0.2 mm, Yantai Chemical Industry Research Institute, Yantai, China). All solvents used in CC were of analytical grade (Tianjin Damao Chemical Plant, Tianjin, China). The analytical high-performance liquid chromatography (HPLC) was performed on a Shimadzu LC-20AB Liquid Chromatography with SPD-M20A Detector, using ODS column (Phenomex Gemini C18 column [ϕ 5 μm, 250 mm × 4.6 mm; Phenomex, Inc., Torrance, CA, USA]) at 220, 254, and 280 nm. The semi-preparative HPLC and preparative HPLC were performed on a Shimadzu LC-6AD Liquid Chromatography with SPD-20A Detector, using ODS columns (YMC-Pack C18 column [ϕ 5 μm, 250 mm × 10.0 mm, YMC, Inc.] and Phenomex Gemini C18 column [ϕ 5 μm, 250 mm × 21.2 mm, Phenomex, Inc.], respectively) at 220, 254 and 280 nm.

3.2 Fungal material

The strain of *Mucor* sp. was collected from the mountain soil of Xinjiang Province of China. The isolate was identified by one of the authors (X.-Z. Liu) and assigned the accession number XJ07027-5 in the culture collection at the Institute of Traditional Chinese Medicine and Natural Products, College of Pharmacy, Jinan University, Guangzhou. The fungal strain was cultured on slants of potato dextrose agar at 15°C for 5 days. Agar plugs were used to inoculate four Erlenmeyer flasks (250 ml), each containing 100 ml of potato dextrose broth. Four flasks of the inoculated media were incubated at 15°C on a rotary shaker at 200 rpm for 5 days to prepare the seed culture. Fermentation was carried out in 20 Erlenmeyer flasks (500 ml), each containing 70 g of rice. Distilled H_2O (105 ml) was added to each flask, and the rice was soaked overnight before autoclaving at 120°C for 30 min. After cooling to room temperature, each flask was inoculated with 5.0 ml of the spore inoculum and incubated at 15°C for 58 days.

3.3 Extraction and isolation

The fermented rice substrate was extracted three times with EtOAc, and the organic solvent was removed under reduced pressure to yield crude extract (54.0 g). The crude extract was suspended in 90% MeOH–H_2O and simultaneously extracted with cyclohexane (1:1, v/v) for three times. The residue of the 90% MeOH–H_2O layer (18.6 g) was subjected to C_{18} medium performance liquid chromatography (MPLC) eluted with MeOH–H_2O (50% and 100%, v/v) to afford two fractions (F1 and F2). The fraction F1

(6.8 g) was subjected to MPLC eluted with MeOH–H_2O in a gradient (from 20% to 100%, v/v) to give five subfractions (F1a–F1e). Subfraction F1c (1898.1 mg) was subjected to Sephadex LH-20 column (MeOH), MPLC (MeOH–H_2O, from 20% to 70%, v/v), and semi-preparative HPLC (18% CH_3CN–H_2O, 4 ml/min, 254 nm) to yield compounds **1** (3.9 mg, $t_R = 43.0$ min) and **3** (3.7 mg, $t_R = 55.3$ min). Subfraction F1e (510.5 mg) was subjected to silica gel CC with a gradient $CHCl_3$–MeOH (from 99% to 90%, v/v) and preparative HPLC (26% CH_3CN–H_2O, 8 ml/min, 254 nm) to give compound **2** (1.5 mg, $t_R = 17.8$ min).

*3.3.1 2-Hydroxy-3-methoxy-8-methyl-1-O-β-D-glucopyranosylnaphthaline (**1**)*

Faint yellow powder; $[\alpha]_D^{26} + 49.1$ ($c = 0.2$, MeOH). UV (MeOH) λ_{max} (log ϵ): 237 (5.36) and 287 (4.44) nm. IR (KBr) ν_{max}: 3361.3, 2935.1, 1623.8, 1579.4, 1512.9, 1478.2, 1384.6, 1247.7, 1064.5, and 819.6 cm^{-1}; ^1H and ^{13}C NMR spectroscopic data, see Table 1. Positive ESI-MS: *m/z* 389 $[M + Na]^+$. Positive HR-ESI-MS: *m/z* 389.1215 $[M + Na]^+$ (calculated for $C_{18}H_{22}O_8Na$, 389.1212).

*3.3.2 1-Hydroxy-3-methoxy-8-methyl-2-O-β-D-glucopyranosylnaphthaline (**2**)*

Faint yellow powder; $[\alpha]_D^{26} + 48.0$ (*c* 0.4, MeOH). UV (MeOH) λ_{max} (log ϵ): 237 (4.42) and 291 (3.72) nm. IR (KBr) ν_{max}: 3376.7, 2926.5, 1649.8, 1592.9, 1456.0, and 1069.3 cm^{-1}. ^1H and ^{13}C NMR spectroscopic data, see Table 1. Positive ESI-MS: *m/z* 389 $[M + Na]^+$. Positive HR-ESI-MS: *m/z* 389.1213 $[M + Na]^+$ (calculated for $C_{18}H_{22}O_8Na$, 389.1212).

*3.3.3 3-(3,3-Dichloro-2-hydroxylpropyl)-6-methoxy-8-O-β-D-glucopyranosyl-1H-isochromen-1-one (**3**)*

White powder; $[\alpha]_D^{26} - 20.5$ (*c* 0.35, MeOH). UV (MeOH) λ_{max} (log ϵ): 245 (5.01) and 341 (3.98) nm. IR (KBr) ν_{max}: 3399.9, 2922.6, 1694.2, 1607.4, 1565.9, 1451.2, 1380.8, 1204.3, and 1065.5 cm^{-1}; ^1H and ^{13}C NMR spectroscopic data, see Table 1. Positive ESI-MS: *m/z* 503 $[M + Na]^+$(100), 505 $[M + Na + 2]^+$(67.5). Positive HR-ESI-MS: *m/z* 503.0474 $[M + Na]^+$ (calculated for $C_{19}H_{22}Cl_2O_{10}Na$, 503.0488).

3.4 Acid hydrolysis of compounds 1–3

D-Glucose (2.0 mg) and L-glucose (2.0 mg) were heated with L-cycteine methyl ester (2.5 mg) in pyridine (1 ml) at 60°C for 60 min and then *o*-tolyl isothiocyanate (10 μl) was added to the mixtures and heated further for 60 min [19]. The reaction mixtures were analyzed by reverse phase (RP) HPLC and detected at 250 nm. Compounds **1**–**3** (each 0.5 mg) were hydrolyzed by heating in 2 M HCl (2 ml) for 120 min at 90°C. After drying under reduced pressure, the mixtures were suspended with H_2O (1 ml) and then extracted three times with isopyknic water-saturated EtOAc. The EtOAc layer was removed. After drying the H_2O layer, the residual mixtures were heated with L-cycteine methyl ester in pyridine at 60°C for 60 min, and then *o*-tolyl isothiocyanate (10 μl) was added to the mixtures and heated further for 60 min. The reaction mixtures were analyzed by RP HPLC and detected at 250 nm. The retention times (t_R) and the flow rate (v) of the glucose derivative were as follows: D-glucose derivative ($t_R = 21.28$ min, $v = 0.8$ ml/min) and L-glucose derivative ($t_R = 19.45$ min, $v = 0.8$ ml/min). By comparing the retention time and the flow rate (compound **1**: $t_R = 21.26$ min, $v = 0.8$ ml/min; compound **2**: $t_R = 21.27$ min, $v = 0.8$ ml/min; compound **3**: $t_R = 21.28$ min, $v = 0.8$ ml/min) with standard glucose derivative, the absolute configurations of the glucopyranose unit in compounds **1**–**3** were determined as D.

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Note

1. These two authors contributed equally to the work reported in this article.

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