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•Special topic•

New furo[3,2-*h*]isochroman from the mangrove endophytic fungus *Aspergillus* sp. 085242

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[ABSTRACT] Four new compounds, asperisocoumarin G (**1**), asperisocoumarin H (**2**), (±)-asperisocoumarin I [(±)-**3**], along with the known pergillin (**4**) and penicisochroman L (**5**) were isolated from a mangrove endophytic fungus *Aspergillus* sp. 085242 by further chemical investigation. The structures of the new compounds, including their absolute configurations, were established by analysis of HR-ESI-MS and NMR spectroscopic data, and ECD calculation. Asperisocoumarins G-I (**1**−**3**) were new isocoumarins belonging to the class of furo[3, 2-h]isocoumarins which are rarely found in natural sources. All of the isolated compounds were evaluated for their *α*-glucosidase inhibitory effects, and compounds **1** and **4** showed moderate *α*-glucosidase inhibitory activity, respectively. In an antimicrobial test, the racemate of **3** showed antibacterial activity against *Salmonella*.

[KEY WORDS] *Aspergillus*; Furo[3, 2-*h*]isocoumarins; *α*-Glucosidase inhibitory activity; Antimicrobial activity
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Introduction

Aspergillus is an important group of mangrove endophytic fungus that is widely recognized as prolific sources of biologically active and structurally unique secondary metabolites [1]. These natural products have diverse chemical structures including, terpenoids, alkaloids and isocoumarin [2-4]. Among them, isocoumarins are a kind of important natural products with diverse structural features, and the furoisocoumarins were relatively rarely isolated in nature [5]. Up to now, there were only several reports on the isolation of angular type furo[3,2-*h*]isocoumarins from natural sources, such as penicisochroman [6], asperisocoumarin [7], coriandrone A [8], pergillin [9], ustusorane [10].

As a part of our efforts to discover structurally interesting and biologically active secondary metabolites from mangrove endophytic fungus [11-13], four new angular type furo[3,2-*h*]isocoumarins, namely asperisocoumarins G−I (**1**−**3**), along with the known pergillin (**4**) and penicisochroman L (**5**) were isolated from a mangrove endophytic fungus *Aspergillus* sp. 085242 (Fig. 1). All the isolates were evaluated for *α*-glucosidase inhibitory activity and the antimicrobial activity against a panel of human pathogenic bacteria. Among them, compounds **1** and **4** exhibited moderate *α*-glucosidase inhibitory activity and the racemate of **3** displayed antimicrobial activity against *Salmonella*.

Results and Discussion

The mangrove endophytic fungus *Aspergillus* sp. 085242 was cultured on solid rice medium with saline water for 28 days. The MeOH extract of the fermentation was fractionated by repeated silica gel chromatography and Sephadex LH-20 column chromatography to yield compounds **1**−**5**.

Asperisocoumarin G (**1**) was isolated as pale yellow oil. Its molecular formula was assigned as $C_{16}H_{16}O_5$ on the basis of HR-ESI-MS at *m/z* 289.1071 $[M + H]^+$ (Calcd. for $C_{16}H_{17}O_5$, 289.1071), indicating nine degrees of unsaturation. The ^{1}H NMR data (Table 1) showed two aromatic protons (δ_H

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These authors have no conflict of interest to declare.

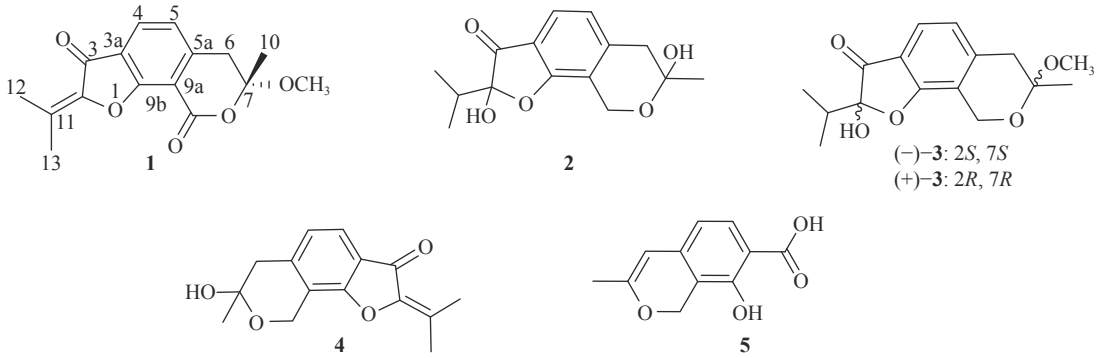

Fig. 1 Structures of compounds 1−5

Table 1 NMR spectroscopic data for compounds 1−3

No.	**1**[a]		**2**[b]		(±)-**3**[b]	
	δ_C	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)
2	145.4, C		109.3, C		109.3, C	
3	182.4, C		201.8, C		201.8, C	
3a	124.5, C		118.9, C		118.9, C	
4	129.7, CH	7.89, d (7.8)	122.7, CH	7.41, d (7.8)	122.8, CH	7.40, d (7.8)
5	122.1, CH	6.99, d (7.8)	124.0, CH	6.85, d (7.8)	123.7, CH	6.83, d (7.8)
5a	146.6, C		145.8,C		145.3, C	
6*α*	40.1, CH_2	3.32,d (16.2); 3.21, d (16.8)	40.1, CH_2	2.97, d (17.4)	40.2, CH_2	2.96, d (17.4)
6*β*				2.87, d (17.4)		2.89, d (17.4)
7	104.4, C		95.4, C		98.7, C	
9*α*	160.3, C		58.2, CH_2	4.89, m	58.6, CH_2	4.79, t (15.2)
9*β*				4.79, t (16.2)		4.61, m
9a	110.5, C		120.3, C		119.9, C	
9b	164.2, C	1.71, s	168.5, C		168.3, C	
10	23.0, CH_3		28.9, CH_3	1.54, s	23.2, CH_3	1.49, s
11	135.1, C		35.2, CH	2.17, m	35.2, CH	2.16, m
12	20.8, CH_3	2.24, s	16.5, CH_3	0.85, d (6.0)	16.5, CH_3	0.84, brs
13	17.8, CH_3	2.39, s	15.9, CH_3	1.07, d (7.2)	15.9, CH_3	1.06, brs
OMe	50.4, CH_3	3.38, s			58.5, CH_3	3.33, s

[a] ^{1}H (600MHz) and ^{13}C (150MHz) in $CDCl_3$; [b] ^{1}H (600MHz) and ^{13}C (150MHz) in CD_3OD

7.89 and δ_H 6.99), one methoxyl group (δ_H 3.38), one methylene group [δ_H 3.32 (H-6*α*), 3.21 (H-6*β*)], and three methyl groups (δ_H 2.39, δ_H 2.24, and δ_H 1.71). The ^{13}C NMR and HSQC spectra showed 16 carbon resonances corresponding to two carbonyl, six aromatic, two olefinic, one methoxyl, one methylene, one quaternary and three methyl carbons. These spectroscopic features illustrated that **1** belongs to the family of isocoumarins. A literature survey suggested that the ^{1}H and ^{13}C NMR data of **1** were very similar to the known asperisocoumarin A isolated from *Aspergillus* sp. 085242 before [7]. Together with the upfield appearance of carbonyl group C-9 (δ_C 160.3), the 7-methyl-7-methoxyisocoumarin unit was confirmed by the key HMBC correlations (Fig. 2) from H-10 to C-6 and C-7, MeO-7 to C-7, H-6 to C-5, C-5a, and C-9a, H-5 to C-3a and C-9a. The HMBC correlations of two methyl protons H-12 and H-13 to C-2, C-3, and C-11, as well as the chemical shifts of these carbons, established a 2-oxy-3-methyl-2-butenoyl moiety. This moiety connected to the aromatic ring at C-3a and 9b was established by the HMBC correlation from H-4 to C-3, C-3a and C-9b. According to the chemical shifts of C-2 (δ_C 145.4) and C-9b (δ_C 164.2) as well as the desired unsaturation, the ether bond between C-2

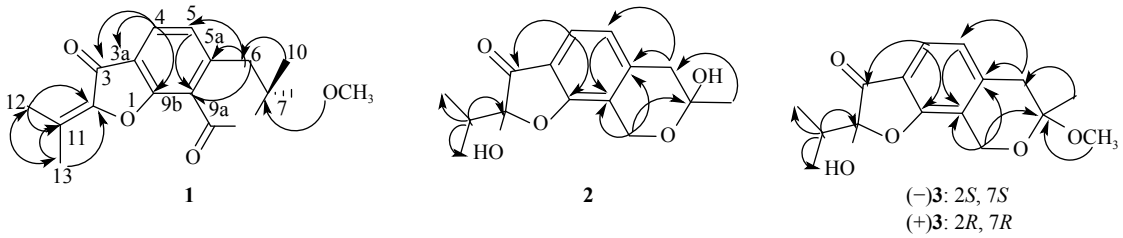

Fig. 2 Key HMBC (arrows) correlations of compounds 1−3

and C-9b was fused into a 3-oxobenzofuran unit. Moreover, the absolute configuration of **1** was assigned by comparison of the experiment ECD spectra with the calculated using Time-dependent Density Functional Theory (TDDFT) at B3LYP/6-311 + G (d, p) level, and the experimental ECD curve of **1** agreed well with the predicted one (*R*) (Fig. 3). Thus, the structure of **1** was identified as (*R*)-7-methoxy-7-methyl-2-(propan-2-ylidene)-6,7-dihydro-2*H*-furo[3,2-*h*]isochromene-3,9-dione, named asperisocoumarin G.

Asperisocoumarin H (**2**) was obtained as a pale yellow amorphous solid. Its molecular formula was determined as $C_{15}H_{18}O_5$ by HR-ESI-MS (*m*/*z* 277.1082 $[M-H]^-$, Calcd. for $C_{15}H_{17}O_5$, 277.1081). A careful comparison of its ^1H and ^{13}C NMR spectra (Table 1) with those of **1** indicated that compound **2** also shared the same isocoumarin skeleton as compound **1**. The main differences were that two hydroxyl group was attached to C-2 (δ_C 109.3) and C-7 (δ_C 95.4), and an additional methylene carbon at δ_C 58.2, whereas one carbonyl carbon at δ_C 160.3, one methoxyl carbon at δ_C 50.4, and a double bond at δ_C 145.4 and δ_C 135.1, were absent in the spectrum of compound **2**. And the ^1H NMR spectrum showed the signal corresponding to C-11. These differences were further supported by the HMBC correlations of H_2-9 to C-7, C-5a and C-9a, and H-11 to C-2, C-12 and C-13 (Fig. 2). Unfortunately, the compound **2** was racemic mixture for the optical rotation value was nearly zero, and it can not be further isolated by positive or reverse chiral HPLC to give optical pure compound. This result may be due to the unstable hemiketal structure at C-7 and C-2 in compound **2**. Therefore, the structure of compound **2** was established as depicted in Fig. 1, and named as asperisocoumarin H.

(±)-Asperisocoumarin I [(±)-**3**] was isolated as a pale yellow amorphous solid and the molecular formula was deduced as $C_{16}H_{20}O_5$ from HR-ESI-MS analysis (*m*/*z* 291.0876 $[M-H]^-$), indicating seven degrees of unsaturation. The ^1H and ^{13}C NMR spectra of compound **3** were quite similar to those of **2** except for a methoxyl instead of a hydroxyl connected to C-7 (δ_C 98.7) and an additional proton signal at δ_H 3.33 (3H, s, MeO-7) (Table 1). Together with the mass information, the strong HMBC correlation of MeO-7 to C-7 further supported the replacement of hydroxyl at C-7 by a methoxyl group in **3** (Fig. 2). So the planar structure of **3** was deduced as the 7-methoxylated **2.** Subsequently separation through chiral HPLC resolved the racemate into (+)-**3** and (−)-**3**. The absolute configuration of (+)-**3** and (−)-**3** were assigned by comparison of the experimental ECD spectra with the calculated one using Time-dependent Density Functional Theory (TDDFT) at B3LYP/6-311 + G (d, p) level, and the calculated ECD curves for 2*S*, 7*S* and 2*R*, 7*R* were well matched with the experimental results of (−)-**3** and (+)-**3,** respectively (Fig. 4). Thus, compounds (−)-**3** and (+)-**3** were named as (−)-(2*S*, 7*S*)-asperisocoumarin I and (+)-(2*R*, 7*R*)-asperisocoumarin I, respectively.

In addition, the known compounds were identified as pergillin (**4**) [9], and penicisochroman L (**5**) [14] by comparison of their physical and spectroscopic data with those reported in the literature.

All isolates **1**−**5** were evaluated for their *α*-glucosidase inhibitory activity using clinical acarbose (IC_{50} of 725.1 μmol·L^{-1}) as a positive control (Table 2). Compounds **1** and **4** showed moderate *α*-glucosidase inhibitory activity with IC_{50} of 392.4 and 428.1 μmol·L^{-1}, respectively. The other tested compounds showed less activity than acarbose. Furthermore, compounds **1**, **2**, (±)−**3**, **4**, and **5** were examined for their antimicrobial activities against several Gram-positive and Gram-

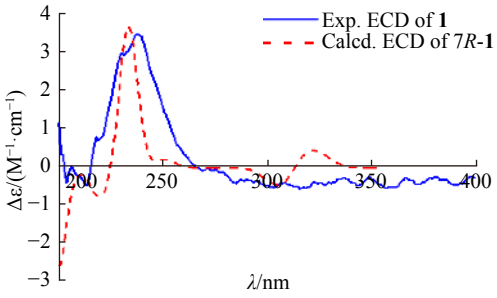

Fig. 3 Comparison of the experimental and calculated ECD spectra of 1

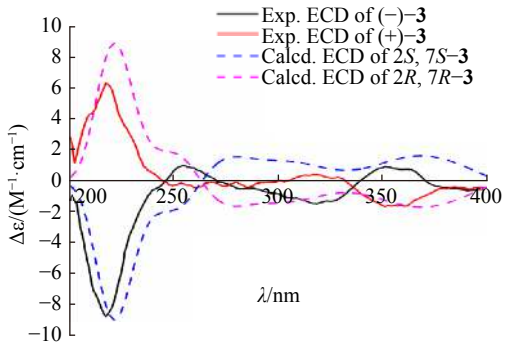

Fig. 4 Comparison of the experimental and calculated ECD spectra of (±)−3

Table 2 Inhibitory activities of *α*-glucosidases

Compounds	**1**	**2**	(+)-**3**	(−)-**3**	**4**	**5**	Acarbose [a]
IC_{50} (μmol·L^{-1})[b]	392.4	1853.4	1736.5	1464.1	428.1	1073.2	725.1

[a] positive control; [b] IC_{50} values are shown as mean from three independent experiments

negative bacterial strains (*S. aureus* ATCC 6538, *B. subtilis* ATCC 6633, *E. coli* ATCC 8739, *P. Aeruginosa* ATCC 9027, *Salmonella* ATCC 14028). The result disclosed the racemate of **3** was moderate active (inhibition zone, 14 mm) against *Salmonella c*ompared to the positive control ciprofloxacin (inhibition zone, 26 mm).

In conclusion, four new furo[3,2-*h*]isocoumarins, asperisocoumarin G, asperisocoumarin H and (±)-asperisocoumarin I (**1**, **2**, (±)-**3**), along with the known pergillin (**4**) and penicisochroman L (**5**) were isolated from a mangrove endophytic fungus *Aspergillus* sp. 085242 by thoroughly chemical investigation. And compounds **1** and **4** exhibited moderate *α*-glucosidase inhibitory activity. Moreover, the racemic compound **3** showed antimicrobial activity against *Salmonella*. Our findings would enrich chemical context of the genus *Aspergillus* and expand the chemical and biological diversity of isocoumarin.

Experimental

General experiment procedures

UV data were recorded on a Shimadzu UV-2700 spectrophotometer (Shimadzu, Kyoto, Japan). Optical rotations were measured on a MCP 500 (Anton Paar, Austria) at 25 ℃. CD spectrum was recorded with a Chirascan spectropolarimeter (Applied Photophysics, U.K). EIMS data were measured on a DSQ EI-mass spectrometer (Thermo, Shanghai, China) and HREIMS data were carried out on a DMAT95XP high-resolution mass spectrometer. ESIMS spectra were recorded on a Finnigan LCQ-DECA mass spectrometer and HR-ESI-MS data were determined on a Shimadzu LCMS-IT-TOF mass spectrometer. NMR spectra were recorded on a Bruker AVANCE NEO 500 MHz spectrometer and Bruker AVANCE NEO 600 MHz spectrometer (Bruker BioSpin, Switzerland). Separation of enantiomers **(±)-3** was carried out on a Phenomenex chiral ND (2) 5 μm column (4.6 mm × 250 mm) using a Shimadzu LC-20 AD liquid chromatograph instrument. Silica gel (200−300 mesh, Qingdao Marine Chemical Factory) and Sephadex LH-20 (Amersham Pharmacia, Piscataway) were used for column chromatography (CC). Thin layer chromatography was performed on precoated silica gel plates (Qingdao Huang Hai Chemical Group Co., G_{60}, F_{254}).

Fungal material

The detailed strain information about *Aspergillus* sp. 085242 has been reported before [2] and a voucher strain was deposited in the China Center for Type Culture Collection with a patent depository number CCTCC M2013081.

Fermentation, extraction, and isolation

The fungus was grown on autoclaved rice solid substrate medium (one hundred 500 mL Erlenmeyer flasks, each containing 50 g rice and 50 mL 3‰ of saline water) at room temperature under static conditions and daylight for 28 days. The mycelia and solid rice medium were extracted with MeOH three times. The extracts were then filtered, combined, and evaporated under reduced pressure, yielding a dark-brown residue (153.3 g). The residue was suspended in distilled water and partitioned with EtOAc successively. The residue was fractionated on a normal-phase silica gel column using petroleum ether and EtOAc as eluent (from 0 : 1 to 0 : 1), obtaining 16 fractions (Fr. 1–Fr. 16). Fr. 1 (24.6 mg) was subjected to a silica gel column (CH_2Cl_2–MeOH, 100 : 1, *V*/*V*) to afford two subfractions (Frs. 1.1–1.2). Fr. 1.1 (14.3 mg) was further purified by Sephadex LH-20 (CH_2Cl_2–MeOH, 1 : 1, *V*/*V*) to obtain **1** (7.5 mg). Compounds **2** (6.5 mg) and **3** (5.9 mg) were obtained from Fr. 3 (19.2 mg) by preparative HPLC (MeOH–H_2O, 70 : 30, *V*/*V*), then the enantiomeric mixtures (**3**) was separated by using chiral HPLC by elution with *n*-hexane–isopropanol (90 : 10, *V*/*V*) to obtain (+)-**3** (2.3 mg) and (−)-**3** (2.1 mg). Fr. 5 (48.5 mg) was divided into two fractions (Frs. 5.1–5.2) through silica gel column (PE–EtOAc, 5 : 1–0 : 1, *V*/*V*). Fr. 5.1 (21.2 mg) was further purified by silica gel column (CH_2Cl_2–MeOH, 100 : 2, *V*/*V*) to afford **4** (15.1 mg), and Fr. 5.2 (13.7 mg) was purified by silica gel column (CH_2Cl_2–MeOH, 100 : 3, *V*/*V*) and Sephadex LH-20 (CH_2Cl_2–MeOH, 1 : 1, *V*/*V*) to obtain **5** (10.1 mg).

Asperisocoumarin G

Pale yellow oil; $[\alpha]_D^{25}$ +13° (*c* 0.1, MeOH); ^1H and ^{13}C NMR data, Table 1; (+)-ESI-MS *m*/*z* 277.2 $[M + H]^+$; (+)-HR-ESI-MS at *m*/*z* 289.1071 $[M + H]^+$ (Calcd. for $C_{16}H_{17}O_5$, 289.1071).

Asperisocoumarin H

Pale yellow amorphous solid; $[\alpha]_D^{25}$ 0° (*c* 0.1, MeOH); ^1H and ^{13}C NMR data, Table 1; (−)-ESI-MS *m*/*z* 277.2 $[M - H]^-$; (−)-HR-ESI-MS at *m*/*z* 277.1082 $[M - H]^-$ (Calcd. for $C_{15}H_{17}O_5$, 277.1081).

(−)-(2S, 7S)-asperisocoumarin I

Pale yellow amorphous solid; $[\alpha]_D^{25}$ −23.4° (*c* 0.1, MeOH); ^1H and ^{13}C NMR data, Table 1; (−)-HR-ESI-MS at *m*/*z* 291.0876 $[M - H]^-$ (Calcd. for $C_{16}H_{19}O_5$, 291.0876).

(+)-(2R, 7R)-asperisocoumarin I

Pale yellow amorphous solid; $[\alpha]_D^{25}$ +57.9° (*c* 0.1, MeOH); ^1H and ^{13}C NMR data, Table 1; (−)-HR-ESI-MS at *m*/*z* 291.0876 $[M - H]^-$ (Calcd. for $C_{16}H_{19}O_5$, 291.0876).

α-Glucosidase inhibitory activity

All the assays were performed according to the modified method of Liu *et al*. [15]. Enzyme solution was prepared to give 2.0 units per mL in 2 mL aliquots. The assay medium

contained phosphate buffer, pH 7.0 (140 mL), 10 mL of enzyme solution, 20 mL DMSO or inhibitor (dissolved in DMSO) and 30 mL of 0.01 μmol·L^{-1} substrate (*p*-nitrophenyl) (PNP) glycoside (3 mg·mL^{-1}). The substrate was immediately added to a 96-well microtiter plate containing enzyme and buffer with inhibitor after 15 min of incubation time at 37 °C. Absorbance was measure at 405 nm, due to the hydrolysis of PNP-G by *α*-glucosidase, was monitored continuously with the microplate reader (iMark, Bio-Rad, USA). All measurements were done in triplicate from two independent experiments. The reported IC_{50} was the average value of two independent experiments.

Antimicrobial activity

Antimicrobial activity against *Salmonella* (ATCC 14028) was measured using the paper disk method. *Salmonella* was cultured in LB liquid medium consisting of 0.5% yeast extract (BD Biosciences), 1% tryptone (BD Biosciences), and 1% NaCl, at 37 °C for 24 h. A paper disk (diameter 6 mm, Whatman) that contained 1 mg·mL^{-1} of **1** (**2**, **3**, **4** and **5**) was placed on an LB agar plate including 0.2% of the liquid culture; the plate was then incubated at 37 °C for 24 h. The antimicrobial activity is expressed as the diameter (mm) of the inhibitory zone. Ciprofloxacin (1 mg·mL^{-1}) as a positive control showed an inhibition zone of 26 mm against *Salmonella*.

Quantum mechanical calculation

Molecular Merck force field (MMFF) and DFT/TD-DFT calculations were carried out with Spartan' 14 software (Wavefunction Inc., Irvine, CA, USA) and Gaussian 09 program, respectively. The corresponding minimum geometries were further fully optimized with the Gaussian 09 (Gaussian, Wallingford, CT, USA) program package at the B3LYP/6-31G(d) as frequency calculations. The obtained stable conformers were submitted to CD calculation by the TDDFT B3LYP/6-311 + G (d, p) method. ECD spectrum were generated by program SpecDis 1.6 (University of Würzburg, Würzburg, Germany) and OriginPro 8.5 (OriginLab, Ltd., Northampton, MA, USA) from dipole-length rotational strengths by applying Gaussian band shapes with sigma = 0.26 eV. All calculations were performed with the High-Performance Grid Computing Platform of Sun Yat-sen University.

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