

Article

Four New Chromones from the Endophytic Fungus *Phomopsis asparagi* DHS-48 Isolated from the Chinese Mangrove Plant *Rhizophora mangle*

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Abstract: Four new chromones, phomochromenones D–G (1–4), along with four known analogues, diaporchromone A (5), diaporchromanone C (6), diaporchromanone D (7), and phomochromenone C (8), were isolated from the culture of *Phomopsis asparagi* DHS-48 from Chinese mangrove *Rhizophora mangle*. Their structures were elucidated on the basis of comprehensive spectroscopic analysis. The absolute configurations of 1 and 4 were assigned on the basis of experimental and calculated electronic circular dichroism (ECD) data, and those of enantiomers 2 and 3 were determined by a modified Mosher's method and basic hydrolysis. To the best of our knowledge, phomochromenones D–F (1–4) possessing a 3-substituted-chroman-4-one skeleton are rarely found in natural sources. Diaporchromone A (5) showed moderate to weak immunosuppressive activity against T and/or B lymphocyte cells with IC₅₀ of 34 μM and 117 μM.

Keywords: mangrove endophytic fungi; *Phomopsis* sp.; chromenones; immunosuppressive activity



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1. Introduction

Marine-derived fungi are morphologically and physiologically adapted to harsh environmental stresses, such as high salinity, high temperature, extreme tides, oxygen pressure, high humidity, and light and air limitations, which have increasingly attracted the attention of both pharmaceutical and natural product chemists in recent decades [1,2]. Fungi colonized in mangrove forests, which comprise the second largest ecological group of marine fungi, have especially adapted their metabolic mechanisms to the unique properties of the marine environment via the generation of a large variety of structurally unprecedented and biologically interesting metabolites of pharmaceutical importance [3–5]. One fungal genus which is especially productive with regard to the accumulation of a diverse array of mostly bioactive compounds is *Phomopsis*. Chemical investigation of this fungal genus has resulted in the discovery of over 70 potentially bioactive secondary metabolites, such as subintestinal vessel plexus (SIV) accelerator phomopsis-H76 A [6], cytotoxic phomopchallasins B and C [7], mycoepoxydiene [8], dicerandrols [9], antibiotic phomoxanthone A [10], phomodiol [11], phompsichalasin [12], antimicrotubule phomosidin [13], and anti-inflammatory phomol [14]. As part of our ongoing investigation on bioactive metabolites from mangrove endophytic fungi [15–19], *Phomopsis asparagi* DHS-48 was isolated from a fresh root of the mangrove plant *Rhizophora mangle*. Four new chromones (1–4), and five known compounds, including diaporchromone A (5) [20], diaporchromanone C (6) [20], diaporchromanone D (7) [20], and phomochromenone C (8) [21] (Figure 1) were isolated from the EtOAc extract of *P. asparagi* after fermentation on a solid rice medium containing sea salt. Herein, we report the isolation, structural elucidation, and exploration on the biological activities of compounds 1–8.

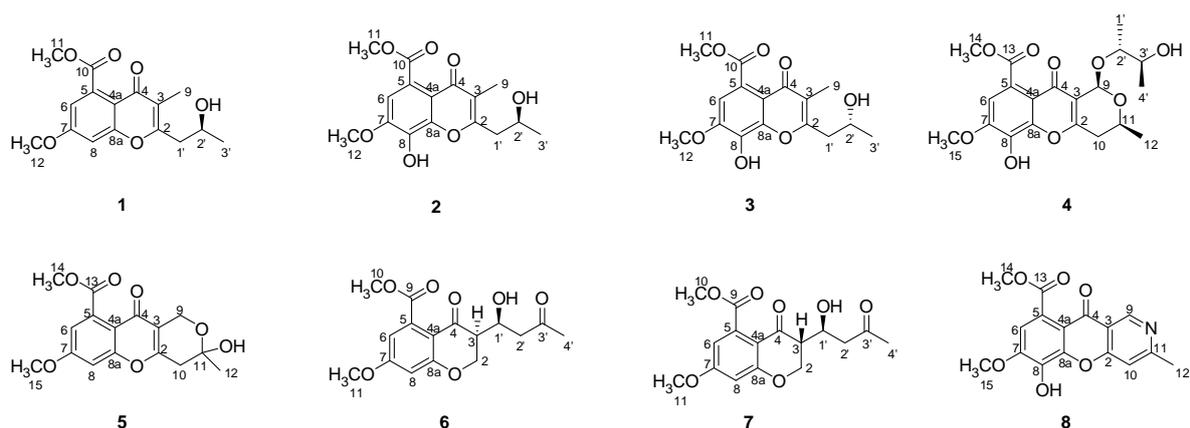


Figure 1. The chemical structures of compounds 1–8.

2. Results and Discussion

Phomochromenone D (**1**), a white amorphous powder, has the molecular formula $C_{16}H_{19}O_6$, established by HR-ESIMS (m/z 307.1139, calcd. for $[M+H]^+$ 307.1182), implying eight degrees of unsaturation. The UV absorption maxima at 219, 245, 295 nm indicated that **1** could be a chromone derivative. The 1D NMR data of **1** (Table 1) indicated that six of the eight units of unsaturation came from four carbon–carbon double bonds and two carbonyls. Therefore, the other two units of unsaturation come from two rings. The 1H NMR spectrum of **1** showed the presence of two meta-coupled aromatic protons at δ_H 6.92 (d, $J = 2.4$ Hz, H-6) and δ_H 7.10 (d, $J = 2.4$ Hz, H-8), one methine at δ_H 4.25 (m, H-2'), one methylene at δ_H 2.92 (dd, $J = 14.0, 8.0$ Hz, H_a-1') and 2.60 (dd, $J = 14.0, 5.1$ Hz, H_b-1'), and two methyl at δ_H 2.02 (s, H₃-9) and δ_H 1.29 (d, $J = 6.3$ Hz, H₃-3'), and two methoxy at δ_H 3.917 (s, H₃-11) and δ_H 3.924 (s, H₃-12). The ^{13}C NMR and DEPT spectra showed 16 carbon resonances corresponding to two sp^2 methine (δ_C 114.52 and 102.3), ten sp^2 quaternary (δ_C 165.1, 164.8, 159.5, 135.9, 119.1, 114.49, one carbonyl at δ_C 171.4, one conjugated carbonyl at δ_C 178.4), one oxygenated methine (δ_C 67.1), two methoxy (δ_C 57.0, 53.5), one methylene (δ_C 42.6), and two methyl (δ_C 23.7, 10.3) carbons. The HMBC correlation (Figure 2) from H-6 to C-5, C-7, and C-10, and from H-8 to C-7, C-4a and C-8a indicated the presence of the chromone moiety. Moreover, HMBC correlations from H₃-9 to C-2, C-3 and C-4; and from H₂-1' to C-2 and C-3, suggested that a 2-hydroxypropyl group was attached to C-2 of the chromone core. The absolute configuration of C-2' of **1** was determined by the comparison of its experimental and time-dependent density functional theory (TDDFT)-calculated electronic circular dichroism spectrum. The experimental ECD spectrum (CH₃OH) for 2'*S*-**1** matched well with the calculated spectrum (Figure 3), which confirmed the unambiguous assignment of the absolute configuration of **1** as *S*, and the trivial name, phomochromenone D, was assigned.

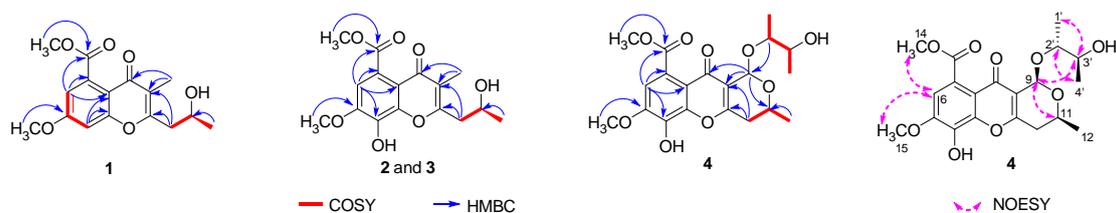
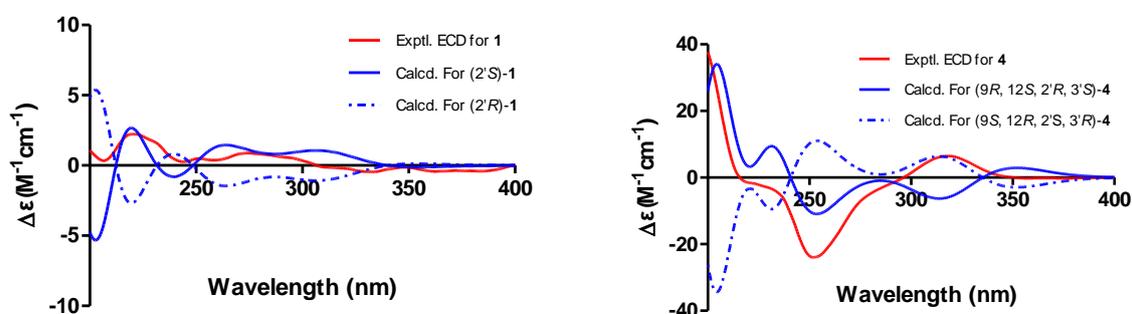


Figure 2. Selected 2D NMR of compounds 1–4.

Table 1. ^1H (400 MHz) and ^{13}C (125 MHz) NMR spectroscopic data for **1–4** in CD_3OD .

Position	1		2/3		4	
	δ_{C} , Type	δ_{H} (J in Hz)	δ_{C} , Type	δ_{H} (J in Hz)	δ_{C} , Type	δ_{H} (J in Hz)
2	164.8, C		164.8, C		166.7, C	
3	119.1, C		118.2, C		116.8, C	
4	178.4, C		178.9, C		176.8, C	
4a	135.9, C		115.4, C		116.5, C	
5	114.5, C		123.2, C		123.0, C	
6	114.6, CH	6.92, d, 2.4	110.0, CH	7.07, s	110.3, CH	7.09, s
7	165.1, C		151.4, C		152.6, C	
8	102.3, CH	7.10, d, 2.4	138.2, C		140.3, C	
8a	159.5, C		147.1, C		147.2, C	
9	10.3, CH_3	2.02, s	10.2, CH_3	2.02, s	96.9, CH	5.73, s
10	171.4, C		172.4, C		35.2, CH_2	H_a 2.77, dd, 17.9, 3.7 H_b 2.68, dd, 17.9, 10.7
11	53.5, CH_3	3.92, s	53.5, CH_3	3.89, s	63.9, CH	4.40, m
12	57.0, CH_3	3.92, s	57.2, CH_3	3.96, s	20.8, CH_3	1.37, d, 6.2
13					172.1, C	
14					53.3, CH_3	3.87, s
15					57.2, CH_3	3.96, s
1'	42.6, CH_2	H_a 2.92, dd, 14.0, 8.0 H_b 2.83, dd, 14.0, 5.1	42.4, CH_2	H_a 2.95, dd, 14.1, 7.9 H_b 2.83, dd, 14.1, 5.1	18.8, CH_3	1.19, d, 6.0
2'	67.1, CH	4.25, m	67.1, CH	4.29, m	83.4, CH	3.60, m
3'	23.7, CH_3	1.29, d, 6.3	23.6, CH_3	1.29, d, 6.3	73.4, CH	3.54, m
4'					18.3, CH_3	1.13, d, 6.0

**Figure 3.** Experimental and calculated ECD spectra of **1** and **4**.

Phomochromenones **E** (**2**) and **F** (**3**) were isolated as a mixture of two enantiomers and shared the same NMR data, ^1H - ^1H COSY and HMBC spectra. Based on the HR-ESIMS ion detected at m/z 321.0989 (calcd. for $[\text{M}-\text{H}]^-$, 321.0974), the mixture (**2/3**) had the same molecular formula of $\text{C}_{16}\text{H}_{18}\text{O}_7$ (i.e., differing from that of **1** by an additional hydroxyl group). The ^1H and ^{13}C NMR spectrum clearly indicated that this hydroxyl group was attached at C-6. Supporting evidence for this assignment was obtained from the downfield chemical shifts of C-8 (δ_{C} 138.2, s) and the absence of the proton signal of H-6 in **2/3** (δ_{H} 7.10, d, $J = 2.4$ Hz for H-8 and δ_{C} 102.3, d for C-8 in **1**, respectively). Moreover, the presence of a hydroxyl group at C-8 was corroborated by the observed HMBC correlation from H-6 (δ_{H} 7.10, d, $J = 2.4$ Hz) to C-8, C-7 (δ_{C} 151.4), C-5 (δ_{C} 123.2), C-4a (δ_{C} 115.4), and C-10 (δ_{C} 172.4). However, the antipode rotation and ECD were detected, which suggested the mixture was not an optically pure compound. Since the chiral phase HPLC (CHIRALPAK IC) did not afford the separation of these two enantiomers, a modified Mosher's experiment was performed to obtain its MPA esters. The products of Mosher's reactions were subsequently

analyzed by UPLC-ESI-MS through a RP-C₁₈ chromatography column to afford two pairs of diastereomeric esters (*R*-MPA-2/*R*-MPA-3 and *S*-MPA-2/*S*-MPA-3) (Figure 4). The spectral non-equivalences in ¹H NMR chemical shifts between (*R*)- and (*S*)-MPA esters ($\Delta\delta = \delta_R - \delta_S$) indicated a 2'-*S* configuration for (+)-**2** (positive for H-9 and negative for H-3') and a 2'-*R* configuration for (−)-**3** (negative for H-9 and positive for H-3') (Figure 4). The (*R*)- and (*S*)-MPA esters of **2/3** was further separated by semi-prep. RP-C₁₈ HPLC (40% CH₃CN/H₂O, 0.2% HCOOH) to afford *R*-MTPA-2/*S*-MTPA-3 (*t*_R 18.8 min) and *R*-MPA-3/*S*-MPA-2 (*t*_R 19.2 min). Compounds **2** and **3** were successfully obtained by chromatography after alkaline hydrolysis of *R*-MPA-2/*S*-MPA-2 and *R*-MPA-3/*S*-MPA-3, respectively. Therefore, enantiomers **2** and **3** were successfully isolated and assigned the names phomochromenone E and phomochromenone F, respectively.

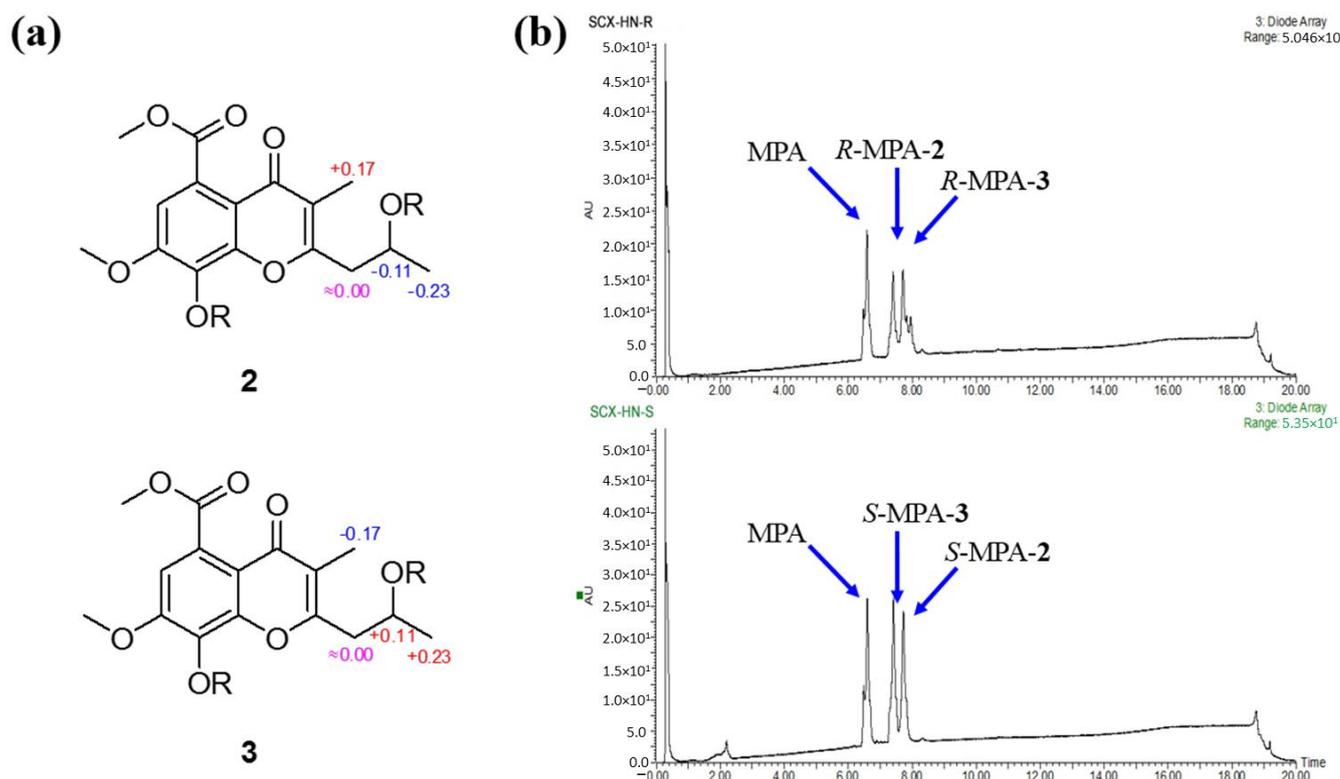


Figure 4. (a) $\Delta\delta (= \delta_R - \delta_S)$ values for (*R*)- and (*S*)- MPA esters of **2** and **3**. (b) UPLC analysis profile of (*R*)- and (*S*)- MPA esters of **2** and **3** over a 20 min gradient as follows: T = 0.0, 5% B; T = 15.0, 95% B; T = 17.0, 100% B, T = 18.0, 100% B, and T = 18.1, 5% B, and T = 20.0, 5% B (A, MQ+0.2% HCOOH; B, MeOH+0.2% HCOOH).

Phomochromenone G (**4**) was obtained as a white amorphous powder and had a molecular formula of C₂₀H₂₄O₉, as determined by its HR-ESIMS (*m/z* 431.1314, calcd. for [M+Na]⁺ 431.1318), indicating nine degrees of unsaturation. The ¹H NMR spectrum showed resonances for one singlet aromatic proton at δ_H 7.09 (s, H-6); four methine protons at δ_H 5.73 (s, H-9), δ_H 4.40 (m, H-11), δ_H 3.60 (m, H-1'), and δ_H 3.54 (m, H-2'); one methylene at δ_H 2.77 (dd, *J* = 17.9, 3.7 Hz, H_a-10) and 2.68 (dd, *J* = 17.9, 10.7 Hz, H_b-10); three methyl at δ_H 1.37 (d, *J* = 6.2 Hz, H₃-12), δ_H 1.13 (d, *J* = 6.0 Hz, H₃-3'), and δ_H 1.19 (d, *J* = 6.0 Hz, H₃-4'); two methoxy groups at δ_H 3.96 (s, H₃-15) and δ_H 3.87 (s, H₃-14). The ¹³C NMR and DEPT spectra showed 20 carbon signals, including a keto group, an ester carbonyl group, eight olefinic carbon signals (including four oxygenated carbons), three oxy-methines, one methylene, two methoxy group, and three methyl group. Comparison of the NMR data of **4** with those of phomochromenone B [21], previously isolated from endophytic fungus *Phomopsis* sp. HNY29-2B derived from mangrove plant *Acanthus ilicifolius* Linn, revealed that both compounds differed with regard to the nature of the side chain at C-1, where

the hydroxyl group of the latter was replaced by the 3-hydroxybutan-2-yloxy group of **3**. This was confirmed through ^1H - ^1H COSY correlations of $\text{H}_3\text{-4}'/\text{H-1}'/\text{H-2}'/\text{H}_3\text{-3}'$ and HMBC correlations (Figure 2) from H-9 to C-11 (δ_{C} 63.9), C-4 (δ_{C} 176.8), C-2 (δ_{C} 166.7), and C-1' (δ_{C} 67.1). The relative configuration of **4** was based on the NOESY correlations as indicated in Figure 2. The NOESY correlations of H-9 to H-11, $\text{H}_3\text{-1}'$ and H-3'; H-11 to $\text{H}_3\text{-1}'$; and $\text{H}_3\text{-4}'$ to H-2' indicated that H-9, H-11, $\text{H}_3\text{-1}'$, and $\text{H}_3\text{-4}'$ were on the opposite side of the $\text{H}_3\text{-12}$, H-2', and $\text{H}_3\text{-4}'$. The absolute configuration of **4** were also determined by comparing experimental and calculated electronic circular dichroism (ECD) spectra for the truncated model (9*R*, 12*S*, 2'*R*, 3'*S*)-**4** and the truncated model (9*S*, 12*R*, 2'*S*, 3'*R*)-**4** using time-dependent density functional theory (TDDFT). The theoretical spectrum of **4** showed an excellent fit with the experimental plot recorded in MeOH (Figure 3), which supported the absolute configuration to be 9*R*, 12*S*, 2'*R*, 3'*S*. Thus, the structure of **4** was determined and named phomochromenone F.

Our primary application of immunosuppressive activity screening indicated that a crude extract of *P. asparagi* DHS-48 showed strong inhibitory of splenic lymphocyte growth with IC_{50} of 6 $\mu\text{g}/\text{mL}$. An immunosuppressive assay showed that compound **5** exhibited moderate to weak inhibitory activity against ConA-induced T and LPS-induced B murine splenic lymphocytes *in vitro* with IC_{50} values of 34 and 117 μM , respectively (Table 2, Figure 5), whereas the other investigated compounds showed no obvious inhibitory effect. The cytotoxicity of **5** was tested in splenocyte cultures for 72 h using the tetrazolium salt-based CCK-8 assay. The results showed that it inhibited splenic lymphocyte growth with relatively lower toxicity (IC_{50} 47 μM), at which the survival of normal splenic cells was slightly influenced in comparison with that of CsA (IC_{50} 11 μM). The results showed that compound **5** with a 1,3,4,10-tetrahydropyrano[4,3-*b*]chromene nucleus displayed significant immunosuppressive activity compared to compounds (**1–3**, **6**, **7**) with a chromone nucleus and compound **8** with a 10*H*-chromeno[3,2-*c*]pyridine nucleus. The additional 11-OH group in **5** is essential for its stimulated splenic lymphocyte inhibitory compared to compound **4**.

Table 2. Immunosuppressive activities of isolated compounds 1–8.

Compound	Cytotoxicity ^a IC_{50} (μM) ^b	ConA-Induced T-Cell Proliferation		LPS-Induced B-Cell Proliferation	
		IC_{50} (μM) ^b		IC_{50} (μM) ^b	
5	47	34	117		
1–4, 6–8	-	-	-		
CsA ^c	11	4	25		

^a Cell viability on murine splenocytes was tested by using CCK-8 method. ^b Data are presented as mean \pm SD ($n = 3$) in μM . ^c Positive control.

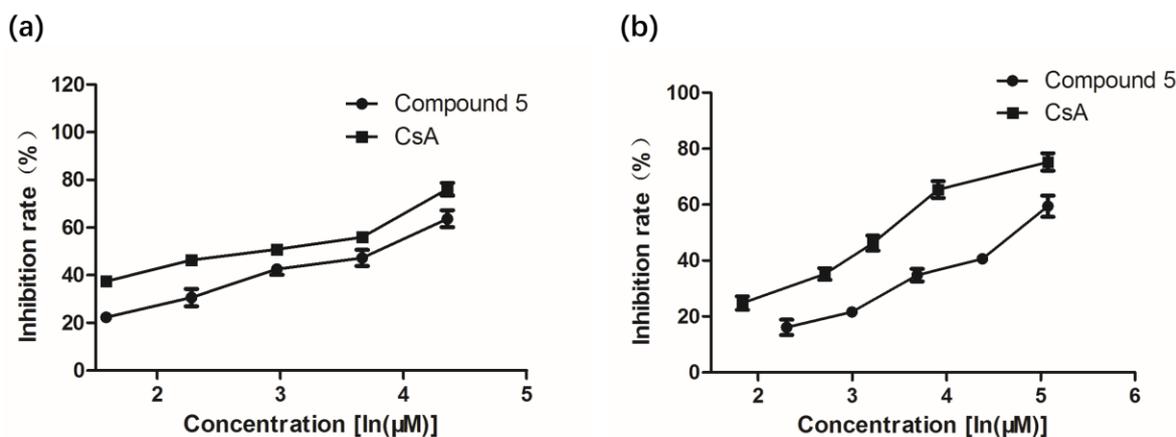


Figure 5. Effect of **5** on mouse splenocytes viability and proliferation. (a) Effect of **5** on the viability of T lymphocyte cells. (b) Effect of **5** on the viability of B lymphocyte cells. All values are expressed as mean \pm SD. $n = 3$.

3. Materials and Methods

3.1. General Procedures

Specific rotations were obtained on a WYA-2S digital Abbe refractometer (Shanghai Physico-optical Instrument Factory, Shanghai, China). UV spectra were determined using a Shimadzu UV-2401 PC spectrophotometer (Shimadzu Corporation, Tokyo, Japan), while CD spectra were measured on a JASCO J-715 spectra polarimeter (Japan Spectroscopic, Tokyo, Japan). ^1H , ^{13}C and 2D NMR spectra were recorded on a Bruker AV 400 NMR spectrometer using TMS as an internal standard. High-resolution ESI-MS were performed on an LTQ Orbitrap XL instrument (Thermo Fisher Scientific, Bremen, Germany) using peak matching. TLC and column chromatography (CC) were carried out over silica gel (200–400 mesh, Qingdao Marine Chemical Inc., Qingdao, China), or a Sephadex-LH-20 (18–110 μm , Merck, Darmstadt, Germany), respectively. UPLC analysis (Waters Corporation, Milford, MA, USA) was recorded using a Waters system equipped in ESI mode on an Acquity UPLC H-Class connected to an SQ Detector 2 mass spectrometer using a BEH RP C₁₈ column (2.1 \times 50 mm, 1.7 μm , 0.5 mL/min). Semi-preparative HPLC was performed using a Waters equipped with a 2998 PDA detector (Waters Corporation, Milford, MA, USA) and a RP C₁₈ column (YMC-Pack ODS-A, 10 \times 250 mm, 5 μm , 3 mL/min).

3.2. Fungal Material

Endophytic fungus *Phomopsis asparagi* was isolated with PDA medium from the fresh root of the mangrove plant *Rhizophora mangle*, collected in October 2015 in Dong Zhai Gang-Mangrove Garden on Hainan Island, China. The fungus (strain no.DHS-8) was identified using a molecular biological protocol by DNA amplification and sequencing of the ITS region (GenBank Accession no.MT126606) [22]. A voucher strain was deposited at one of the authors' laboratories (J.X.).

3.3. Extraction Isolation

The fungus was fermented onto auto autoclaved rice solid-substrate medium (thirty 1000 mL Erlenmeyer flasks, each containing 100 g of rice and 100 mL of 0.3% of saline water) and incubated for 28 days at 28 °C. In total, 140 flasks of culture were extracted three times with EtOAc and the filtrate was evaporated under reduced pressure to yield crude extract (65 g). The crude extract was partitioned with petroleum ether (PE), dichloromethane, ethyl acetate (EA), and n-butyl alcohol (BA). The dichloromethane fraction and ethyl acetate fraction were combined (30 g), then chromatographed on silica gel column chromatography using gradient elution with a CH₂Cl₂-MeOH mixture of increasing polarity (100:0–0:100, *v/v*) to afford 8 fractions (Fr. 1–Fr. 8). Fr. 2 was subjected to open silica gel CC using gradient elution with CH₂Cl₂-EtOAc (4:1–1:1, *v/v*) to yield fractions Fr. 2.1–2.6. Fr. 2.4 and Fr. 2.5 were purified by semi-preparative reversed-phase HPLC using MeOH-H₂O (60:40, *v/v*) to afford **8** (5.0 mg) and **5** (4.8 mg), respectively. Fr. 3.4 was separated by silica gel CC using CH₂Cl₂-EtOAc (2:1, *v/v*) and was subsequently subjected to Sephadex LH-20 CC using MeOH as an eluent to give Fr. 3.4.5, followed by gradient elution MeOH-H₂O (70:30–0:100, *v/v*) with semi-preparative reversed-phase HPLC to obtain **1** (1.2 mg) and a mixture of diaporchromanone C (**6**) and D (**7**) (5.1 mg). Purification of Fr. 4 was isolated using CC over silica gel CC using CH₂Cl₂-EtOAc (1:1, *v/v*) to afford Fr. 4.1–Fr. 4.6. Fr. 4.5 was purified using RP-18 with a MeOH–H₂O (70:30, *v/v*) and then separated by semi-preparative reversed-phase HPLC with MeOH-H₂O (50:50, *v/v*) to yield **2/3** (5.1 mg). Fr. 6.2, collected from Fr. 6, was subjected to silica gel CC with gradient elution of CH₂Cl₂-EtOAc (100:6–100:8, *v/v*) to give Fr. 6.2.1–Fr. 6.2.3. Fr. 6.2.2 was further separated by semi-preparative HPLC (MeOH-H₂O, 55:45, *v/v*) to obtain **4** (3.0 mg).

3.4. Compound Characterization Data

Phomochromenone D (**1**): white amorphous powder (MeOH); $[\alpha]_{\text{D}}^{20} - 13$ (c 0.001, MeOH); UV (MeOH) λ_{max} 219, 245, 295 nm; ^1H and ^{13}C NMR data, see Tables 1 and 2, respectively; HR-ESI-MS *m/z* 307.1179 [M+H]⁺ (calcd. for C₁₆H₁₉O₆, 307.1182).

Phomochromenone E (**2**): yellow gum (MeOH); $[\alpha]_D^{20} +17$ (c 0.001, MeOH); UV (MeOH) λ_{\max} 220, 245, 294 nm; ^1H NMR data, see Table 1; HR-ESI-MS m/z 321.0989 $[\text{M}-\text{H}]^-$ (calcd. for $\text{C}_{16}\text{H}_{17}\text{O}_7$, 321.0974).

Phomochromenone F (**3**): yellow gum (MeOH); $[\alpha]_D^{20} - 19$ (c 0.0008, MeOH); UV (MeOH) λ_{\max} 220, 245, 294 nm; ^1H NMR data, see Table 1; HR-ESI-MS m/z 321.0989 $[\text{M}-\text{H}]^-$ (calcd. for $\text{C}_{16}\text{H}_{17}\text{O}_7$, 321.0974).

Preparation of MPA Esters of **2/3** by Mosher's Method. The mixture of compounds **2** and **3** (1.5 mg) was treated with (*R*)- or (*S*)-MPA (1.0 mg) with DCC (1.0 mg) and DMAP (0.3 mg) in anhydrous CH_2Cl_2 (0.5 mL). After being stirred at room temperature for 4 h at 0 °C, the solvent from the reaction mixture was removed in vacuo to furnish a residue, which was then subjected to semi-preparative RP-HPLC eluting with $\text{CH}_3\text{CN}-\text{H}_2\text{O}$ (40:60, 0.2% HCOOH) to obtain *R*-MTPA-**2**/*S*-MTPA-**3** (t_R 18.8 min) and *R*-MPA-**3**/*S*-MPA-**2** (t_R 19.2 min).

(*R*)-MPA ester of **2**: yellow gum (MeOH); ^1H NMR(400 MHz, CD_3OD) δ_{H} 7.63–7.41 (m, 5H), 7.25–7.18 (m, 5H), 7.22 (s, 1H), 5.26 (s, 1H), 4.98 (m, 1H), 4.68 (s, 1H), 3.94 (s, 3H), 3.92 (s, 3H), 3.55 (s, 3H), 3.20 (s, 3H), 2.77 (m, 1H), 2.62 (m, 1H), 1.92 (s, 3H), 1.09 (d, $J = 6.2$, 3H); HR-ESI-MS m/z 619.2164 $[\text{M} + \text{H}]^+$ (calcd. for $\text{C}_{34}\text{H}_{35}\text{O}_{11}$, 619.2179).

(*S*)-MPA ester of **2**: yellow gum (MeOH); 7.72–7.40 (m, 5H), 7.16 (s, 1H), 7.05–6.82 (m, 5H), 5.30 (s, 1H), 4.64 (s, 1H), 4.28 (m, 1H), 3.96 (s, 3H), 3.85 (s, 3H), 3.55 (s, 3H), 3.27 (s, 3H), 2.88 (m, 1H), 2.61 (m, 1H), 1.75 (s, 3H), 1.32 (d, $J = 6.1$, 3H); HR-ESI-MS m/z 619.2172 $[\text{M} + \text{H}]^+$ (calcd. for $\text{C}_{34}\text{H}_{35}\text{O}_{11}$, 619.2179).

(*R*)-MPA ester of **3**: yellow gum (MeOH); ^1H NMR(400 MHz, CD_3OD) δ_{H} 7.65–7.42 (m, 5H), 7.16 (s, 1H), 7.05–6.82 (m, 5H), 5.30 (s, 1H), 4.64 (s, 1H), 4.28 (m, 1H), 3.96 (s, 3H), 3.85 (s, 3H), 3.55 (s, 3H), 3.27 (s, 3H), 2.88 (m, 1H), 2.62 (m, 1H), 1.75 (s, 3H), 1.32 (d, $J = 6.2$, 3H); HR-ESI-MS m/z 619.2184 $[\text{M} + \text{H}]^+$ (calcd. for $\text{C}_{34}\text{H}_{35}\text{O}_{11}$, 619.2179).

(*S*)-MPA ester of **3**: yellow gum (MeOH); ^1H NMR(400 MHz, CD_3OD) δ_{H} 7.63–7.41 (m, 5H), 7.25–7.19 (m, 5H), 7.22 (s, 1H), 5.26 (s, 1H), 4.98 (m, 1H), 4.68 (s, 1H), 3.94 (s, 3H), 3.92 (s, 3H), 3.55 (s, 3H), 3.20 (s, 3H), 2.77 (m, 1H), 2.60 (m, 1H), 1.92 (s, 3H), 1.09 (d, $J = 6.2$, 3H); HR-ESI-MS m/z 619.2157 $[\text{M} + \text{H}]^+$ (calcd. for $\text{C}_{34}\text{H}_{35}\text{O}_{11}$, 619.2179).

Preparation of phomochromenones E(**2**) and F(**3**). (*R*)- and (*S*)-MPA ester of **2** were combined (1.2 mg) and dissolved in 10% NaOH (1.0 mL) and stirred for 1 h at room temperature (rt). The reaction mixture was extracted with EtOAc repeatedly and the organic layer was evaporated in vacuo to give optically pure (+)-**2** (0.7 mg). Similarly, (–)-**3** (0.8 mg) was prepared from the alkaline hydrolysis of (*R*)- and (*S*)-MPA ester of **3** (totally 1.0 mg) in the same manner.

Phomochromenone G (**4**): white amorphous powder (MeOH); $[\alpha]_D^{20} - 15$ (c 0.001, MeOH); UV (MeOH) λ_{\max} 205, 241, 303 nm; ^1H and ^{13}C NMR data, see Tables 1 and 2, respectively; HR-ESI-MS m/z 431.1314 $[\text{M} + \text{Na}]^+$ (calcd. for $\text{C}_{20}\text{H}_{24}\text{O}_9\text{Na}$, 431.1318).

Diaporchromone A (**5**): yellow gum (CHCl_3); $[\alpha]_D^{20} - 40$ (c 0.001, MeOH); UV (MeOH) λ_{\max} 219, 294 nm; ^1H NMR(400 MHz, CDCl_3) δ_{H} 6.89(1H, d, $J = 2.3\text{Hz}$, H-6), 6.87(1H, d, $J = 2.3\text{Hz}$, H-8), 4.72(2H, m, H-9), 3.98(3H, s, H-14), 3.90(3H, s, H-15), 2.86(1H, dd, $J = 17.4\text{Hz}$, $J = 2.8\text{Hz}$, 10-Ha), 2.76(1H, dd, $J = 17.4\text{Hz}$, $J = 9.8\text{Hz}$, 10-Hb), 1.63(3H, s, H-12); ^{13}C -NMR (100 MHz, CDCl_3) δ_{C} 173.7 (C-4), 169.8 (C-13), 163.2 (C-7), 158.4 (C-2), 158.0 (C-8a), 134.7 (C-5), 115.6 (C-3), 114.4 (C-4a), 113.0 (C-6), 101.7 (C-8), 95.4 (C-11), 57.5 (C-9), 56.2 (C-15), 53.2 (C-14), 37.1 (C-10), 29.4 (C-12). ESI-MS m/z 321.09 $[\text{M} + \text{H}]^+$.

Diaporchromanone C (**6**): yellow gum (CHCl_3); UV (MeOH) λ_{\max} 215, 239, 275, 315 nm; ^1H NMR(400MHz, CDCl_3) δ_{H} 6.54(1H, d, $J = 2.4\text{Hz}$, H = 6), 6.46(1H, d, $J = 2.4\text{Hz}$, H = 8), 4.71(1H, dd, $J = 11.6\text{Hz}$, $J = 6.3\text{Hz}$, 2-Ha), 4.57(1H, dd, $J = 11.6\text{Hz}$, $J = 3.9\text{Hz}$, 2-Hb), 4.38 (1H, ddd, $J = 8.5\text{Hz}$, $J = 8.5\text{Hz}$, $J = 2.9\text{Hz}$, H-1'), 3.94(3H, s, H-10), 3.84(3H, s, H-11), 2.66(1H, ddd, $J = 8.5\text{Hz}$, $J = 6.3\text{Hz}$, $J = 3.9\text{Hz}$, H-3), 2.88(1H, dd, $J = 18.1\text{Hz}$, $J = 2.9\text{Hz}$, 2'-Ha), 2.71(1H, dd, $J = 18.1\text{Hz}$, $J = 8.5\text{Hz}$, 2'-Hb), 2.18(3H, s, H-4'); ^{13}C NMR (125MHz, CDCl_3) δ_{C} 210.0 (C-3'), 190.2 (C-4), 169.7 (C-9), 165.5 (C-8a), 164.0 (C-7), 136.3 (C-5), 111.7 (C-4a), 109.8 (C-6), 102.0 (C-8), 68.2 (C-2), 64.0 (C-1'), 56.1 (C-11), 53.1 (C-10), 50.6 (C-3), 47.7 (C-2'), 30.9 (C-4'). ESI-MS m/z 323.09 $[\text{M} + \text{H}]^+$.

Diaporchromanone D (7): yellow gum (CHCl₃); UV (MeOH) λ_{\max} 215, 239, 275, 315 nm; ¹H NMR(400MHz, CDCl₃) δ_{H} 6.56(1H, d, $J = 2.4$ Hz, H = 6), 6.46(1H, d, $J = 2.4$ Hz, H = 8), 4.60(1H, dd, $J = 11.5$ Hz, $J = 5.1$ Hz, 2-Ha), 4.51(1H, ddd, $J = 8.7$ Hz, $J = 5.1$ Hz, $J = 3.6$ Hz, H-1'), 4.47 (1H, dd, $J = 11.5$ Hz, $J = 9.6$ Hz, 2-Hb), 3.93(3H, s, H-10), 3.85(3H, s, H-11), 2.96(1H, ddd, $J = 9.6$ Hz, $J = 5.1$ Hz, $J = 5.1$ Hz, H-3), 2.80(1H, dd, $J = 17.3$ Hz, $J = 8.7$ Hz, 2'-Ha), 2.74(1H, dd, $J = 17.3$ Hz, $J = 3.6$ Hz, 2'-Hb), 2.18(3H, s, H-4'); ¹³C NMR (125MHz, CDCl₃) δ_{C} 208.8 (C-3'), 190.9 (C-4), 169.7 (C-9), 165.6 (C-8a), 164.1 (C-7), 136.3 (C-5), 112.3 (C-4a), 109.8 (C-6), 102.2 (C-8), 68.6 (C-2), 66.4 (C-1'), 56.1 (C-11), 53.1 (C-10), 49.6 (C-3), 46.8 (C-2'), 31.0 (C-4'). ESI-MS m/z 323.09 [M+H]⁺.

Phomochromenone C (8): yellow gum (MeOH); UV (MeOH) λ_{\max} 206, 236, 308 nm; ¹H NMR(400 MHz, CD₃OD) δ_{H} 9.34(1H, s, H-1), 7.30(1H, s, H-4), 6.98(1H, s, H-8), 4.06(3H, s, H-14), 4.02(3H, s, H-13), 2.70(3H, s, H-3); ¹³C-NMR (125MHz, CD₃OD) δ_{C} 176.3 (C-10), 171.7(C-12), 165.4 (C-3), 162.9 (C-4a), 153.9 (C-7), 150.1 (C-1), 146.6 (C-5a), 139.1 (C-6), 124.6 (C-9), 116.7 (C-10a), 115.4 (C-9a), 112.7 (C-4), 109.6 (C-8), 57.3 (C-14), 53.4 (C-13), 24.4 (C-11). ESI-MS m/z 316.12 [M+H]⁺.

3.5. Computational Analyses

Conformational analysis of the enantiomers of compounds 1 and 4 established by NOESY analyses were carried out using optimization and spectrum calculation. Conformational searches were carried out by means of the MM+ method in Hyper Chem 8.0 software (Hyperchem Release 8.0, Hypercube, Inc., Gainesville, FL, USA). The lowest energy conformers within 2 kcal/mol were subjected to further DFT calculations. The geometries of the conformers were optimized at the B3LYP/6-31+G(d,p) level in the gas phase using the Gaussian 09 program (Gaussian Inc., Wallingford, CT, USA). The theoretical calculation of ECD was conducted with IEFPCM solvent model for CH₃OH using TDDFT at the B3LYP/6-31+G(d,p) level for all conformers of compounds (see Supplementary Materials). Boltzmann-weighted ECD spectra was obtained using SpecDis1.70.1 software (University of Würzburg, Würzburg, Germany).

3.6. Preparation of Spleen Lymphocytes

Female BALB/c mice (6–8 weeks old, 20 ± 2 g) were purchased from the Department of Laboratory Animal Science (Hainan Medicinal University, China). The mice were sacrificed by cervical dislocation, and their spleens were collected in complete RPMI 1640 medium, which was minced with surgical scissors in a germ-free condition. The suspension was then filtered through a sterile sieve mesh to obtain the single cell suspension. After centrifugation (1000 rpm at 4 °C for 5 min), the resulting cells were treated with erythrocytes lysis buffer, followed by washing twice with cold phosphate-buffered saline (PBS). Then, the cells were adjusted to the concentration of 5 × 10⁶ cells/mL and resuspended in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS).

3.7. Cell Viability Assay

Cell viability of compounds 1–8 were measured using the tetrazolium salt-based CCK-8 assay according to a previously described protocol with some modifications [23]. Spleen lymphocytes were seeded into 96-well plates at a density of 2 × 10⁶ cells/mL onto 96-well plates containing 100 μ L of RPMI1640 complete medium (triplicate wells). Then, the cells were treated with 100 μ L of various concentrations (3 μ g/mL, 6 μ g/mL, 12 μ g/mL, 25 μ g/mL, 50 μ g/mL) of isolated compounds or cyclosporine (CsA) at 37 °C with a 5% CO₂ incubator for 44 h. At the end of the culture, the cell culture plate was removed and observed under an inverted microscope followed by 20 μ L of CCK-8 reagent (5 mg/mL). The cells were further incubated for 4 h at 37 °C with 5% CO₂. The optical density was measured at 450 nm on a microplate reader.

3.8. Immunosuppressive Assay

Compounds 1–8 were evaluated for immunosuppressive activity against the proliferation of concanavalin A (ConA)-induced T and lipopolysaccharide (LPS)-induced B murine splenic lymphocyte in vitro using a CCK-8 method according to previously reported methods [24]. Cyclosporine A was used as a positive control.

Supplementary Materials: The following are available online at <https://www.mdpi.com/article/10.3390/md19060348/s1>. Figures S1–S36: Copies of HR-ESI-MS, 1D- and 2D-NMR spectra of 1–4. Tables S1–S4: Gibbs free energies^a and equilibrium populations^b of low-energy conformers of 1 and 4; Cartesian coordinates for the low-energy reoptimized MMFF conformers of 1 and 4 at B3LYP/6-31G(d,p) level of theory in gas.

Author Contributions: J.X. conceived and designed the experiments; C.W. and Z.F. isolated the metabolites; C.S. elucidated structures; X.Z. performed the bioactivity assays; J.X. and C.W. wrote the paper. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: All experiments conducted on animal material were approved by the Ethics Committee of the Hainan University (HNUAUCC-2021-00082, Feb. 19th, 2021), and all methods used were compliant with the regulations of the Ethics Committee.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data is contained within the article or Supplementary Materials.

Conflicts of Interest: The authors declare no conflict of interest.

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