

Article

Meroterpenes from Endophytic Fungus A1 of Mangrove Plant *Scyphiphora hydrophyllacea*

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Received: 3 July 2012; in revised form: 10 August 2012 / Accepted: 4 September 2012 /

Published: 17 September 2012

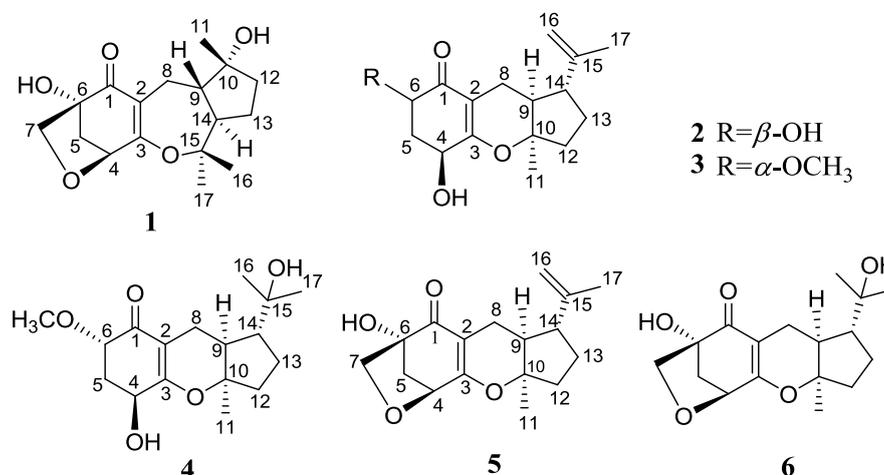
Abstract: Four new meroterpenes, guignardones F–I (**1–4**), together with two known compounds guignardones A (**5**) and B (**6**) were isolated from the endophytic fungus A1 of the mangrove plant *Scyphiphora hydrophyllacea*. Their structures and relative configurations were elucidated by spectroscopic data and single-crystal X-ray crystallography. A possible biogenetic pathway of compounds **1–6** was also proposed. All compounds were evaluated for inhibitory activity against methicillin-resistant *Staphylococcus aureus* (MRSA) and *Staphylococcus aureus*.

Keywords: marine endophyte; *Scyphiphora hydrophyllacea*; meroterpenes; *Guignardia* sp.; MRSA; *Staphylococcus aureus*

1. Introduction

Marine-derived fungi have recently become a research focus as one of the richest sources of new and bioactive secondary metabolites in the marine environment [1,2]. The mangrove plant *Scyphiphora hydrophyllacea* Gaertn. F. (Rubiaceae) growing in tropical and subtropical intertidal habitats is a rich source of new iridoids, which have been found to possess cytotoxic activity [3–6]. The metabolites produced by endophytic fungi from *S. hydrophyllacea* collected in Hainan attracted our attention [7], a new fatty acid glycoside and two new meroterpenes have been found from the fungus A1 of *S. hydrophyllacea* [8,9]. Further phytochemical investigation of the secondary metabolites from the culture broth of the fungus A1 led to the isolation of four new meroterpenes, guignardones F–I (**1–4**) (Figure 1), and two known compounds, guignardones A (**5**) and B (**6**) [10]. This paper reports the isolation and structural elucidation, as well as biological activities and plausible biogenetic pathway of these compounds.

Figure 1. The structures of compounds **1–6**.



2. Results and Discussion

Guignardone F (**1**) was isolated as colorless needle crystals, and possessed a molecular formula of C₁₇H₂₄O₅ as established by HREIMS (m/z 308.1625 [M]⁺), indicating six degrees of unsaturation. The IR spectrum displayed hydroxyl (3463 cm⁻¹), conjugated carbonyl (1657 cm⁻¹) and double bond (1606 cm⁻¹) absorptions. In the ¹³C NMR and DEPT spectra (Table 1), a total of 17 carbon resonances were found and classified into three methyls, five methylenes (one oxygenated), three methines (one oxygenated), and six quaternary carbons (one α,β -unsaturated carbonyl unit, three oxygenated). In the HMBC spectrum (Figure 2), the correlations from H₂-8 (δ_H 3.03, 1.92) to C-1 (δ_C 200.2), C-2 (δ_C 114.7), and C-3 (δ_C 173.9), from H-4 (δ_H 4.46) to C-2 and C-6 (δ_C 81.8), from H₂-5 (δ_H 2.31, 1.91) to C-1 and C-3, and from H₂-7 (δ_H 3.80, 3.45) to C-4 (δ_C 81.3), C-5 (δ_C 42.5), and C-6 were found and led to the establishment of partial structure **1a**, which was identical to that of guignardone B (**6**) [10]. In the ¹H-¹H COSY spectrum, a proton spin system (H-8/H-9/H-14/H-13/H-12) was found as drawn with bond lines in Figure 2. In addition, the HMBC cross-peaks from H₃-11 (δ_H 1.35) to C-9 (δ_C 46.1), C-10 (δ_C 80.5), and C-12 (δ_C 39.3), and from both H₃-16 (δ_H 1.17) and H₃-17 (δ_H 1.45) to C-14 (δ_C 54.1) and C-15 (δ_C 86.9) determined the establishment of substructure **1b**. Careful comparison of the ¹³C NMR data of **1** with

those of **6**, the upfield shift of C-10 ($\Delta -10.2$ ppm) and the downfield shift of C-15 ($\Delta +14.0$ ppm) were observed. So it was proposed that C-3 connected with C-15 via an ether bond to form a seven-membered ring in **1**, instead of the C-3 connected with C-10 via an ether bond to form a six-membered ring in **6**. This also gave a good explanation for the obvious difference ($\Delta 10.8$ ppm) of chemical shifts between the 16-CH₃ and the 17-CH₃, since their stereochemistry circumstance was quite different in the structure of **1**. Thus, the gross structure of **1** was elucidated and further confirmed by X-ray crystallography which also established its relative configuration (Chart 1). Therefore, the structure of **1** was established as shown in Figure 1, named Guignardone F (**1**).

Table 1. ¹³C NMR data (100 MHz, CDCl₃) for **1–4**.

position	1	2	3	4
1	200.2 s	198.7 s	194.9 s	195.0 s
2	114.7 s	105.0 s	105.8 s	105.5 s
3	173.9 s	167.1 s	167.7 s	168.5 s
4	81.3 d	66.7 d	65.7 d	65.7 d
5	42.5 t	36.9 t	34.5 t	34.6 t
6	81.8 s	67.1 d	79.1 d	79.0 d
7	69.3 t			
8	21.9 t	16.2 t	16.1 t	18.7 t
9	46.1 d	43.3 d	43.2 d	41.2 d
10	80.5 s	88.2 s	87.7 s	88.9 s
11	26.5 q	23.5 q	22.3 q	22.1 q
12	39.3 t	37.7 t	37.4 t	38.3 t
13	25.9 t	27.0 t	26.9 t	24.5 t
14	54.1 d	48.7 d	48.9 d	51.1 d
15	86.9 s	145.4 s	145.4 s	72.9 s
16	19.2 q	111.3 t	111.2 t	28.6 q
17	30.0 q	19.2 q	19.2 q	27.5 q
OCH ₃			58.3 q	58.3 q

Figure 2. ¹H-¹H COSY and key HMBC correlations of **1–4**.

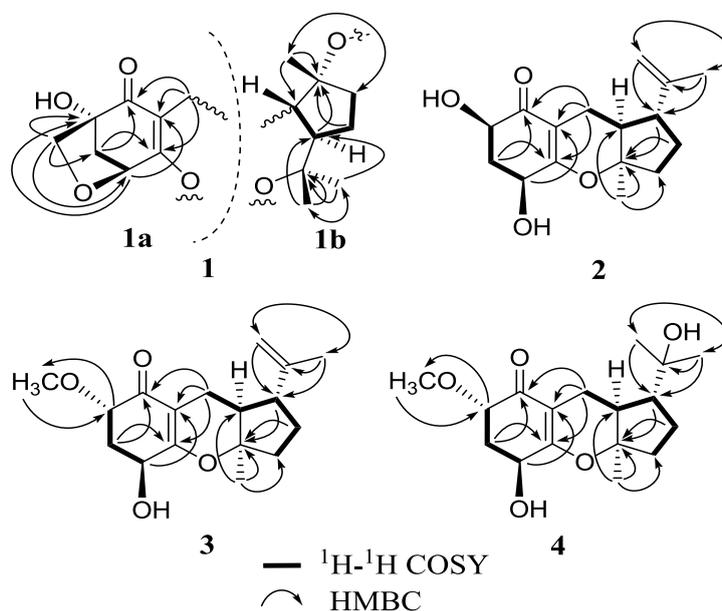
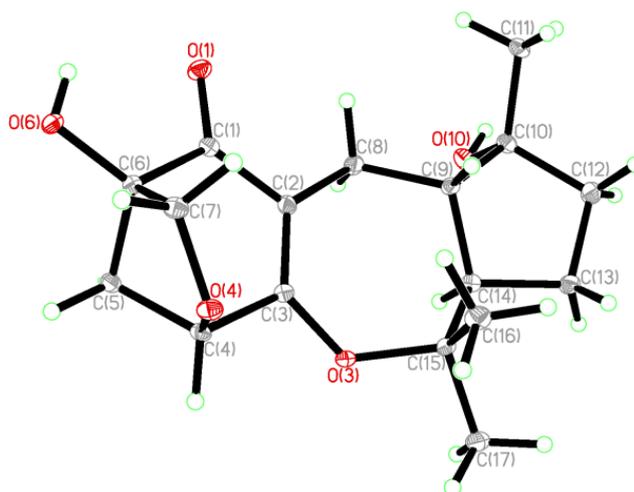
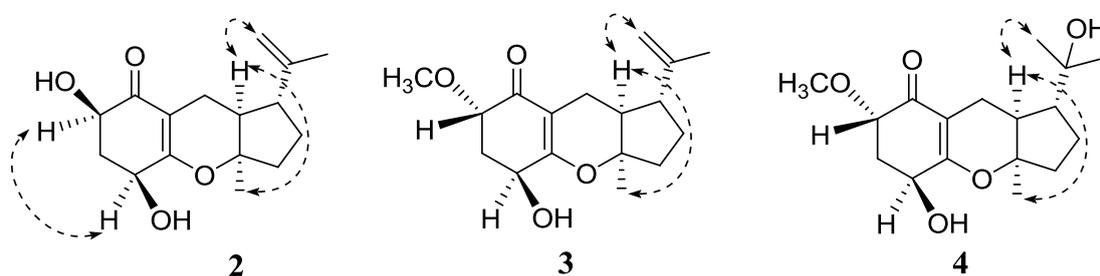


Chart 1. The ORTEP view of compound 1.



Guignardone G (**2**) was obtained as a colorless oil. Its molecular formula was determined as $C_{16}H_{22}O_4$ from $[M]^+$ ion peak at m/z 278.1513 in the HREIMS, with six degrees of unsaturation. IR absorptions implied the presence of hydroxyl (3440 cm^{-1}), conjugated carbonyl (1653 cm^{-1}) and double bond (1622 cm^{-1}) groups. The ^{13}C NMR and DEPT spectra (Table 1) revealed 16 carbon signals, including one α,β -unsaturated carbonyl group, one terminal double bond, one oxygenated quaternary carbon, four methines (two oxygenated), four methylenes, and two methyls. Apart from one carbonyl and two double bonds, the remaining degrees of unsaturation indicated that **2** possessed a tricyclic system. Detailed analysis of its ^1H NMR and ^{13}C NMR spectra showed that compound **2** was analogous to guignardone A (**5**) [10], except for the absence of a methylene (C-7), a quaternary carbon (C-6) in **5** and the presence of a methine (C-6) in **2**. In addition, the ^{13}C NMR signals of C-4 upshifted from δ_{C} 78.5 to δ_{C} 66.7 and C-5 from δ_{C} 44.0 to δ_{C} 36.9. Base on the above evidence, it was supposed that the C-7 of **5** was degraded and the corresponding ring was broken to form the structure of **2**, which was confirmed by 2D NMR experiments. The relative configuration of **2** was assigned by a ROESY experiment and comparison with **5**. H-9 (δ_{H} 1.95) showed correlations to both H₃-11 (δ_{H} 1.32) and H₂-16 (δ_{H} 4.64), which indicated that when H-9 and CH₃-11 possess α -orientation, H-14 (δ_{H} 2.27) will possess β -orientation, as in accordance with the relative configuration of **5** (Figure 3). The α -orientation of H-4 (δ_{H} 4.35) was also tentatively established as same as that of **5** for the biogenetic pathway consideration. H-6 (δ_{H} 4.48, dd, $J = 11.9, 5.4\text{ Hz}$) exhibited ROESY correlation with H-4, and the large vicinal coupling constant ($J = 11.9\text{ Hz}$) established the pseudoaxial position for H-6. So H-6 was established as α -oriented. Thus, the structure of **2** was established and named guignardone G.

Figure 3. Selected ROESY correlations of 2–4.



Guignardone H (**3**) was isolated as a colorless oil. The HRTOFMS of **3** showed a pseudo-molecular ion peak at m/z 293.1745 $[M + H]^+$ corresponding to the molecular formula $C_{17}H_{24}O_4$, implying six degrees of unsaturation. The 1H NMR and ^{13}C NMR spectra of **3** (Tables 1 and 2) were strikingly similar to those of **2**, with the only obvious difference being the presence of a methoxy (δ_H 3.47, 3H, s; δ_C 58.3) group in **3**. The HMBC correlations of the methoxy protons to C-6 (δ_C 79.1) and from H-6 (δ_H 3.70) to the methoxy carbon indicated that the methoxy group located at C-6. β -orientations of OH-4 and H-14 and α -orientations of H-9 and CH₃-11 were established by ROESY correlations and comparison with **2** and proved to be the same as **2**. There was no ROESY correlation between H-6 (δ_H 3.70, dd, $J = 6.8, 3.6$ Hz) and H-4 (δ_H 4.24), and the proton coupling constants between H-6 and H₂-5 (δ_H 2.35, 2.23) were 6.8 and 3.6 Hz, differed from those of H-6 in **2**. Hence, H-6 was deduced to be β -oriented. Thus, the structure of **3** was established as shown (Figure 1) and named guignardone H.

Table 2. 1H NMR data (400 MHz, $CDCl_3$) for **1–4**.

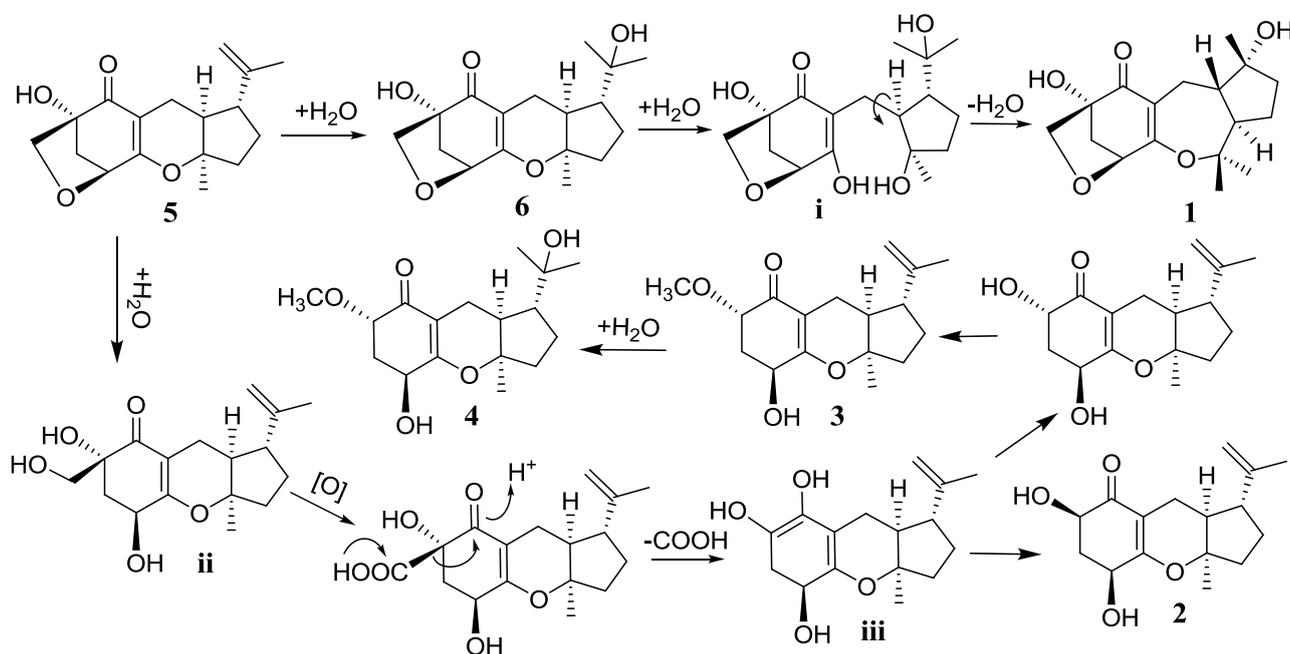
position	1	2	3	4
4	4.46, d (5.2)	4.35, br t (2.8)	4.24, br s	4.26, br s
5	2.31, m	2.53, dq (13.4, 5.4)	2.35, m	2.38, dt (13.6, 4.2)
	1.91, m	2.01, m	2.23, m	2.23, m
6		4.48, dd (11.9, 5.4)	3.70, dd (6.8, 3.6)	3.71, dd (7.0, 3.6)
7	3.80, d (8.0)			
	3.45, d (8.0)			
8	3.03, dd (15.0, 3.2)	2.32, m	2.33, d (17.3)	2.62, d (17.6)
	1.92, m	2.28, m	2.15, m	2.26, m
9	1.40, dd (12.0, 3.2)	1.95, m	1.94, m	2.04, m
11	1.35, s, 3H	1.32, s, 3H	1.33, s, 3H	1.33, s, 3H
12	1.76, m, 2H	2.10, m; 1.81, m	2.13, m; 1.79, m	2.01, m; 1.59, m
13	1.95, m; 1.22, m	1.94, m; 1.57, m	1.91, m; 1.53, m	1.79, m; 1.58, m
14	2.33, m	2.27, m	2.17, m	1.58, m
16	1.17, s, 3H	4.73, m	4.72, m	1.20, s, 3H
		4.64, m	4.62, m	
17	1.45, s, 3H	1.66, s, 3H	1.65, s, 3H	1.18, s, 3H
OCH ₃			3.47, s, 3H	3.47, s, 3H

Guignardone I (**4**) was obtained as a colorless oil. The molecular formula was deduced as $C_{17}H_{26}O_5$ on the basis of its HREIMS ion peak at m/z 310.1783 $[M]^+$ with five degrees of unsaturation. The 1H NMR and ^{13}C NMR data (Tables 1 and 2) of **4** closely resembled those of **3**, except for the disappearance of an exocyclic double bond and the presence of an oxygenated quaternary carbon (C-15, δ_C 72.9) and a methyl group (C-16, δ_C 28.6) in **4**. Therefore, it was supposed that compound **4** may derived from **3** by hydroxylation at the exocyclic double bond, which was confirmed by the HMBC correlations of H₃-16 (δ_H 1.20) with C-14 (δ_C 51.1), C-15 (δ_C 72.9), and C-17 (δ_C 27.5). The ROESY correlations and chemical shifts at chiral centers of **4** were similar to those of **3**, which showed the same relative configurations at C-4, C-6, C-9, C-10, and C-14.

A possible biogenetic pathway of compounds **1–6** was proposed as shown in Scheme 1. Guignardone A (**5**), a major product isolated in this experiment, was taken as the biogenetic precursor. Two ether bonds were suitable for the enzymatic opening that resulted in a 3,10-dihydroxy

intermediate (i) and a 4,7-dihydroxy intermediate (ii). The five-membered ring and the connected isopropanol group of intermediate **i** could turn upside-down around the bond C-8–C-9 to generate **1**. The intermediate **ii** underwent an oxidation and decarboxylation to give an intermediate (iii). Afterward, the intermediate (iii) converted to its C-1 ketone isomer. This assumption reasonably accounted for the simultaneous occurrences of β -orientation of OH-6 in **2** and α -orientation of OCH₃ in **3**.

Scheme 1. Plausible biosynthetic pathway of 1–6.



All compounds were tested for antibacterial activities against methicillin-resistant *Staphylococcus aureus* (MRSA) and *Staphylococcus aureus*. Guignardone I (**4**) exhibited inhibition zones of 9.0 and 11.0 mm in diameter (the diameter of sterile filter paper discs was 6 mm) toward MRSA and *S. aureus* at 65 μ M, respectively. Guignardone B (**6**) gave an inhibition zone of 8.0 mm against MRSA at 65 μ M. The other compounds have not show any antibacterial action under the same conditions. 10 μ L 0.08 mg mL⁻¹ kanamycin sulfate was used as positive control, for which the diameter of the inhibition zone was 30 mm.

3. Experimental Section

3.1. General Experimental Procedures

Optical rotations were taken on a Rudolph Autopol III. IR spectra were measured on a Nicolet 380 FT-IR instrument with KBr pellets. NMR spectra were recorded on a Bruker AM-400 spectrometer at 400 MHz for ¹H NMR and at 100 MHz for ¹³C NMR using TMS as an internal standard. MS spectra were obtained on a VG Auto Spec-3000 mass spectrometer (VG, Manchester, UK). Column chromatography was performed on silica gel (200–300 mesh; Qingdao Marine Chemical Inc., Qingdao, People's Republic of China). Sephadex LH-20 for chromatography was purchased from Merck (Germany). TLC was performed with silica gel GF254 (Marine Chemical Inc, Qingdao, China), and spots were visualized by heating silica gel plates sprayed with 10% H₂SO₄ in EtOH.

3.2. Fungal Material and Fermentation

The marine-derived fungus A1 was isolated from the leaves of mangrove plant *Scyphiphora hydrophyllacea* Gaertn. F. (Rubiaceae) (No. SH20081205), which were collected in Wenchang County in Hainan Province (China) in December 2008. The strain, which was identified as a *Guignardia* sp. based on the ITS sequences, was deposited in the Institute of Tropical Bioscience and Biotechnology, Chinese Academy of Tropical Agricultural Sciences, Haikou, China, and maintained on potato dextrose agar (PDA) slant at 4 °C. The marine-derived fungus A1 was grown on PDA at room temperature for 5 days. Three pieces of mycelial agar plugs (0.5 × 0.5 cm²) were inoculated into 1 L Erlenmeyer flasks containing 300 mL potato dextrose broth. The cultivation was shaken at 120 rpm at room temperature for 7 days, and then kept in still at room temperature for 45 days.

3.3. Extraction and Isolation

The culture broth (130 L) was filtered to give the filtrate and mycelia. The filtrate was evaporated *in vacuo* to small volume and then partitioned in succession between H₂O and petroleum ether, EtOAc, *n*-Butanol. The EtOAc solution was evaporated under reduced pressure to give a crude extract (10.5 g), which was separated into 11 fractions (Fr.1–Fr.11) on a silica-gel column using a step gradient elution of CHCl₃/MeOH (1:0→0:1). The Fr.1 (732 mg) was firstly subjected to Sephadex LH-20 (CHCl₃/MeOH, 1:1) CC, then subjected to silica gel CC (Pet/EtOAc 12:1, Pet/acetone 10:1) repeatedly to give **5** (80.6 mg) and **3** (12.5 mg). The Fr.3 (583 mg) was subjected to silica gel CC (Pet/Acetone 8:1 to 1:1, Pet/EtOAc 6:1, CHCl₃/EtOAc 6:1) repeatedly, and further purified by Sephadex LH-20 (CHCl₃/MeOH, 1:1) CC to give **1** (11.3 mg), **2** (8.5 mg), and **6** (6.2 mg). The Fr.4 (468 mg) was purified by silica gel CC (Pet/Acetone 6:1 to 1:1, Pet/EtOAc 4:1, CHCl₃/MeOH 40:1) repeatedly to give **4** (9.2 mg).

Guignardone F (**1**): Colorless needle crystals; $[\alpha]_D^{32}$ −42 (*c* 0.20, MeOH); IR (KBr) ν_{\max} 3463, 3285, 2973, 2942, 2877, 1657, 1606, 1455, 1375, 1232, 1186, 1131, 1092, 1021 cm^{−1}; ¹H and ¹³C NMR, see Tables 1 and 2; HREIMS *m/z* 308.1625 [M]⁺ (calcd for C₁₇H₂₄O₅, 308.1624).

Guignardone G (**2**): Colorless oil; $[\alpha]_D^{32}$ +3.5 (*c* 0.32, MeOH); IR (KBr) ν_{\max} 3440, 2955, 2925, 2854, 1653, 1622, 1458, 1378, 1167, 1099, 1059 cm^{−1}; ¹H and ¹³C NMR, see Tables 1 and 2; HREIMS *m/z* 278.1513 [M]⁺ (calcd for C₁₆H₂₂O₄, 278.1518).

Guignardone H (**3**): Colorless oil; $[\alpha]_D^{32}$ +8.9 (*c* 0.34, MeOH); IR (KBr) ν_{\max} 3439, 2927, 1658, 1616, 1442, 1394, 1378, 1168, 1077, 1019, 890 cm^{−1}; ¹H and ¹³C NMR, see Tables 1 and 2; HRTOFMS *m/z* 293.1745 [M + H]⁺ (calcd for C₁₇H₂₅O₄, 293.1752).

Guignardone I (**4**): Colorless oil; $[\alpha]_D^{32}$ −32 (*c* 0.24, MeOH); IR (KBr) ν_{\max} 3440, 2968, 2930, 1654, 1613, 1458, 1396, 1379, 1364, 1281, 1164, 1131, 1077, 1020, 887 cm^{−1}; ¹H and ¹³C NMR, see Tables 1 and 2; HREIMS *m/z* 310.1783 [M]⁺ (calcd for C₁₇H₂₆O₅, 310.1780).

Crystallographic data for Guignardone F (**1**): C₁₇H₂₄O₅; MW = 308.36; Monoclinic, space group C2; 9.7072 (17) Å, *b* = 9.7382 (16) Å, *c* = 16.752 (3) Å, $\alpha = \beta = \gamma = 90^\circ$, *V* = 1583.6 (5) Å³, *Z* = 4, *d* = 1.293 g/cm³, crystal dimensions 0.08 × 0.20 × 0.70 mm³.

3.4. Antibacterial Activity

Compounds **1–6** were tested for antibacterial activity against *S. aureus* and MRSA strains (obtained from the Food and Drug Administration of Hainan Province, Haikou, China) using the filter paper disc agar diffusion method [11]. The strains were cultured using nutrient agar. Fifty microliters (28 mM) of the compound were impregnated on sterile filter paper discs (6-mm diameter), and then, aseptically applied to the surface of the agar plates. Ten microliters (0.08 mg/mL) of kanamycin sulfate were used as positive control. Then the diameters of inhibition zones were measured after 24 h incubation at room temperature. Experiments were done in triplicate, and the results presented as mean values of the three measurements.

4. Conclusions

Four new meroterpenes, guignardones F–I (**1–4**), together with two known compounds guignardones A (**5**) and B (**6**), were isolated from the endophytic fungus A1 *Guignardia* sp. of mangrove plant *Scyphiphora hydrophyllacea*. The plane structure of guignardone G was same as coibanol A, one of the three tricyclic meroterpenes isolated from an endophytic fungus *Pycnoporus sanguineus*. The H-9 and CH₃-11 possess α -orientations in compounds **1–6**, different from the β -orientation in coibanols A–C [12]. The plane structure of guignardone H was same as tricycloalternarene F, another tricyclic meroterpene isolated from an endophytic fungus *Guignardia mangiferae*, the stereochemistry of which has not been clarified [13]. Guignardones A (**5**) and B (**6**) with an additional tetrahydrofuran ring in their structure have been isolated from a fungus *Guignardia mangiferae* associated with normal *Ilex cornuta* leaves [10]. Especially, compound **1** possessed a novel structure, beside an additional tetrahydrofuran ring, the six-membered ring possessing an oxygen atom was changed to a seven-membered ring. Antibacterial tests demonstrated that guignardone I (**4**) showed modest inhibitory effects on *Staphylococcus aureus* and MRSA, diameters of inhibition zones of which were 9.0 and 11.0 mm, respectively. Guignardone B (**6**) exhibited weak antibacterial activity against MRSA with a diameter of inhibition zones of 8.0 mm.

Acknowledgments

This work was supported by National Natural Science Foundation of China (No.30560018, No.20862020), and National Non-profit Institute Research Grant of ITBB (ITBBZD0841). The authors also thank the members of the analytical group of the State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, for the spectral measurements.

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Samples Availability: Available from the authors.

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