Dihydroisocoumarins from the Mangrove-Derived Fungus *Penicillium citrinum*

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Abstract: Three new dihydroisocoumarin penicimarins G–I (1–3), together with one known dihydroisocoumarin (4) and three known meroterpenoids (5–7), were obtained from a fungus *Penicillium citrinum* isolated from the mangrove *Bruguiera sexangula var. rhynchopetala* collected in the South China Sea. Their structures were elucidated by the detailed analysis of spectroscopic data. The absolute configuration of 1 was determined by the X-ray diffraction analysis using Cu Kα radiation. The absolute configurations of 2 and 3 were determined by comparison of their circular dichroism (CD) spectra with the literature. All compounds were evaluated for their antibacterial activities and cytotoxic activities.

Keywords: *Penicillium citrinum*; metabolites; dihydroisocoumarin; antibacterial activity; cytotoxic activity

1. Introduction

Marine-derived fungi have attracted increasing attention from those seeking new pharmaceutically useful natural products in recent years [1]. In particular, secondary metabolites isolated from the marine-derived fungi in the genus *Penicillium* can produce various bioactive metabolites, such as antifouling polyketides [2], antibacterial communal A [3], cytotoxic penicinoline [4], antioxidant terrestrols [5], and antileukemic sorbicillactone A [6]. These results have drawn the attention of both pharmaceutical and natural product chemists. In our search for new bioactive natural products from marine fungi in the South China Sea, we have found several bioactive compounds, including anthraquinone derivatives, cytochalasins, lumazine peptides, benzopyrans, stemphol sulfates, triterpenes, and a-pyrone derivatives [7–13]. Our previous investigation on the endophytic fungus *Penicillium citrinum* has resulted in the discovery of one new benzopyran derivative (2R*,4R*)-3,4-dihydro-5-methoxy-2-methyl-2H-1-benzopyran-4-ol [10]. Further, chemical investigation of the fermentation broth of the *Penicillium citrinum* resulted in the isolation of three new dihydroisocoumarin penicimarins (G–I (1–3)), together with one known dihydroisocoumarin (aspergillumarin A (4)) [14], and three known meroterpenoids (dehydroaustin (5) [15], 11β-acetoxyisoaustinone (6) [15], and austinol (7) [16]) (Figure 1). All isolated metabolites (1–7) were evaluated for their antibacterial and cytotoxic activities. Herein we report the isolation, structure elucidation, and biological activities of these compounds.
2. Results and Discussion

Compound 1 was obtained as colorless crystals. Its molecular formula of C_{15}H_{20}O_{5} (six degrees of unsaturation) was determined by HR-ESI–MS and combined with ^1H and ^13C NMR spectroscopic data. In the ^1H NMR spectrum (Table 1), the signals and the coupling constants at δ^H 7.05 (d, J = 8.4 Hz) and 6.90 (d, J = 8.4 Hz) indicated the presence of a 1,2,3,4-tetrasubstituted benzene system. Two oxygenated methine proton signals at δ^H 4.39 (m) and 3.75 (m), one methoxyl group at δ^H 3.82 (s), one methyl group at δ^H 1.17 (d, J = 6.2 Hz), and four methylene groups at δ^H 3.16 (dd, J = 16.8, 3.0 Hz) and 2.58 (dd, J = 16.8, 11.6 Hz)), (1.84 (m) and 1.70 (m)), 1.59 (m) and 1.55 (m) were also observed. The ^1H NMR spectra indicated that this compound included four methylenes, two methines, one methoxy, and one methyl. The ^13C NMR spectrum revealed the signals for six aromatic carbons and one carbonyl carbon, including one lactone carbonyl carbon at δ_C 165.8. Thus, these spectroscopic features suggested that 1 was very similar to penicimarin B [17]. The obvious difference in the ^1H NMR spectrum was the absence of one aromatic proton signal at δ^H 6.79 (d, J = 7.2 Hz, H-5) in 1. Furthermore, in the ^13C NMR spectra, the C-5 signal moved downfield significantly [δ_C 148.2 (C) in 1 vs 119.3 (CH)] in penicimarin B, indicating an aromatic proton is replaced by a hydroxyl group. The ^1H,^1H-COSY, HMQC, and HMBC spectra allowed the complete assignment for 1 (Figure 2). The relative configuration of 1 could, however, not be confirmed unambiguously. Crystallization of 1 from MeOH afforded colorless crystals, which gave an X-ray crystal structure with a Flack parameter of 0.0 (3) (Figure 3). The absolute configuration of C-3 was also determined by circular dichroism (CD) spectroscopy (Figure 4). The negative circular dichroism at 259 nm (Figure 4) suggested the R configuration at C-3, by comparison with data for dihydroisocoumarins described in the literature [18]. Thus, compound 1 was named as penicimarin G, and the absolute configuration of 1 was defined as (3R, 4′R).

![Figure 1. Structures of compounds 1–7.](image-url)
| Position | 1 \(^a\) | 2 \(^a\) | 3 \(^b\) |
|----------|---------|---------|---------|
| 3\(’\)   | 39.8, CH\(_3\) | 1.55 (m) | 43.6, CH\(_3\) | 2.58 (m) | 173.2 |
| 4\(’\)   | 68.3, CH | 3.75 (m) | 211.4, CH | 51.8, CH\(_3\) | 3.70 (s) |
| 5\(’\)   | 22.3, CH\(_3\) | 1.17 (d, 6.2) | 29.8, CH\(_3\) | 2.15 (s) |
| 8-OMe    | 56.7, CH\(_3\) | 3.82 (s) | 56.7, CH\(_3\) | 3.81 (s) |
| 8-OH     | 2.15 (s) | 2.58 (m) | 173.2 |

\(^a\) Record in Methanol-\(d\(_4\)\); \(^b\) Record in CDCl\(_3\).

Figure 2. \(^1\)H–\(^1\)H COSY correlations and key HMBC correlations of Compounds 1–3.

Figure 3. X-ray structure of Compound 1.

Figure 4. Circular dichroism (CD) spectra of Compounds 1–4.
Compound 2 was isolated as colorless crystals with the molecular formula assigned as C_{13}H_{18}O_{5} (seven degrees of unsaturation) on the basis of its HR-ESI–MS and combined with ^{1}H and ^{13}C NMR spectroscopic data. The 1D NMR data of 2 was very similar with that of 1, except for the presence of a carbonyl signal (δ_{C} 211.4) for C-4′ in 2 and the absence of one oxygenated methine proton signal (δ_{H} 68.3) for C-4′ in 1. This was corroborated by the HMBC correlations from H-3′ and H-5′ to C-4′ (Figure 2). The ^{1}H–^{1}H-COSY, HMQC, and HMBC spectra allowed the complete assignment for 1. Thus, compound 2 was named as penicimarin H, and the absolute configuration of C-3 was also determined to be R by CD spectra (Figure 4) [18].

Compound 3 was obtained as a colorless oil. Its molecular formula of C_{13}H_{14}O_{5} (seven degrees of unsaturation) was determined by HR-ESI–MS and combined with ^{1}H and ^{13}C NMR spectroscopic data. In the ^{1}H NMR spectrum (Table 1), the proton signals and the coupling constants at δ_{H} 7.42 (dd, J = 8.0, 7.6 Hz), 6.89 (d, J = 8.0 Hz), and 6.70 (d, J = 7.6 Hz) indicated the presence of an 1,2,3-trisubstituted benzene system. One hydrogen-bonded hydroxyl group at δ_{H} 10.9 (s), one oxygenated methine proton signal at δ_{H} 4.65 (m), one methoxyl group at δ_{H} 3.70 (s), and three methylene groups at δ_{H} 2.92 (m), 2.62 (m), and 2.13 (m) were also observed. The ^{1}H NMR and ^{13}C NMR spectra included three methylenes, one methine, and one methoxy. The ^{13}C NMR data exhibited the presence of six aromatic carbons and two carbonyl carbons, including two lactone carbonyl carbons at δ_{C} 173.2 (C) and 169.7 (C). Thus, these spectroscopic features suggested that 3 was very similar to aspergillumarin A (4) [14]. The obvious differences in the ^{1}H NMR spectra were the absence of one methylene signal at δ_{H} 1.76 (m), a singlet methyl signal at δ_{H} 2.16 (s), and the presence of a methoxy signal at δ_{H} 3.70 (s) in 3. The position of this methoxy group was confirmed by the HMBC correlation from 4′-OMe to C-3′ (Figure 1). The ^{1}H,^{1}H-COSY, HMQC, and HMBC spectra allowed the complete assignment for 3. Thus, compound 3 was named as penicimarin I, and the absolute configuration of C-3 was also determined to be R by CD spectra (Figure 4) [18].

By comparing physical and spectroscopic data with literature values, the four known compounds were identified as aspergillumarin A (4) [14] and three meroterpenoids: dehydroaustin (5) [15], 11β-acetoxyisoaustinone (6) [15], and austinol (7) [16]. The absolute configuration of C-3 in compound 4 was determined to be R by comparison with the CD spectrum and the optical rotation data of those reported in the literature [14,18].

More detailed spectra of new compounds are available in the Supplementary Materials (Figures S1–S18).

All the isolated compounds were evaluated for their antibacterial activities against five terrestrial pathogenic bacteria and two marine pathogenic bacteria. Compounds 1, 2, and 5–7 exhibited selective antibacterial activity (Table 2). Compounds 2 and 7 showed moderate activity against Staphylococcus epidermidis and S. aureus with the same minimum inhibitory concentration (MIC) values of 10 µM. Compounds 1 and 2 showed a broad spectrum of antibacterial activity against the five pathogenic bacteria S. epidermidis, S. aureus, Escherichia coli, Bacillus cereus, and Vibrio alginolyticus. All compounds were also tested for cytotoxic activity against HeLa, MCF-7, and A549 cells, however, these compounds showed no cytotoxic activity (IC_{50} > 50 µM).

Table 2. Antibacterial activity for Compounds 1, 2 and 5–7.

| Compound | Minimum Inhibitory Concentration (MIC, µM) |
|----------|-------------------------------------------|
|          | S. epidermidis | S. aureus | E. coli | B. cereus | V. alginolyticus |
| 1        | 20            | 20        | 20      | 20        | 20               |
| 2        | 10            | 20        | >20     | >20       | >20              |
| 5        | 20            | >20       | >20     | >20       | >20              |
| 6        | 20            | >20       | >20     | >20       | >20              |
| 7        | 10            | >20       | >20     | 20        | >20              |
| Ciprofloxacin | 0.30        | 0.30      | 0.60    | 1.20      | 1.25             |

*a* Ciprofloxacin was used as a positive control.
3. Materials and Methods

3.1. General Experimental Procedures

Optical rotations were measured on a JASCO P-1020 digital polarimeter. IR spectra were recorded on a Thermo Nicolet 6700 (using KBr disks) spectrophotometer (Thermo Scientific, Madison, WI, USA). Both 1D and 2D NMR spectra were measured on a Bruker AV-400 (Bruker Corporation, Switzerland) instrument with TMS as the internal standard. HR-ESI–MS spectra were obtained on the Bruker Daltonics Apex-Ultra 7.0 T (Bruker Corporation, Billerica, MA, USA) and the Q-TOF Ultima Global GAA076 LC mass spectrometer. Single-crystal data: Agilent Gemini Ultra X-ray single crystal diffractometer (Cu Kα radiation). CD spectra were recorded on a MOS-450 spectrometer. Prep. HPLC were used for Agilent 1260 prep-HPLC system with an Agilent C18 analytical HPLC column (4.6 × 250 mm, 5 µm) and semi-preparative column (9.4 × 250 mm, 7 µm). Sephadex LH-20 (Pharmacia Co. Ltd, Sandwich, UK) and Silica gel (200–300 mesh, 300–400 mesh Qingdao Marine Chemical Factory, Qingdao, China) were used for column chromatography (CC). All solvents were purchased from Xilong Chemical Reagent Factory (Guangzhou, China).

3.2. Fungal Materials

The fungal strain *Penicillium citrinum* HL-5126 was isolated from the mangrove *Bruguiera sexangula* var. *rhynchopetala* collected in the South China Sea in August, 2012. The strain was deposited in the Key Laboratory of Tropical Medicinal Plant Chemistry of Ministry of Education, College of Chemistry and Chemical Engineering, Hainan Normal University, Haikou, China, with the access code HL-5126. The fungal strain was cultivated in 20 L potato glucose liquid medium (15 g of glucose and 30 g of sea salt in 1 L of potato infusion, in 1 L Erlenmeyer flasks each containing 300 mL of culture broth) at 25 °C without shaking for 4 weeks.

3.3. Identification of Fungus

The fungus was identified according to its morphological characteristics and by comparison of the ITS sequence amplification, primer pair ITS1F and ITS4 and sequencing of the internal transcribed spacer (ITS) region. The sequence data has been submitted to GenBank, with an accession number KJ466981, and the fungal strain was identified as *Penicillium citrinum*.

3.4. Extraction and Isolation

The fungal cultures were filtered through cheesecloth, and the filtrate was extracted with EtOAc (3 × 20 L, 24 h each). The organic extracts were concentrated in vacuo to yield an oily residue (10.2 g), which was subjected to silica gel column chromatography (CC) (petroleum ether, EtOAc v/v, gradient 100:0–0:100) to generate six fractions (Fr. 1–Fr. 5). Fr. 3 was isolated by CC on silica gel eluted with petroleum ether–EtOAc (v/v, gradient 100:10–100:100), and then subjected to Sephadex LH-20 CC eluting with mixtures of Petroleum ether:CHCl₃:MeOH (2:1:1), and further purified by using HPLC on an ODS semi-preparative column (Agilent C18, 9.4 × 250 mm, 7 µm, 2 mL/min) eluted with 80% MeOH/H₂O to obtain compound 1 (5.0 mg). Fr. 4 was isolated by CC on silica gel eluted with petroleum ether–EtOAc (v/v, gradient 100:10–10:100), and then subjected to Sephadex LH-20 CC eluting with mixtures of Petroleum ether:CHCl₃:MeOH (1:1) to obtain Fr. 4.2 (400.0 mg), Fr. 4.3 (106.0 mg). Fr. 4.2 was subjected to repeated Sephadex LH-20 CC (CHCl₃/MeOH, v/v, 1:1) and further purified by using HPLC on an ODS semi-preparative column (Agilent C18, 9.4 × 250 mm, 7 µm, 2 mL/min) eluted with 70% MeOH/H₂O to obtain compounds 4 (6.0 mg) 5 (8.0 mg), 6 (10.0 mg), and 7 (7.0 mg). Fr. 4.3 was subjected to repeated Sephadex LH-20 CC (MeOH) and further purified by using HPLC on an ODS semi-preparative column (Agilent C18, 9.4 × 250 mm, 7 µm, 2 mL/min) eluted with 60% MeOH/H₂O to obtain compounds 2 (5.0 mg) and 3 (6.2 mg).
Penicimarin G (1): Colorless crystals. [α]D24 +24.6 (c = 0.25, MeOH). mp 136.9–137.6 °C. λmax (log ε) 220 (3.02), 246 (2.50), 312 (1.62) nm; CD (5.2 × 10⁻⁴ mol/L, MeOH) λmax (Δε) 239 (+2.00), 257 (−8.60), 290 (−1.52), 329 (−3.10); IR (KBr) νmax 1380, 1318, 1272, 1250, 1230, 1112, 1082, 1064, 798 cm⁻¹; 1H and 13C NMR see Table 1; HRESIMS m/z 281.1383 [M + H]+ (calcd. for C15H21O5, 281.1383).

Penicimarin H (2): Colorless crystals. [α]D24 +24.6 (c = 0.25, MeOH). mp 136.9–137.6 °C. λmax (log ε) 220 (3.02), 246 (2.50), 312 (1.62) nm; CD (5.2 × 10⁻⁴ mol/L, MeOH) λmax (Δε) 239 (+2.00), 257 (−8.60), 290 (−1.52), 329 (−3.10); IR (KBr) νmax 1380, 1318, 1272, 1250, 1230, 1112, 1082, 1064, 798 cm⁻¹; 1H and 13C NMR see Table 1; HRESIMS m/z 579.2200 [2M + Na]+ (calcd. for C30H36O10Na, 579.2200).

Penicimarin I (3): Colorless crystals. [α]D24 −12.0 (c = 0.25, MeOH). λmax (log ε) 220 (3.02), 246 (2.50), 312 (1.62) nm; CD (4.0 × 10⁻⁴ mol/L, MeOH) λmax (Δε) 240 (+9.60), 260 (−2.65), 328 (−0.16); IR (KBr) νmax 3518, 1712, 1602, 1250, 1230, 1112, 1082, 1064, 798 cm⁻¹; 1H and 13C NMR see Table 1; HRESIMS m/z 273.0733 [M + Na]+ (calcd. for C13H14O3Na, 273.0740).

X-ray Crystallographic Analysis of 1. Colorless crystals of 1 were obtained from MeOH. Single-crystal X-ray diffraction data were collected on a Xcalibur, Atlas, Gemini ultra diffractometer with Cu Kα radiation (λ = 1.54180 Å) at 120.01(10) K, respectively. The structure was solved by direct methods (ShelXS) and refined with the ShelXL refinement package using least squares minimization. All non-hydrogen atoms were refined anisotropically, and all hydrogen atoms were placed in idealized positions and refined relatively isotropically with a riding model. Crystallographic data for 1 has been deposited in the Cambridge Crystallographic Data Centre with the deposition number CCDC 1489691, respectively. Copies of the data can be obtained, free of charge, on application to the Director, CCDC, 12 Union Road, Cambridge CB21EZ, UK (Fax: +44-(0)1223-366033, or e-mail: deposit@ccdc.cam.ac.uk).

Crystal data for 1: C15H21O5·H2O, M_r = 298.33, orthorhombic, a = 8.1247(2) Å, b = 11.8703(4) Å, c = 15.5875(5) Å, α = 90°, β = 90°, γ = 90°, V = 1503.30(8) Å³, space group P2₁2₁2₁, Z = 4, D_x = 1.318 mg/mm³, μ (Cu Kα) = 0.848 mm⁻¹, and F(000) = 640. Independent reflections: 2681 (R_int = 0.083). The final R1 values were 0.0389, wR2 = 0.0979 (l > 2σ(l)). Flack parameter = 0.0(3).

3.5. Biological Assays

Antibacterial activity was determined against five terrestrial pathogenic bacteria, including Staphylococcus epidermidis, S. aureus, Escherichia coli, Pseudomonas aeruginosa and Bacillus cereus, and two marine pathogenic bacteria Vibrio parahaemolyticus and V. alginolyticus by the microtitre plate assay method [19]. Ciprofloxacin was used as the positive control. Cytotoxic activities of all compounds against HeLa, MCF-7 and A549 cell lines were evaluated by the MTT method [20]. Epirubicin was used as a positive control.

4. Conclusions

Seven secondary metabolites, including three new dihydroisocoumarin penicimarins G–I (1–3), together with one known dihydroisocoumarin (4) and three known meroterpenoids (5–7), were obtained from a fungus P. citrinum isolated from the mangrove B. sexangula var. rhynchopetala collected in the South China Sea. Their structures were elucidated by the detailed analysis of spectroscopic data. The absolute configuration of 1 was determined by the X-ray diffraction analysis using Cu Kα radiation. The absolute configurations of 2–3 were determined by comparison of their CD spectra with literature. All compounds were evaluated for their antibacterial activities and cytotoxic activities.

Supplementary Materials: The following are available online at www.mdpi.com/1660-3397/14/10/177/s1. Figure S1: 1H NMR (400 MHz, MeOD-d₄) spectrum of Compound 1, Figure S2: 13C NMR (100 MHz, MeOD-d₄) spectrum of Compound 1, Figure S3: HMOC (MeOD-d₄) spectrum of Compound 1, Figure S4: 1H–1H COSY (MeOD-d₄) spectrum of Compound 1, Figure S5: HMBC spectrum (MeOD-d₄) of compound 1, Figure S6: HRESIMS spectrum of Compound 1, Figure S7: 1H NMR (400 MHz, MeOD-d₄) spectrum of Compound 2, Figure S8: 13C NMR (100 MHz, MeOD-d₄) spectrum of Compound 2, Figure S9: HMOC (MeOD-d₄) spectrum of Compound 2, Figure S10: 1H–1H COSY (MeOD-d₄) spectrum of Compound 2, Figure S11: HMBC (MeOD-d₄) spectrum of Compound 2, Figure S12: HRESIMS spectrum of Compound 2, Figure S13: 1H NMR (400 MHz, CDCl₃) spectrum
of Compound 3, Figure S14: 13C NMR (100 MHz, CDCl3) spectrum of Compound 3, Figure S15: HMQC (CDCl3) spectrum of Compound 3, Figure S16: 1H–1H COSY (CDCl3) spectrum of Compound 3, Figure S17: HMBC (CDCl3) spectrum of Compound 3, Figure S18: HRESIMS spectrum of Compound 3.

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