Phyllomeroterpenoids A–C, Multi-biosynthetic Pathway Derived Meroterpenoids from the TCM Endophytic Fungus *Phyllosticta* sp. and their Antimicrobial Activities

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Phyllomeroterpenoids A–C (1–3), multi-biosynthetic pathway derived meroterpenoids from amino acid/pentose phosphate/terpenoid pathways, were isolated from the TCM endophytic fungus *Phyllosticta* sp. J13-2-12Y, together with six biosynthetically related compounds (4–9). All structures were determined by extensive spectroscopic analysis, chemical derivatization, and ECD experiments. A plausible biosynthetic pathway of 1–3 was proposed. In addition, the antimicrobial activities of all isolated compounds were evaluated against *Staphylococcus aureus* 209P (bacterium) and *Candida albicans* FIM709 (fungus).

Meroterpenoids, such as fumagillin¹, mycophenolic acid², avinosol³, merochlorin A⁴, cochlearol B⁵, and others, have received much attention from chemists and pharmacologists⁶ for their remarkable structural diversity and varied biological activities. In general, meroterpenoids originate from a dual-biosynthetic pathway⁶,⁷. This is composed of a non-terpenoid pathway and a terpenoid pathway, such as the polyketide/terpenoid and shikimate/terpenoid pathways.

*Acorus tatarinowii* is a common and important medicinal plant, and its dried rhizomes have a long history of being used as traditional Chinese medicine (TCM) as Shi Chang Pu (*Acori Tatarinowii Rhizoma*) to treat many diseases, such as nervous ailments, dysentery, bronchitis, intermittent fevers etc ⁸. *A. tatarinowii* is rich with asarones, which show antimicrobial activity⁹,¹⁰. The micro-environment of *A. tatarinowii* is special due to the existence of abundant antimicrobial asarones, and the microorganisms living in this habitat should be distinctive. During our recent search for bioactive compounds from microorganisms¹¹–¹⁴, chemical investigation on a TCM endophytic fungal strain of *Phyllosticta* sp. J13-2-12Y from the leaves of *A. tatarinowii* was carried out. Through this investigation, three unusual meroterpenoids, phyllomeroterpenoids A–C (1–3), were isolated, along with six biosynthetically related compounds (4–9) (Fig. 1). Phyllomeroterpenoids A–C (1–3) are multi-biosynthetic pathway derived meroterpenoids, whose structures are composed of one guignardianone unit from the amino acid pathway, one C7 unit from the pentose phosphate pathway, and one monoterpene unit from the terpenoid pathway. The guignardianone unit is the skeleton of the guignardianone derivative, while the C7 unit and the monoterpene unit compose the guignardone-type meroterpenoids. In this study, we report the isolation and structural elucidation of 1–3 as well as their antimicrobial activities. In addition, a plausible biogenetic pathway of 1–3 is proposed.
Results

The known compounds, including three guignardianones (4–6) and three guignardone-type meroterpenoids (7–9) were identified as (S, Z)-guignardianone C (4)\(^{15,16}\), (S, Z)-botryosphaerinc B (5)\(^17\), (S, Z)-phenguignardic acid methyl ester (6)\(^{15,16}\), (4S, 6R, 9S, 10R, 14R)−17-hydroxylated guignardone A (7)\(^{18}\), (4S, 6R, 9S, 10R, 14R)-guignardone B (8)\(^{18}\), and (4S, 6R, 9S, 10S, 12S, 14R)−12-hydroxylated guignardone A (9)\(^{18}\) by comparisons of their NMR (recorded in CDCl\(_3\)) and ECD data with references. In addition, the NMR data of (4) were reported for the first time.

Phyllomeroterpenoid A (1) was obtained as a yellowish oil. The positive ion at \(m/z\) 551.2277 [M + H]\(^+\) (calcd. for C\(_{31}\)H\(_{30}\)O\(_5\)), indicated the molecular formula of C\(_{31}\)H\(_{30}\)O\(_5\) (index of hydrogen deficiency = 15). In the \(^1\)H NMR spectrum of 1, the characteristic signals of five aromatic protons [\(\delta\)\(_H\) 7.67 (2H), 7.41 (2H), 7.35 (1H)], three olefinic protons [\(\delta\)\(_H\) 6.50 (1H, s), 5.11 (1H, br s), 4.91 (1H, br s)], one O-methylene [\(\delta\)\(_H\) 4.54 (1H, d, \(J = 5.5\) Hz)], two O-methylene [\(\delta\)\(_H\) 4.64 (2H, br s), 3.79 (1H, d, \(J = 7.9\) Hz), 3.47 (1H, d, \(J = 7.9\) Hz)], and three methyls [\(\delta\)\(_H\) 1.29 (3H, s), 1.07 (6H, d, \(J = 6.9\) Hz)] were observed. Among them, the five aromatic protons indicated the existence of a mono-substituted benzene ring moiety in 1. Combined with the DEPT-135 spectrum, 31 signals were observed in the \(^1\)C NMR spectrum, which can be assigned to eight sp\(^3\) quaternary carbons (including one ketone carbonyl and two ester carbons), six sp\(^2\) methine carbons, one sp\(^2\) methylene carbon, three sp\(^2\) O- quaternary carbons, four sp\(^3\) methine carbons (including one O-methine carbon), six sp\(^3\) methylene carbons (including two O-methylene carbons), and three methyl carbons. Based on the analysis of \(^1\)H−\(^1\)H COSY experiment, four subunits (C-4—C-5, C-8—C-9—C-14—C-13—C-12, C-5′—C-6′—C-7′—C-8′—C-9′, and C-13′—C-12′—C-14′) were revealed as shown in Fig. 2. Combined with the analysis of \(^1\)H−\(^1\)H COSY, the HMBC correlations (Fig. 2) from H-4 to C-2/C-3/C-6/C-7, from Ha-5/Hb-5 to C-1/C-3/C-6/C-7, from Ha-7/Hb-7 to C-1/C-4/C-5/C-6, from Ha-8/Hb-8 to C-1/C-2/C-3/C-10/C-14, from H-9 to C-10, from H-11 to C-9/C-10/C-12, from Ha-16/Hb-16 to C-14/C-15/C-17, and from H-17 to C-14/C-15/C-16 revealed a guignardone-type meroterpenoid moiety in 1. In addition, the HMBC correlations (Fig. 2) from H-3′ to C-1′/C-2′/C-5′/C-9′, from H-5′/H-9′ to C-3′, from H-6′/H-8′ to C-4′, from H-7′ to C-5′/C-9′, from H-12′ to C-10′/C-11′, from H-13′ to C-11′/C-12′/C-14′, and from H-14′ to C-11′/C-12′/C-13′ revealed a guignardianone moiety in 1, combined with a comparison of NMR data with (S, Z)-guignardianone C (4)\(^{15,16}\) and the above analysis of \(^1\)H−\(^1\)H COSY. Based on the molecular formula and the key HMBC correlation from H-17 to C-10′, these two moieties can be combined, and the planar structure was established as shown in Fig. 2. This is the ester of a guignardone-type meroterpenoid and a guignardianone, and the assignments of NMR data can be found in Table 1.

The key NOESY correlations (Table S1) between H-5′/H-9′ and H-13′/H-14′ indicated that the configuration of the double bond of Δ\(^8\) as Z. Furthermore, the \(^1\)C NMR data of guignardianone unit in 1 were quite similar to those of (S, Z)-guignardianone C (4)\(^{15,16}\), which confirmed the above deduction. In addition, the alkaline hydrolysis of 1 gave a major reaction product (1a) that was identified as (4S, 6R, 9S, 10R, 14R)−17-hydroxylated

Figure 1. Chemical structures of 1–9.
be the same as those in C-9 with MIC values of 4 g/mL. (bacterium) and R, 14, S1′, 12S described in S, the absolute configuration of 3 was determined as 4, 6′, 14′, 1′, 14, S1. According to the structure, the guignardone moiety in 3 was the sum of the experimental ECD data of the enantiomer of 3, which was the sum of the experimental ECD curve of 1 (Fig. 3), therefore we deduced that the configuration of C-11′ in 1 should be the same as that in 4. Thus, the absolute configuration of 1 was determined as 4S, 6R, 9S, 10R, 14R, 11′S.

Phyllomeroterpenoid B (2) was obtained as a yellowish oil. The positive ion at m/z 553.2455 [M + H]+ (calcd. for C28H34O8 553.2438) from HRESIMS indicated the molecular formula of C28H34O8 (index of hydrogen deficiency = 14). The detailed 2D NMR analysis (Table S2) and the comparison of NMR data with (S, Z)-guignardianone C (4) (Table S4) revealed that 2 was the ester of a guignardone-type meroterpenoid and a guignardianone unit. A precise comparison of 1D NMR data of 2 (Table 1) with (4S, 6R, 9S, 10R, 14R)-guignardone B (8) (Table S5) showed an obviously downfield shifted carbon at C-15, which suggested that the esterification was at C-15 in 2. Therefore, the planar structure of 2 was established as shown in Fig. 1. Combined with the carbon NMR data comparison with (S, Z)-guignardianone C (4) (Table S4), the key NOESY correlations (Table S2) between H-5′/H-9′ and H1-13′/H1-14′ revealed the configuration of the double bond of Δ2 as Z. With the same alkaline hydrolysis experiment (Figures S4–S6) and the comparison analysis of the simulated ECD with the experimental ECD data (Fig. 4) as described in 1, the absolute configuration of 2 was determined as 4S, 6R, 9S, 10R, 14R, 11′S.

Phyllomeroterpenoid C (3) was obtained as a yellowish oil, and its molecular formula was the same as that of 1 (C28H34O8) as determined by HRESIMS. Based on comparison of the NMR data with (S, Z)-guignardianone C (4)15,16 and detailed NMR analyses (Table S3), the planar structure of 3 was established as shown in Fig. 1, and the assignments of NMR data can be found in Table 1.

Combined with the carbon NMR data comparison with (S, Z)-guignardianone C (4)15,16, the key NOESY correlations (Fig. 5) between H-5′/H-9′ and H1-13′/H1-14′ indicated the configuration of the double bond of Δ2 as Z. In addition, the key NOESY correlations (Fig. 5) between H-11 and H-9/Hb-8, between Hb-13 and H-9, between Ha-8 and H-14, between H-12 and H-14, and between H-14 and Hb-7, and the coupling constants of JH12-Hb13 (2.2 Hz) and JH12,Ha13 (6.9 Hz) in 3 were the same as those in 9 (Table S6), indicating that the relative configuration of the guignardone moiety in 3 is 4S, 6R, 9S, 10S, 12S, 14R, which is the same as 9. Since 3 and 9 coexist in Phyllosticta sp. J13-2-12Y, the configurations of C-4, C-6, C-9, C-10, C-12, and C-14 in 3 should be the same as those in 9.

With the same comparison analysis of the simulated ECD with the experimental ECD data (Fig. 6) as described in 1, the absolute configuration of 3 was determined as 4S, 6R, 9S, 10S, 12S, 14R, 11′S.

The antimicrobial activities of the isolated compounds were evaluated against Staphylococcus aureus 209P (bacterium) and Candida albicans FIM709 (fungus). All compounds exhibited different antimicrobial activities (Table 2). Especially, displayed obvious antimicrobial activities against S. aureus 209P and C. albicans FIM709 with MIC values of 4 μg/mL.

Discussion
Guignardone-type meroterpenoids are a rare kind of meroterpenoids that are composed of one C7 unit and one monoterpenic unit derived from the pentose phosphate/terpenoid pathways26. Up to present, about 30 members18,21–26 with tricyclic or tetracyclic skeletons have been reported from Guignardia sp.,26 Pycnoporus sanguineus,27 and Aspergillus sp.,28 and they showed antifungal18, antibacterial23, cytotoxic25, and Toll-Like Receptor 3 regulating activities26. Guignardianones are a special kind of fungal-derived benzylidene dioxolanes derived from the amino acid pathway15,29,30, and they exhibit antifungal16 and antibacterial31 activities. Up to now, only 13 natural guignardianones have been reported from Guignardia sp.,15,16,18,29,31, Botryosphaeria sp.,17 and Aspergillus sp.30. On the basis of our chemical investigation, three known guignardianones (4–6), and three known guignardone-type meroterpenoids (7–9) were isolated from the TCM endophytic fungal strain of Phyllosticta sp. J13-2-12Y. In addition, unusual meroterpenoids (1–3), the heterozygotes of guignardianone and guignardone-type meroterpenoid were also obtained. Phyllomeroterpenoids A–C (1–3) are composed of one

![Figure 2. Key 1H-1H COSY, HMBC, and NOESY correlations of 1.](image-url)
guignardianone unit, one C7 unit, and one monoterpene unit, and they are multi-biosynthetic pathway derived meroterpenoids from the amino acid/pentose phosphate/terpenoid pathways. They could originate from phenylalanine30, 2-epi-5-epi-valiolone (EEV)33, and a monoterpenoid as shown in Fig. 7.

Materials and Methods

General Experimental Procedures. The detail of instruments applied in this work are provided in supporting information.

Fungal Material. The strain numbered as J13-2-12Y was isolated from the leaves of A. tatarinowii collected from Guangxi Medicinal Botanical Garden, Guangxi Province, People’s Republic of China. The details of isolation and identification can be found in supporting information.

Extraction and Isolation. The fermented material was extracted with EtOAc for three times, and the solvent was evaporated to dryness under vacuum to obtain a crude extract (42.8 g). Then the crude extract was separated by silica gel CC (4 × 15 cm) with a elution system of cyclohexane-MeOH (100:0 and 0:100, v/v) to yield a cyclohexane portion (C, 24.7 g) and a MeOH portion (W, 15.7 g). The MeOH portion (W, 15.7 g) was subjected to ODS CC (4 × 30 cm) eluting with MeOH–H2O (50:50, 70:30, 85:15, and 100:0, v/v) to yield 4 fractions (W1–W4). Fraction W2 (3.2 g) was further separated by MPLC on ODS CC (4 × 45 cm) with a elution system of MeOH-H2O.

| No. | δc (ppm) | δh (J in Hz) | δc (ppm) | δh (J in Hz) | δc (ppm) | δh (J in Hz) | δc (ppm) | δh (J in Hz) |
|-----|----------|-------------|----------|-------------|----------|-------------|----------|-------------|
| 1   | 198.6, C | 200.1, C    | 198.7, C | 104.0, C    | 198.7, C | 200.1, C    | 171.8, C | 104.0, C    |
| 2   | 4.54, d (5.5) | 79.9, CH | 4.52, d (5.5) | 78.0, CH | 4.53, d (5.5) | 78.0, CH | 4.53, d (5.5) |
| 3   | 4.00, CH2 | 2.45, dd (10.7, 5.5), Ha | 2.30, dd (10.7, 5.5), Ha | 43.9, CH2 | 2.45, Ha | 2.02, d (10.7), Hb | 2.07, d (10.7), Hb | 2.00, d (10.7), Hb |
| 4   | 81.7, C | 83.3, C    | 81.9, C | 81.9, C    | 81.9, C | 81.9, C    | 81.9, C | 81.9, C    |
| 5   | 70.5, CH2 | 3.79, d (7.9), Ha | 72.3, CH2 | 3.70, d (7.9), Ha | 70.5, CH2 | 3.80, d (7.9), Ha | 3.47, d (7.9), Hb | 3.49, d (7.9), Hb | 3.45, d (7.9), Hb |
| 6   | 15.5, CH2 | 2.33, br d (17.1), Ha | 18.7, CH2 | 2.55, dd (17.1, 1.3), Ha | 15.0, CH2 | 2.29, Ha | 2.21, Hb | 2.21, dd (17.1, 5.9), Hb | 2.04, Hb |
| 7   | 43.5, CH | 1.99 | 42.3, CH | 2.11, ddd (10.0, 5.9, 1.3) | 40.7, CH | 2.06 | 88.8, C | 91.7, C | 88.7, C |
| 8   | 23.0, CH3 | 1.29, s | 22.9, CH3 | 1.28, s | 18.4, CH3 | 1.14, s |
| 9   | 37.0, CH2 | 2.07, ddd (14.1, 9.2, 4.0), Ha | 38.6, CH2 | 1.89, ddd (13.1, 7.1, 1.0), Ha | 80.8, CH | 5.23, ddd (6.8, 2.1, 1) | 1.77, ddd (14.1, 11.7, 6.2), Hb | 1.67, Hb |
| 10  | 27.4, CH2 | 1.99, Ha | 25.3, CH2 | 1.79, Ha | 35.2, CH2 | 2.54, ddd (15.3, 9.8, 6.9), Ha | 1.51, Hb | 1.55, Hb | 1.51, ddd (15.3, 8.1, 2.2), Hb |
| 11  | 45.3, CH | 2.21 | 51.1, CH | 1.93, td (10.0, 4.8) | 47.1, CH | 2.16 | 142.7, C | 90.3, C | 143.3, C |
| 12  | 114.9, CH2 | 5.11, br s, Ha | 24.3, CH2 | 1.51, s | 113.0, CH2 | 4.75, br s, Ha | 4.91, br s, Hb | 4.59, br s, Hb |
| 13  | 67.4, CH3 | 4.64, br s | 24.2, CH2 | 1.50, s | 18.5, CH2 | 1.60, br s |
| 14  | 162.7, C | 164.2, C | 162.7, C |
| 15  | 135.6, C | 137.3, C | 135.6, C |
| 16  | 109.8, CH | 6.50, s | 110.2, CH | 6.52, s | 110.0, CH | 6.53, s |
| 17  | 132.1, C | 133.7, C | 132.0, C |
| 18  | 129.9, CH | 7.67 | 130.9, CH | 7.70 | 129.8, CH | 7.65 |
| 19  | 128.8, CH | 7.41 | 129.9, CH | 7.41 | 128.9, CH | 7.40 |
| 20  | 129.1, CH | 7.35 | 130.3, CH | 7.35 | 129.4, CH | 7.36 |
| 21  | 165.0, C | 165.6, C | 165.4, C |
| 22  | 108.4, C | 109.9, C | 108.3, C |
| 23  | 32.9, CH | 2.67, sept (6.9) | 33.8, CH | 2.61, sept (6.9) | 32.5, CH | 2.71, sept (6.9) |
| 24  | 15.2, CH3 | 1.07, d (6.9) | 15.6, CH3 | 1.06, d (6.9) | 15.2, CH3 | 1.11, d (6.9) |
| 25  | 14.5, CH3 | 1.07, d (6.9) | 14.8, CH3 | 1.04, d (6.9) | 14.6, CH3 | 1.11, d (6.9) |

Table 1. NMR data of 1−3 (δ in ppm, J in Hz). *The data recorded in CDCl3 (600 MHz for 1H and 150 MHz for 13C). **The data recorded in CD3OD (600 MHz for 1H and 150 MHz for 13C). *Indiscernible signals from overlap or complex multiplicity are reported without designating multiplicity. "The assignment maybe exchanged in each group."
Figure 3. The experimental ECD spectra of 1, 4, and 7 and the simulated ECD spectrum of 1 (the sum of 4 and 7).

Figure 4. The experimental ECD spectra of 2, 4, and 8 and the simulated ECD spectrum of 2 (the sum of 4 and 8).

Figure 5. Key NOESY correlations of 3.
W1-2 (0.7 g) was subjected to silica gel CC with an elution system of cyclohexane-EtOAc (100:0 to 0:100, v/v) to obtain 4 fractions (W1-2-1–W1-2-4). 8 (tR: 18.8 min, 44.0 mg) was isolated from fraction W1-2-3 (122.0 mg) by preparative HPLC using MeCN–H2O (25:75, v/v) at 3 mL/min to yield. 7 (tR: 14.7 min, 4.0 mg) and 9 (tR: 15.5 min, 5.0 mg) were isolated from fraction W1-2-2 (87.0 mg) by preparative HPLC using MeCN–H2O (28:72, v/v) at 3 mL/min.

Spectroscopic data of 1–3.

Phyllomeroterpenoid A (1): yellowish oil; [α]27 D –38.3 (c 0.10, MeOH); UV (MeOH) λmax (log ε) 204 (3.67), 222 (3.44), 269 (3.66), 295 (3.68), 308 (3.60); IR (KBr) νmax 3441, 2938, 1796, 1755, 1658, 1616, 1450, 1364, 1256, 1178, 1029, 977, 758, 689 cm⁻¹; ECD λnm (Δε) (c 0.9 × 10⁻⁴ mol/L, MeOH) 222 (+9.53), 255 (+12.77), 292 (–14.64) nm; ESI-MS (positive): m/z 1123 [2M+Na]+, 573 [M+Na]+; HRESIMS (positive): m/z 551.2277 [M+H]+ (calcd. for C31H35O9, 551.2281).

Phyllomeroterpenoid B (2): yellowish oil; [α]27 D –18.7 (c 0.10, MeOH). UV (MeOH) λmax (log ε) 204 (3.67), 222 (3.46), 267 (3.66), 295 (3.61), 309 (3.55); IR (KBr) νmax 3447, 2979, 2935, 1799, 1746, 1655, 1619, 1450, 1370, 1299, 1249, 1181, 1124, 1036, 977, 758, 693 cm⁻¹; ECD λnm (Δε) (c 0.9 × 10⁻⁴ mol/L, MeOH) 221 (+10.02), 258 (+11.33), 295 (–9.88) nm; ESI-MS (positive): m/z 575 [M+Na]+, 553 [M+H]+; HRESIMS (positive): m/z 553.2455 [M+H]+ (calcd. for C31H37O9, 553.2438).

Phyllomeroterpenoid C (3): yellowish oil; [α]27 D –45.0 (c 0.10, MeOH). UV (MeOH) λmax (log ε) 204 (3.67), 222 (3.46), 267 (3.66), 295 (3.61), 309 (3.55); IR (KBr) νmax 3447, 2979, 2935, 1799, 1746, 1655, 1619, 1450, 1370, 1299, 1249, 1181, 1124, 1036, 977, 758, 693 cm⁻¹; ECD λnm (Δε) (c 0.9 × 10⁻⁴ mol/L, MeOH) 221 (+14.16), 260 (+24.27), 293 (–18.84) nm; ESI-MS (positive): m/z 1123 [2M+Na]+, 573 [M+Na]+; HRESIMS (positive): m/z 551.2285 [M+H]+ (calcd. for C31H35O9, 551.2281).

Alkaline hydrolysis of 1 and 2. A sample of 1 (1 mg) was treated with 2 N KOH (200 μL), THF (200 μL), and CH3OH (200 μL), and stirred at 25 °C for 4 h. After neutralizing with 10% HCOOH and extracting with EtOAc, the EtOAc layer was evaporated to dryness and dissolved in MeOH. Then, 1a (0.4 mg) was isolated from the mixture by analytical HPLC.
Figure 7. Plausible biosynthetic pathway of compounds 1–3.

(Phenomenex Gemini C18 column, 5μm, 4.6 × 250 mm) with MeOH-H2O (67:33, v/v) at 1 mL/min, and its 1H NMR spectrum and ECD spectrum were identical with those of 8 (Figures S5 and S6).

Antimicrobial Assay. The antimicrobial activities against S. aureus 209P and C. albicans FIM709 were measured in sterile 96-well plates using the broth microdilution method4,13, and the detail can be found in supporting information.

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Author Contributions

Prof. Dr. Hao Gao and Xin-Sheng Yao initiated the project. Prof. Dr. Hao Gao designed and coordinated the project. Mr Heng-Gang Yang, Dr. Huan Zhao, and Miss Shao-Meng Chen performed the extraction, isolation, and structural identification of the compounds. Dr. Guo-Dong Chen and Mr Jian Zou performed the quantum chemical calculation. Dr. Sheng-Ying Qin performed the paper antimicrobial assay. Dr. Chuan-Xi Wang and Miss Jiao-Jiao Li performed the isolation of fungus from *A. tatarinowii*. Dr. Dan Hu conducted the sequence analysis of the fungal strain (No. J13–2–12Y). Dr. Guo-Dong Chen and Miss Lang-Ming Mou performed the fermentation of the fungal strain (No. J13–2–12Y). Prof. Dr. Hao Gao and Dr. Sheng-Ying Qin wrote this paper. All authors approved the final version of the manuscript.

Additional Information

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