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Secondary Metabolites and Bioactivities of *Aspergillus ochraceopetaliformis* Isolated from *Anthurium brownii*

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Cite This: *ACS Omega* 2020, 5, 20991−20999

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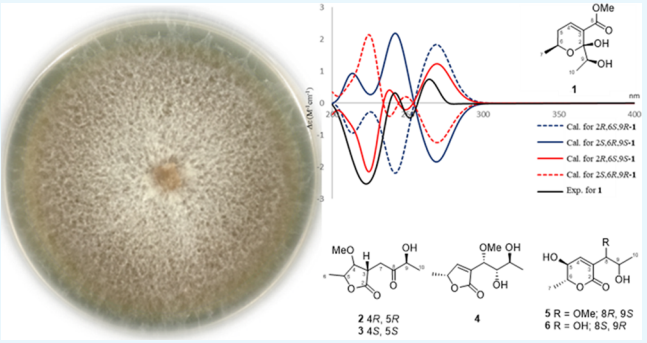
ABSTRACT: Five new polyketides, asperochrapyran (**1**) and asperochralactones A−D (**2**−**5**), along with 12 known polyketides (**6**−**17**), were obtained from the fungal strain *Aspergillus ochraceopetaliformis*. Structures of all isolates were elucidated by their spectroscopic parameters. The relative configurations of the new compounds were deduced by the data of coupling constants and NOESY spectra. The absolute configurations were determined by the comparison of experimental and calculated ECD spectra. Moreover, the plausible biosynthesis pathway of major isolates was proposed as well. Anti-inflammatory activity of compounds **5** and **7**−**17** were evaluated with human neutrophils in response to the stimulation of formyl-methionyl-leucyl phenylalanine (fMLP). Asperlactone (**9**), aspyrone (**13**), and (−)-(3*R*)-mellein (**14**) exerted superoxide anion inhibition at 30 ± 9%, 29 ± 9%, and 26 ± 12%, respectively, at 10 μM. The capacities of asperlactone (**9**), aspilactonol B (**10**), penicillic acid (**12**), and (−)-(3*R*)-mellein (**14**) in elastase release inhibition were revealed as 25 ± 4%, 38 ± 8%, 25 ± 5%, and 34 ± 9%, respectively, at 10 μM.



INTRODUCTION

Fungi generate diverse groups of secondary metabolites with intriguing activities.[1] Several bioactive secondary metabolites have been applied in agriculture or pharmaceutical industries.[1] Following the cooperation of a high-throughput screening project with the Dr. Cecilia Koo Botanic Conservation Center (KBCC) in Taiwan providing extraordinary plant materials, we obtained a fungal strain, isolated from leaves of *Anthurium brownii* (*A. brownii*) Mast, which was identified as *Aspergillus ochraceopetaliformis* (*A. ochraceopetaliformis*) Bat. and Maia. The *Aspergillus* genus contains a large number of species and is widely distributed in natural environments. This genus was reported as a prolific source in producing bioactive secondary metabolites, such as alkaloids,[2] glycosides,[3] peptides,[4] polyketides,[5] steroids,[6] and terpenoids.[7] These molecules exhibited diverse biological activities, including antibacterial,[8,9] antifungal,[10] cytotoxic,[11] nematicidal,[12] radical scavenging,[13] ovicidal, and insect growth-regulating[14] activities. The chemical diversity and broad spectrum of bioactivities make this genus to be a potential resource in the discovery of new drug development. For instance, the renowned case of cholesterol-lowing drug, lovastatin, was discovered from *Aspergillus terreus* (*A. terreus*). It was proved as a trigger of HMG-CoA reductase inhibition and clinically used as a hypercholesterolemia and cardiovascular disease ameliorator.[15] Moreover, simvastatin, a derivative synthesized and modified substance generated from *A. terreus*, is another clinical drug used for a similar purpose of lovastatin.[15] These cases attracted our attention and encouraged us on exploring new potential bioactive molecules from the *Aspergillus* genus.

Although *A. ochraceopetaliformis* was considered as not a common pathogen to humans, it had once been found in human skin lesions and reported as an invader to cause onychomycosis.[16,17] Notably, this fungal species was also found in ocean sponges and even discovered from an Antarctic soil sample.[5,18,19] Furthermore, Wang et al. reported that this fungal species would produce various types of bioactive sesquiterpenoids with fascinating activities such as those against of influenza viruses or lipopolysaccharide-induced NO release in RAW 264.7 cell lines.[5,18]

In a current study, we fermented *A. ochraceopetaliformis* through a liquid fermentation methodology and the ethyl acetate extract of *A. ochraceopetaliformis* was found to possess an anti-inflammatory effect on inhibiting superoxide anion

Received: May 27, 2020
Accepted: July 28, 2020
Published: August 14, 2020

generation and elastase release at 10 μg/mL (103 ± 1% and 107 ± 6%, respectively).

Continuing our efforts on discovery of the chemical diversity and biological activities of natural products, we performed further chemical and biological investigation on this fungus and identified 17 polyketide secondary metabolites, including 5 new polyketides (**1**−**5**) and 12 known polyketides (**6**−**17**).

Herein, we described the structural elucidation of new secondary metabolites (**1**−**5**) and the extensive determination of the absolute configurations by computational approaches. We evaluated the isolated compounds for several bioactivity assays, including those for cytotoxicity and anti-inflammatory properties. Moreover, we proposed the plausible biosynthesis pathway of the isolated polyketide secondary metabolites.

RESULTS AND DISCUSSION

Five new polyketides, asperochrapyran (**1**) and asperochralactones A−D (**2**−**5**), together with 12 known polyketides, (5*S*,6*R*,8*S*,9*R*)-8,9-dihydroxy-8,9-deoxyaspyrone (**6**), aspyronol (**7**), dihydroaspyrone (**8**), asperlactone (**9**), aspilactonol B (**10**), asperochrin B (**11**), penicillic acid (**12**), aspyrone (**13**), (−)-(3*R*)-mellein (**14**), aspinonediol (**15**), aspinotriols A (**16**), and aspinotriols B (**17**) were isolated from the ethyl acetate extract of *A. ochraceopetaliformis* (Figure 1). New

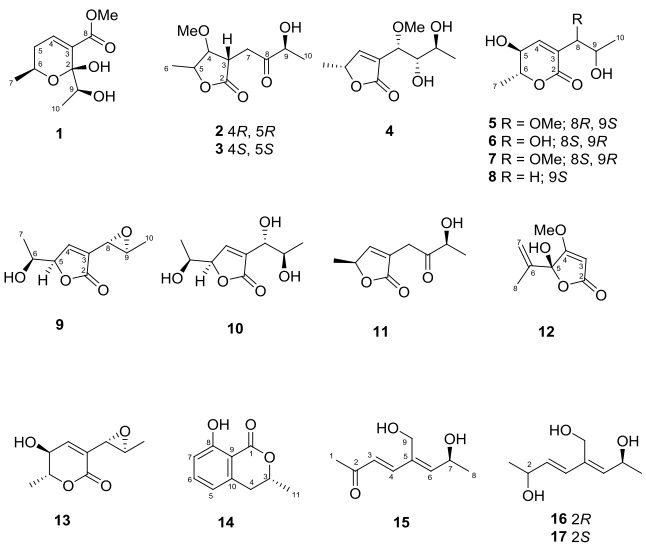


Figure 1. Structures of all isolates (**1**−**17**).

compounds were elucidated and identified by their spectroscopic data as well as by analyzing their stereochemistries with experimental and electronic circular dichroism (ECD) calculations and conformational searches to establish their absolute configurations.

Asperochrapyran (**1**) was obtained as a colorless oil with a specific rotation of $[\alpha]_D^{24}$ = −83 (*c* 0.08, MeOH). The molecular formula ($C_{10}H_{16}O_5$) was confirmed by the analysis of its ^{13}C NMR and HR-ESI-MS data (*m*/*z* 239.08887 [M + Na]$^+$), implying 3 degrees of unsaturation. The infrared (IR) spectrum presented prominent absorption bands for hydroxyl (3415 cm^{-1}), conjugated ester carbonyl (1708 cm^{-1}), and C−O functional groups (1087 cm^{-1}). The UV spectrum showed an absorption at 218 nm. The ^{1}H NMR data of **1** (Table 1) indicated two methyls at δ_H 1.18 (d, *J* = 6.5 Hz) and 1.29 (d, *J* = 7.0 Hz), one olefinic methane at δ_H 6.88 (dd, *J* = 2.3, 1.6 Hz), two oxymethines at δ_H 3.67 (q, *J* = 6.5 Hz) and 4.53 (qdd, *J* = 7.0, 3.8, 3.4 Hz), one methoxy at δ_H 3.75 (s), and one methylene at δ_H 2.26 (ddd, *J* = 17.6, 3.4, 1.6 Hz) and 2.31 (ddd, *J* = 17.6, 3.8, 2.3 Hz). Moreover, the ^{13}C NMR and DEPT data (Table 1) revealed 10 carbon signals. These signals resulted from one ester carbonyl (δ_C 168.5), one olefinic methine (δ_C 141.7), one nonprotonated sp^2 carbon (δ_C 126.4), one hemiacetal carbon (δ_C 97.9), two oxygenated methines (δ_C 66.1 and 73.5), one methylene (δ_C 29.9), one methoxy (δ_C 52.3), and two methyls (δ_C 16.7 and 20.1). According to its MS and NMR data, 2 degrees of unsaturation, C═C double bond and carbonyl functionalities, were disclosed and reduced to one degree of unsaturation. By comparing these data with a previous study, **1** would be inferred as a polyketide framework secondary metabolite.[20]

In interpretation of the COSY spectrum, two fragments, H-4 (δ_H 6.88)/H$_2$-5 (δ_H 2.26 and 2.31)/H-6 (δ_H 4.53)/H-7 (δ_H 1.29) and H-9 (δ_H 3.67)/H-10 (δ_H 1.18), were observed (Figure 2). The HMBC correlations were found from H$_2$-5 to C-3 (δ_C 126.4). These correlations established a 3,6-dihydro-2*H*-pyran ring system. The side-chain moiety of C-9 (δ_C 73.5) and C-10 (δ_C 16.7) was linked to the pyran ring by HMBC cross peaks from both H-9 and H-10 to C-2 (δ_C 97.9). Further, the HMBC correlations from 8-OMe (δ_H 3.75), and H-4 (δ_H 6.88) to C-8 (δ_C 168.5) were used to predict the linkage of ester carbonyl to C-3 (δ_C 126.4) (Figure 2). Based on the above data, the gross structure of **1** was established.

In terms of the stereochemistry of **1**, the C-6 can be scripted by the coupling constants ($J_{5,6}$ = 3.8 and 3.4 Hz). As a result, the H-6 and CH$_3$-7 were sited in pseudo-equatorial and pseudo-axial positions, respectively. All the possible absolute results of **1**, 2*S*,6*S*,9*S*-**1**, 2*R*,6*R*,9*R*-**1**, 2*S*,6*R*,9*S*-**1**, 2*R*,6*S*,9*R*-**1**, 2*S*,6*S*,9*R*-**1**, 2*R*,6*R*,9*S*-**1**, 2*S*,6*R*,9*R*-**1**, and 2*R*,6*S*,9*S*-**1**, were computed by the conformational search algorithms. The experimental $J_{5,6}$ coupling constants of these conformers are composed with an anti and a gauche orientation. However, the calculated results showed the data with both gauche orientations. Hence, the prediction of calculated conformers, 2*S*,6*S*,9*S*-**1**, 2*R*,6*R*,9*R*-**1**, 2*S*,6*S*,9*R*-**1**, and 2*R*,6*R*,9*S*-**1**, cannot match to the $J_{5,6}$ coupling constants. On the other hand, all the other conformers, such as 2*S*,6*R*,9*S*-**1**, 2*R*,6*S*,9*R*-**1**, 2*S*,6*R*,9*R*-**1**, and 2*R*,6*S*,9*S*-**1**, not only conform with the $J_{5,6}$ coupling constants but also meet the data of the absence in NOESY correlation between H$_3$-7 and H-10 (Figure S6). Thus, the absolute configuration of **1** was further determined by comparing the calculated and experimental ECD spectra with Gaussian 09 software. The ECD spectra of possible conformers, 2*S*,6*R*,9*S*-**1**, 2*R*,6*S*,9*R*-**1**, 2*S*,6*R*,9*R*-**1**, and 2*R*,6*S*,9*S*-**1**, are shown in Figure 3. The experimental ECD spectrum of **1** was approximate to 2*R*,6*S*,9*S*-**1**, which exhibited a calculated ECD spectrum with positive Cotton effects at 240 and 270 nm and negative Cotton effects at 223 and 246 nm. Therefore, the structure and absolute stereochemistry of **1** were completely elucidated as descripted, and it was named asperochrapyran (**1**).

Asperochralactone A (**2**) was isolated as a colorless oil, and the high-resolution ESI-MS data showed a molecule peak at *m*/*z* 239.08889 [M + Na]$^+$, which indicated the molecular formula as $C_{10}H_{16}O_5$ and 3 as the index of hydrogen deficiency. The γ-lactone, ketone, and C−O signals were found in the IR spectrum at 1766, 1720, and 1078 cm^{-1}, respectively. In the ^{1}H NMR spectrum of **2** (Table 1), the proton resonances suggested two methyls at δ_H 1.43 (d, *J* = 7.2 Hz) and 1.56 (d, *J* = 6.4 Hz), three oxymethines at δ_H 3.65

https://dx.doi.org/10.1021/acsomega.0c02489
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Table 1. ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR Spectroscopic Data of Compound 1 in CD_3OD and Compounds 2, 4, and 5 in $CDCl_3$; ^{1}H (600 MHz) and ^{13}C (150 MHz) NMR Spectroscopic Data of Compound 3 in $CDCl_3$

	1		2		3		4		5	
no.	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C
2		97.9, C		175.2, C		175.8, C		169.7, C		165.8, C
3		126.4, C	3.01, dt (7.6, 4.8)	43.7, CH	3.08, dt (6.6, 4.6)	41.1, CH		126.8, C		129.7, C
4	6.88, dd (2.3, 1.6)	141.7, CH	3.65, dd (7.6, 6.4)	86.7, CH	3.90, t (6.6)	81.5, CH	7.00, dd (6.6, 1.8)	150.1, CH	6.84, dd (2.6, 0.8)	147.7, CH
5			4.38, quint (6.4)	79.9, CH	4.80, quint (6.6)	77.5, CH	4.65, quint (6.6)	66.5, CH	4.23, dd (8.5, 2.6)	68.4, CH
5a	2.31, ddd (17.6, 3.8, 2.3)	29.9, CH_2								
5b	2.26, ddd (17.6, 3.4, 1.6)									
6	4.53, qdd (7.0, 3.8, 3.4)	66.1, CH	1.56, d (6.4)	20.2, CH_3	1.38, d (6.6)	14.3, CH_3	1.39, d (6.6)	22.9, CH_3	4.33, dq (8.5, 6.3)	80.8, CH
7	1.29, d (7.0)	20.1, CH_3					4.81, dd (1.8, 1.8)	74.8, CH	1.43, d (6.3)	18.4, CH_3
7a			3.09, dd (17.6, 4.8)	36.0, CH_2	3.02, dd (18.2, 4.6)	35.5, CH_2				
7b			2.93, dd (17.6, 4.8)		2.82, dd (18.2, 4.6)					
8		168.5, C		210.0, C		209.8, C	4.33, dd (4.5, 1.8)	85.2, CH	3.89, dd (4.4, 0.8)	83.5, CH
9	3.67, q (6.5)	73.5, CH	4.32, q (7.2)	72.9, CH	4.29, q (7.1)	73.1, CH	3.95, qd (6.6, 4.5)	67.8, CH	3.80, qd (6.4, 4.4)	70.2, CH
10	1.18, d (6.5)	16.7, CH_3	1.43, d (7.2)	20.0, CH_3	1.43, d (7.1)	19.9, CH_3	1.33, d (6.6)	18.9, CH_3	1.17, d (6.4)	19.5, CH_3
4-OMe			3.41, s	58.5, CH_3	3.39, s	58.1, CH_3				
7-OMe							3.42, s	55.7, CH_3		
8-OMe	3.75, s	52.3, CH_3							3.32, s	57.9, CH_3
9-OH			3.25, br s							

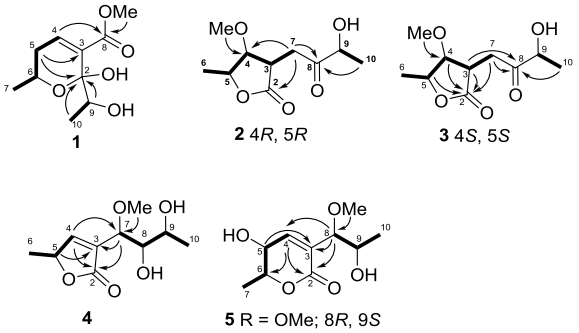

Figure 2. Key COSY ($^{1}H-^{1}H$) and HMBC (H → C) correlations of 1−5.

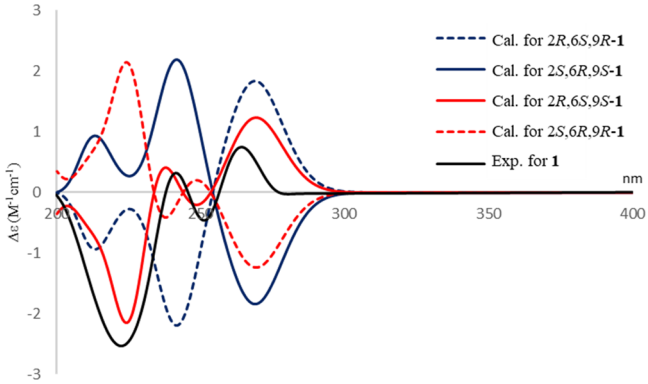

Figure 3. Calculated and experimental ECD spectra of 1.

(dd, J = 7.6, 6.4 Hz), 4.32 (q, J = 7.2 Hz), and 4.38 (quint, J = 6.4 Hz), one methylene at δ_H 2.93 (dd, J = 17.6, 4.8 Hz) and 3.09 (J = 17.6, 4.8 Hz), one methine at δ_H 3.01 (dt, J = 7.6, 4.8 Hz), and one methoxy at δ_H 3.41 (s). Furthermore, the ^{13}C and DEPT NMR data of **2** (Table 1) denoted one carbonyl (δ_C 210.0), one ester carbonyl (δ_C 175.2), three oxygenated methines (δ_C 72.9, 79.9, and 86.7), one methoxy (δ_C 58.5), one methylene (δ_C 36.0), one methine (δ_C 43.7), and two methyls (δ_C 20.0 and 20.2). According to the measured IR, MS, NMR, and UV (212 nm) spectral data analysis and referring to a previous report,[20] compound **2** was identified as a polyketide secondary metabolite with 3 unsaturated degrees counted in γ-lactone and ketone moieties.

The COSY correlations between H_2-7 (δ_H 2.93 and 3.09)/H-3 (δ_H 3.01)/H-4 (δ_H 3.65)/H-5 (δ_H 4.38)/H_3-6 (δ_H 1.56) and H-9 (δ_H 4.32)/H_3-10 (δ_H 1.43) indicated two partial fragments (Figure 2). The HMBC correlations of H_2-7/C-2 (δ_C 175.2) and 4-OMe (δ_H 3.41)/C-4 (δ_C 86.7) and aforementioned COSY fragments were used to elaborate the γ-lactone moiety (Figure 2). Moreover, the HMBC correspondence from H_2-7 and H_3-10 to C-8 (δ_C 210.0) and the remaining COSY fragment suggested the connection with the γ-lactone moiety (Figure 2). Thus, the planar structure of **2** was established. Additionally, the NOESY correlation of H-3/H-4/H-5 was observed (Figure S12), which indicated that those protons were at the same orientation.

https://dx.doi.org/10.1021/acsomega.0c02489
ACS Omega 2020, 5, 20991−20999

The absolute configuration was elucidated with ECD spectroscopies. Four possible candidates, 3*S*,4*R*,5*R*,9*S*-**2**, 3*R*,4*S*,5*S*,9*S*-**2**, 3*R*,4*S*,5*S*,9*R*-**2**, and 3*S*,4*R*,5*R*,9*R*-**2**, were computed by molecular modeling software, Spartan 16, to conduct the results of the conformation search. ECD spectra of each conformer were further calculated by Gaussian 09 as well. The results showed that the experimental ECD spectrum exhibited positive Cotton effects at 221 and 278 nm and negative ones at 243, 316, and 377 nm. The data displayed high similarity to the calculated ECD pattern of 3*S*,4*R*,5*R*,9*S*-**2** (Figure S34). Therefore, the absolute stereochemistry of compound **2** was deduced, and the name of the new compound, asperochralactone A, was given.

Asperochralactone B (**3**) was purified as a colorless oil with a specific optical rotation of $[\alpha]_D^{24} = -74$ (*c* 0.03, MeOH) and possessed a molecular formula of $C_{10}H_{16}O_5$ and 3 degrees of unsaturation deduced from HR-ESI-MS data (*m*/*z* 239.08889 $[M + Na]^+$). Two methyl groups [δ_H 1.38 (d, *J* = 6.6 Hz) and 1.43 (d, *J* = 7.2 Hz)], three oxymethines [δ_H 3.90 (t, *J* = 6.6 Hz), 4.29 (q, *J* = 7.1 Hz), and 4.80 (quint, *J* = 6.6 Hz)], one methylene [δ_H 2.82 (dd, *J* = 18.2, 4.6 Hz) and 3.02 (dd, *J* = 18.2, 4.6 Hz)], one methine [δ_H 3.08 (dt, *J* = 6.6, 4.6 Hz)], and one methoxy [δ_H 3.39 (s)] were revealed from the ^{1}H NMR spectrum of **3** (Table 1). The ^{13}C and DEPT NMR spectra of **3** (Table 1) indicated that 10 carbons can be categorized into one carbonyl (δ_C 209.8), one ester carbonyl (δ_C 175.8), three oxygenated methines (δ_C 73.1, 77.5, and 81.5), one methoxy (δ_C 58.1), one methylene (δ_C 35.5), one methine (δ_C 41.1), and two methyls (δ_C 14.3 and 19.9). The IR, MS, NMR, and UV data of **3** shared high similarity to compound **2**, implying that these two molecules possess the same framework.

The relative configuration of **3** was assigned on the basis of NOESY correlations. The NOESY cross peaks of H3-6/H-3/4-OMe and H-4/H-5 suggested that those two groups showed different orientations (Figure S18). The absolute configuration of **3** was further determined by comparing the ECD spectra between computational and experimental results to **2**. Two possible conformers, 3*S*,4*S*,5*S*,9*S*-**3** and 3*R*,4*R*,5*R*,9*S*-**3**, were simulated by Gaussian 09, which provided the calculated ECD spectra (Figure S35) for conformation search. The pattern of ECD curves of 3*S*,4*S*,5*S*,9*S*-**3** demonstrated high similarity to the experimental ECD spectrum of **3**. Therefore, on the basis of the above data, the structure of **3** was identified and named asperochralactone B.

Asperochralactone C (**4**) was obtained as a colorless oil. Three degrees of unsaturation was estimated on the basis of its molecular formula ($C_{10}H_{16}O_5$), which was determined by the analysis of its HR-ESI-MS data (*m*/*z* 239.08889 $[M + Na]^+$). The hydroxyl (3420 cm^{-1}), *α*,*β*-unsaturated-*γ*-lactone (1748 and 1666 cm^{-1}),[20] and C−O (1087 cm^{-1}) signals were observed in the IR spectrum. The ^{1}H NMR of **4** (Table 1) displayed two methyl groups at δ_H 1.33 (d, *J* = 6.6 Hz) and 1.39 (δ_H, *J* = 6.6 Hz), one olefinic methane at δ_H 7.00 (dd, *J* = 6.6,1.8 Hz), four oxymethines at δ_H 3.95 (qd, *J* = 6.6, 4.5 Hz), 4.33 (dd, *J* = 4.5, 1.8 Hz), 4.65 (quint, *J* = 6.6 Hz), and 4.81 (dd, *J* = 1.8, 1.8 Hz), and one methoxy at δ_H 3.42 (s). The ^{13}C and DEPT NMR spectra (Table 1) revealed one ester carbonyl (δ_C 169.7), one olefinic methine (δ_C 150.1), one non-protonated sp^2 carbon (δ_C 126.8), four oxygenated methines (δ_C 66.5, 67.8, 74.8, and 85.2), one methoxy (δ_C 55.7), and two methyls (δ_C 18.9 and 22.9).

The ^{1}H−^{1}H COSY spectrum (Figure 2) showed correlations between H-4 (δ_H 7.00)/H-5 (δ_H 4.65)/H_3-6 (δ_H 1.39) and H-7 (δ_H 4.81)/H-8 (δ_H 4.33)/H-9 (δ_H 3.95)/H_3-10 (δ_H 1.33). The *α*,*β*-unsaturated-*γ*-lactone moiety was established by the COSY fragment (H4/H5/H_3-6) and the HMBC correlations of H-4/C-2 (δ_C 169.7) and H-5/C-3 (δ_C 126.8) (As shown in Figure 2). Furthermore, the side-chain fragment was attached to the *α*,*β*-unsaturated-*γ*-lactone by HMBC cross-peak correlations (H-4/C-7 and H-7/C-2 and C-3). The methoxy group was substituted to C-7, which was confirmed by an HMBC correlation of 7-OMe/C-7. According to aforementioned data, the planar structure of **4** was determined.

The relative configuration of **4** was assigned by coupling constant analyses and experimental ECD. According to a previous report for xylogibloactone,[20] the coupling constant of *erythro* was more than 4.0 Hz and that of the *threo* was less than 2.5 Hz.[20,21] The coupling constants of H-7/H-8 (1.8 Hz) and H-8/H-9 (4.5 Hz) exhibited *threo* and *erythro* relative configurations, respectively. Notably, the absolute configuration at the *γ* site with a methyl or methoxy substitution on *α*,*β*-unsaturated-*γ*-lactone can be determined by the Cotton effect between 200−235 and 235−270 nm. The positive Cotton effect at 200−235 nm and a negative one at 235−270 nm represent a *β* configuration, and the negative Cotton effect at 200−235 nm and a positive one at 235−270 nm show an *α* configuration.[22−24] According to the experimental ECD spectra (Figure S35), the H-5 of **4** displayed an *α* orientation and the configuration was in the *R* form. Additionally, the calculated ECD spectra of 5*R*,7*R*,8*R*,9*R*-**4** and 5*R*,7*S*,8*S*,9*S*-**4** were conducted by Gaussian 09. The absolute stereochemistry of **4** was assigned to be 5*R*,7*S*,8*S*,9*S* by ECD data (Figure S36). Finally, the structure of compound **4** was elucidated and named asperochralactone C.

Asperochralactone D (**5**) was isolated as a colorless oil. The molecular formula of $C_{10}H_{16}O_5$ was deduced for **5** based on a pseudo ion peak at *m*/*z* 239.08881 $[M + Na]^+$ in the HR-ESI-MS and indicated 3 degrees of unsaturation. The IR spectrum illustrated hydroxyl (3409 cm^{-1}), *α*,*β*-unsaturated-*δ*-lactone (1721 and 1650 cm^{-1}),[25] and C−O (1084 cm^{-1}) functionalities. Furthermore, in analyzing its UV (208 nm) and 1D and 2D NMR spectral data, this compound exhibited high similarity to compound **7**.[20] However, the opposite optical rotation values of compounds **5** (+64) and **7** (−41.6)[20] implied that these two compounds have different stereochemistries. The relative configurations of C-5, C-6, C-8, and C-9 were further determined by coupling-constant analyses.

Considering the conformation between H-5 and H-6, the coupling constant of the *trans* form was usually detected around 7.0 Hz, and the *cis* form would be observed near 3.0 Hz.[2,25−27] The coupling constant of H-5/H-6, calculated as 8.5 Hz, suggested the existence of a *trans* conformation. On the other hand, according to previous studies, the conformation between H-8/H-9 should be regarded as *threo*, while the coupling constant was more than 6.3 Hz. Furthermore, a coupling constant less than 5.0 Hz would be *erythro*.[20,28−30] The *J* value between H-8/H-9 was found to be 4.4 Hz, and the conformation would be considered as *erythro*. In addition, the absolute configuration of **5** was elucidated by comparing the experimental ECD spectra with the calculated results. The experimental ECD spectrum of **5** showed a negative Cotton effect near 270 nm (*n* to *π**), which suggested an *S* configuration at C-5,[31] and C-6 was assigned to an *R* configuration accordingly. Due to the relative conformation between C-8 and C-9 assigned as *erythro*, the possible absolute stereochemistry of **5** would be 5*S*,6*R*,8*S*,9*R* or 5*S*,6*R*,8*R*,9*S*.

https://dx.doi.org/10.1021/acsomega.0c02489
ACS Omega 2020, 5, 20991−20999

Compound **7** was reported to possess a 5*S*,6*R*,8*S*,9*R* configuration,[20] which implied that the structure of **5** should be 5*S*,6*R*,8*R*,9*S* according to the aforementioned information. In order to deduce the absolute configuration, the computational procedures were carried out. The pattern of the ECD curve of the experimental result was approximate to the ECD curve simulated for 5*S*,6*R*,8*R*,9*S*-**5** (Figure S37) and conformation search result was calculated by carbon chemical shifts (Figure S42). Consequently, the structure and absolute configuration of **5** were elucidated, and the name asperochralactone D was given.

Because the absolute configuration of compound **6** has not been established yet,[32] a series of computational experiments were carried out for determining this issue. The experimental ECD displayed a negative Cotton effect at 260 nm. It suggested an *S* configuration at C-5. Moreover, the experimental ECD spectrum of **6** was compared with **5** (8*R*,9*S*) and **7** (8*S*,9*R*).[25] The pattern of the ECD curve of **6** exhibited high similarity to that of **7** (Figure S37). Thus, this suggested that the absolute stereochemistry of **6** can be assigned as 5*S*,6*R*,8*S*,9*R* and named (5*S*,6*R*,8*R*,9*R*)-8,9-dihydroxy-8,9-deoxyaspyrone.

In this study, the conformational search was used to simulate the dynamic balance of compounds in the solvent phase. The conformational search conformers including structures with a flexible chain were also considered and calculated. Due to insufficient samples for chemical modification to help in determination of stereochemistry, absolute configurations of isolated compounds were speculated by spectral data.

Fungi produce many secondary metabolites that exhibit a wide range of biological activities.[33] Polyketides are a class of fungal secondary metabolites, and many of them demonstrate fascinating biological activities.[34] Therefore, the isolated compounds were tested for several bioactivities such as cytotoxicity and anti-inflammation. Due to limited sample amounts, compounds **1**−**4** and **6** were not able to be evaluated in their anti-inflammation assay. Compounds **5** and **7**−**17** were tested for anti-inflammation activity against the response of human neutrophils stimulated by formyl-methionyl-leucyl phenylalanine (fMLP). Compounds **9**, **13**, and **14** exerted an anti-inflammatory effect on inhibiting superoxide anion generation with 30 ± 9%, 29 ± 9%, and 26 ± 12%, respectively, at a concentration of 10 μM (Table 2). Furthermore, the capacities of elastase release inhibition after administrations of compounds **9**, **10**, **12**, and **14** were revealed as 25 ± 4%, 38 ± 8%, 25 ± 5%, and 34 ± 9%, respectively, at a concentration of 10 μM (Table 2). In these assays, LY294002 was taken as the positive control and showed 99 ± 1% superoxide anion inhibition and 73 ± 1% elastase release inhibition at a concentration of 10 μM.[35]

In addition, the cytotoxicity of compounds **1**−**17** was evaluated against three human cancer cell lines, including HepG2 (hepatoma), MDA-MB-231 (human breast carcinoma), and A549 (human lung adenocarcinoma). Compounds **9** and **12** were active in the cytotoxicity evaluation against the HepG2 cancer cell line (IC_{50} = 42.9 ± 0.5 and 32.9 ± 0.0 μM, respectively). Moreover, compound **12** displayed cytotoxic activity against MDA-MB-231 and A549 cancer cell lines with the IC_{50} values of 39.4 ± 0.0 and 25.9 ± 1.8 μM, respectively (Table 3).

The plausible biosynthesis pathway (Figure 4) was proposed in that compounds **1**−**7**, **11**, and **13** were composed of 3-oxobutanoic acid with 3,5-dioxohexanoic acid or 3-oxopentanoic acid. All new compounds were derived from the polyketide synthase (PKS) pathway with a series of condensation, cyclization, decarbonation (acetyl-CoA carboxylase), dehydration (dehydratase), methylation (methyl transferase), reduction (enoyl reductase), and oxidation (oxidase).[36]

Table 2. Anti-Inflammatory Results of Compounds 5 and 7−17

compound	superoxide anion inhibition (%)	elastase release inhibition (%)
5	0 ± 6	14 ± 5*
7	16 ± 8	12 ± 6
8	2 ± 3	11 ± 3*
9	30 ± 9*	25 ± 4
10	8 ± 3	38 ± 8**
11	8 ± 6	21 ± 7
12	3 ± 1**	25 ± 5
13	29 ± 9*	12 ± 8
14	26 ± 12	34 ± 9
15	7 ± 6	1 ± 5
16	0 ± 6	17 ± 6
17	13 ± 3 *	1 ± 8
LY294002[a]	99 ± 1***	73 ± 1***

[a]LY294002 was used as the positive control.[35] Percentage of inhibition (Inh %) at 10 μM concentration. Results are presented as mean ± SEM (n = 3−4). *P < 0.05, **P < 0.01 compared with the control (DMSO).

Table 3. Cytotoxicity Results (IC_{50}, μM)[a]

compound[b]	HepG2	MDA-MB-231	A549
9	42.9 ± 0.5	>100	>100
12	32.9 ± 0.0	39.4 ± 0.0	25.9 ± 1.8
doxorubicin[c]	0.4 ± 0.0	0.8 ± 0.2	0.2 ± 0.0

[a]IC_{50} values are taken as mean ± SD (n = 3). [b]Compounds **1**−**8**, **10**−**11**, and **13**−**17** were inactive with IC_{50} values of >100 μM. [c]Positive control.

CONCLUSIONS

In the current study, five new polyketides, asperochrapyran (**1**) and asperochralactones A−D (**2**−**5**), together with 12 known secondary metabolites (**6**−**17**) were isolated from the fungal strain *A. ochraceopetaliformis*. The structure of each new compound possesses at least three chiral centers, which causes difficulty to determine their absolute stereochemistry. Previous research had never reported the determination of the absolute configurations from this type of polyketide secondary metabolites. Thus, this unusual issue is an imperative task that needs to be clarified. Additionally, the new compounds exhibited an oil-like appearance, whose configurations are difficult to elucidate by a single crystal X-ray analysis method. Therefore, coupling constant, NOESY, experimental, and calculated ECD spectroscopic analyses were used extensively to assign the absolute configuration. Our findings suggested the stereochemistry in a series of special fungal polyketide secondary metabolites, and the plausible biosynthesis pathway of key isolates was proposed.

MATERIALS AND METHODS

General Experimental Procedures. Polymerase chain reaction (PCR) amplifications were reacted by FlexCycler PCR. Optical rotations were measured with a JASCO P-2000

https://dx.doi.org/10.1021/acsomega.0c02489
ACS Omega 2020, 5, 20991−20999

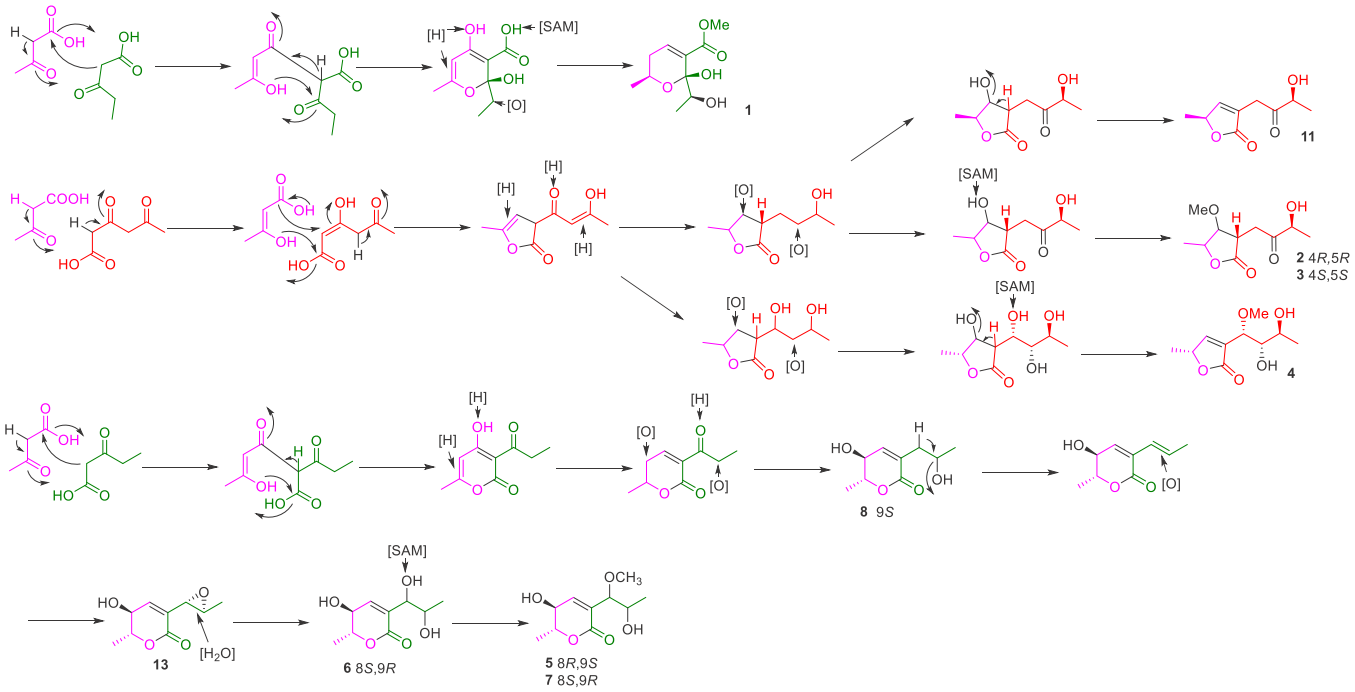

Figure 4. Plausible biosynthesis pathway of new compounds and their analogues.

polarimeter. UV spectra were recorded on a JASCO V-530 UV−vis spectrophotometer, and experimental ECD spectra were measured on a JASCO J-815 spectropolarimeter. IR spectra were obtained on a JASCO FT/IR-4600 Fourier transform infrared spectrometer. 1D and 2D NMR spectra were performed on a JEOL JNM-ECS 400 MHz NMR spectrometer (^{1}H, 400 MHz; ^{13}C, 100 MHz), Varian Mercury Plus 400 MHz FT-NMR (^{1}H, 400 MHz; ^{13}C, 100 MHz), and Varian VNMRS 600 MHz FT-NMR (^{1}H, 600 MHz; ^{13}C, 150 MHz) in $CDCl_3$, CD_3OD, and CD_3COCD_3, respectively. Mass spectra were obtained from a Waters 2695 separation module (ESI-MS) and Bruker FT-MS SolariX (HR-ESI-MS). Column chromatography was carried out on silica gel 60 (0.063−0.200 mm and 0.040−0.063 mm, Merck) and Sephadex LH-20 (Fine Chemicals AB, Uppsala, Pharmacia). Thin-layer chromatography (TLC) analyses were performed using silica gel 60, F254, and RP-18, F254S (0.20 nm, Merck, Germany). Semi-preparative HPLC was performed on Shimadzu LC-10 AD, Shimadzu LC-20AT, or Jasco PU-980 pumps, an SPD-M10A diode array, SPD-10A UV−vis or UV-970 UV−vis detectors, and SCL-10A or CBM-20A controllers with Luna phenylhexyl, 100 Å, 250 × 10 mm, Phenomenex or Luna CN, 100 Å, 250 × 10 mm, Phenomenex columns.

Fungal Material. The fungus, *A. ochraceopetaliformis*, was isolated from *A. brownii* collected from the Dr. Cecilia Koo Botanic Conservation Center (KBCC), Pingtung, Taiwan. KBCC is the biggest botanical garden and deposits over 30,000 living plant materials. The leaves of *A. brownii* were washed and air-dried. The dry leaves were soaked in 0.01% Tween 20 (aq), ddH_2O, and 0.01% bleach (aq) to clean the surface. The washed leaves were moved into a laminar flow after the treatment of 75% alcohol (aq), and we used sterilized scissors and tweezers to cut the central part of leaf (5 mm × 5 mm). The mesophyll of the leaf was bisected and seeded on the potato dextrose agar (PDA) plate. Then, the plates were incubated in a 25 °C incubator. After repeated purification, the pure strains were stored in 2 mL cryogenic vials (Nalgene, Thermo) with 1.5 mL of potato dextrose broth (PDB) and 0.2 mL of sterilized glycerol and stored in a −80 °C refrigerator. The fungal strain was identified by D.-Y.Y. and C.-Y.L. A voucher specimen (code no. K004643) was deposited in the Graduate Institute of Natural Products, College of Pharmacy, Kaohsiung Medical University, Kaohsiung, Taiwan.

Species Identification. The fungus *A. ochraceopetaliformis* was identified on the basis of its morphology and a pair of internal transcribed spacers (ITS1-5.8S-ITS2) rRNA gene analysis using universal fungal primers. DNA was extracted by using the AxyPrep Multisource Genomic DNA miniprep kit (AxyPrep, #02815KC1) following the manufacturer's protocol. PCR amplifications were accomplished by using FlexCycler[2] (Analytik Jena, Germany) with the following conditions: 95 °C (5 min), 30 cycles of 95 °C (30 s), 55 °C (30 s), and 72 °C (40 s), with the last extension at 72 °C (7 min). The PCR products were sent to the Mission Biotech Co., Ltd. for sequencing services after purification. The results of 18S rRNA gene sequences were blasted with the National Center for Biotechnology Information (NCBI) database for species identification. The reversal and forwarding of the 18S rRNA gene sequence displayed 99% sequence identity with *A. ochraceopetaliformis* (GenBank accession no. FJ7976981).

Fermentation, Extraction, and Isolation. The fungus *A. ochraceopetaliformis* was cultivated by using 120 Erlenmeyer flasks (500 mL) with each flask containing 300 mL of PDB medium. These flasks were incubated on the rotator shaker at 150 rpm at 25 °C for 7 days. The whole broth was filtered to give the filtrate from mycelia. The filtrate was extracted by ethyl acetate (EtOAc), and the EtOAc layer was concentrated by rotary evaporators to obtain the crude extract (10.9 g). The crude extract was loaded to the Sephadex LH-20 column and eluted with methanol (MeOH) to yield five fractions (Fr. 1−5). Compound **14**[25] (3.8 mg) was precipitated from Fr. 5. Fr. 3 was isolated by a silica gel column stepwise eluted with dichloromethane (CH_2Cl_2) and MeOH from 29:1 to 0:1 to give 10 fractions (Fr. 3.1−3.10). Fr. 3.3 was further separated

https://dx.doi.org/10.1021/acsomega.0c02489
ACS Omega 2020, 5, 20991−20999

by a silica gel column eluted with CH_2Cl_2 and MeOH from 24:1 to 0:1 to furnish nine fractions (Fr. 3.3.1−3.3.9). Fr. 3.3.5 (100.4 mg) was purified by reversed-phase (RP) HPLC (Luna phenyl-hexyl, 100 Å, 250 × 10 mm, Phenomenex, flow rate of 2.0 mL/min, 35% MeOH (aq)) to afford compounds **9**[25] (20.4 mg) and **13**[12] (4.5 mg). Fr. 3.5 was submitted to silica gel column chromatography eluted with CH_2Cl_2 and MeOH from 39:1 to 0:1 to get 10 fractions (Fr. 3.5.1−3.5.10). Fr. 3.5.5 (16.8 mg) was subjected to RP-HPLC (Luna phenyl-hexyl, 100 Å, 250 × 10 mm, Phenomenex, flow rate of 2.0 mL/min, 32% MeOH (aq)) to give compound **2** (1.0 mg). Fr. 3.5.6 (80.3 mg) was isolated by RP-HPLC (Luna phenyl-hexyl, 100 Å, 250 × 10 mm, Phenomenex, flow rate of 2.0 mL/min, 33% MeOH (aq)) to yield compound **11**[8] (3.2 mg) and three fractions (Fr. 3.5.6.2−3.5.6.4). Compound **3** (1.1 mg) was purified by normal-phase (NP) HPLC (Luna CN, 100 Å, 250 × 10 mm, Phenomenex, flow rate of 2.0 mL/min, hexanes and EtOAc = 1:1). Fr. 3.5.7 (85.3 mg) was isolated by RP-HPLC (Luna phenyl-hexyl, 100 Å, 250 × 10 mm, Phenomenex, flow rate of 2.0 mL/min, 40% MeOH (aq)) to obtain compounds **1** (1.6 mg) and **12**[12] (56.2 mg). Fr. 3.5.9 was separated by a silica gel column (CH_2Cl_2 and MeOH from 24:1 to completely MeOH) to give eight fractions (Fr. 3.5.9.1−3.5.9.8). Compounds **4** (2.5 mg), **5** (4.8 mg), and **7**[22] (3.0 mg) were isolated by RP-HPLC (Luna phenyl-hexyl, 100 Å, 250 × 10 mm, Phenomenex, flow rate of 2.0 mL/min, 25% MeOH (aq)) from Fr. 3.5.9.5 (36.1 mg). Furthermore, Fr. 3.7 (107.7 mg) was subjected to RP-HPLC (Luna phenyl-hexyl, 100 Å, 250 × 10 mm, Phenomenex, flow rate of 2.0 mL/min, 25% $MeOH_{(aq)}$) to afford compounds **8**[37] (30.4 mg) and **15**[11] (1.6 mg). Compounds **6**[32] (3.0 mg), **10**[35] (1.6 mg), **16**[11] (6.4 mg), and **17**[11] (6.5 mg) were purified by RP-HPLC (Luna phenyl-hexyl, 100 Å, 250 × 10 mm, Phenomenex, flow rate of 2.0 mL/min, 15% MeOH (aq)) from Fr. 3.9 (98.9 mg).

Asperochrapyran (**1**): colorless oil; $[\alpha]_D^{24}$ = −83 (*c* 0.08, MeOH); UV $(MeOH)_{\lambda max}$ (log *ε*) 218 (4.00) nm; ECD (6.5 × 10^{-5} M, $MeOH)_{\lambda max}$ (Δ*ε*): 289 (−0.25), 265.5 (+0.19), 251.0 (−0.95), 223.5 (−2.71) nm; IR (ATR) ν_{max}: 3415, 2979, 2918, 1708, 1659, 1557, 1438, 1263, 1235, 1087, 885 cm^{-1}; ^1H and ^{13}C NMR spectroscopic data, see Table 1; HR-ESI-MS *m*/*z* 239.08887 $[M + Na]^+$ (calcd for $C_{10}H_{16}O_5Na$, 239.08899).

Asperochralactone A (**2**): colorless oil; $[\alpha]_D^{24}$ = −53 (*c* 0.06, MeOH); UV $(MeOH)_{\lambda max}$ (log *ε*) 212 (3.92) nm; ECD (6.5 × 10^{-5} M, $MeOH)_{\lambda max}$ (Δ*ε*): 279.5 (+0.57), 242.5 (−1.03), 221.0 (+1.01) nm; IR (ATR) ν_{max}: 3381, 2928, 2898, 1766, 1720, 1449, 1282, 1189, 1140, 1078, 883 cm^{-1}; ^1H and ^{13}C NMR spectroscopic data, see Table 1; HR-ESI-MS *m*/*z* 239.08905 $[M + Na]^+$ (calcd for $C_{10}H_{16}O_5Na$, 239.08899).

Asperochralactone B (**3**): colorless oil; $[\alpha]_D^{24}$ = −74 (*c* 0.03, MeOH); UV $(MeOH)_{\lambda max}$ (log *ε*) 212 (3.91) nm; ECD (6.5 × 10^{-5} M, $MeOH)_{\lambda max}$ (Δ*ε*): 310 (−0.50), 274 (+1.23), 237.0 (−0.09), 220.5 (+0.72), 207 (−0.23) nm; IR (ATR) ν_{max}: 3414, 2979, 2920, 1762, 1716, 1650, 1455, 1373, 1234, 1082, 889 cm^{-1}; ^1H and ^{13}C NMR spectroscopic data, see Table 1; HR-ESI-MS *m*/*z* 239.08889 $[M + Na]^+$ (calcd for $C_{10}H_{16}O_5Na$, 239.08899).

Asperochralactone C (**4**): colorless oil; $[\alpha]_D^{24}$ = −26 (*c* 0.05, MeOH); UV $(MeOH)_{\lambda max}$ (log *ε*) 215 (3.64) nm; ECD (6.5 × 10^{-5} M, $MeOH)_{\lambda max}$ (Δ*ε*): 263.5 (+0.30), 228.0 (−1.05) nm; IR (ATR) ν_{max}: 3420, 2978, 2916, 1748, 1666, 1584, 1401, 1382, 1234, 1087, 883 cm^{-1}; ^1H and ^{13}C NMR spectroscopic data, see Table 1; HR-ESI-MS *m*/*z* 239.08882 $[M + Na]^+$ (calcd for $C_{10}H_{16}O_5Na$, 239.08899).

Asperochralactone D (**5**): colorless oil; $[\alpha]_D^{24}$ = +64 (*c* 0.05, MeOH); UV $(MeOH)_{\lambda max}$ (log *ε*) 208 (4.24) nm; ECD (6.5 × 10^{-5} M, $MeOH)_{\lambda max}$ (Δ*ε*): 270.0 (−8.55), 236.0 (+7.53), 212 (−1.55) nm; IR (ATR) ν_{max}: 3409, 2987, 2912, 1721, 1650, 1446, 1378, 1232, 1084, 1042, 888 cm^{-1}; ^1H and ^{13}C NMR spectroscopic data, see Table 1; HR-ESI-MS *m*/*z* 239.08881 $[M + Na]^+$ (calcd for $C_{10}H_{16}O_5Na$, 239.08899).

***In Silico* Calculations.** Structures were built, and we optimized the minimized energy conformers in the MM2 level. After the gross optimization, the data were used to output *xyz* type files and input files for calculating conformational results at MMFF94 by Spartan 16 software (Wavefunction Inc.; Irvine, CA, U.S.A.). These data were submitted into Gaussian 09 software (Gaussian Inc.; Wallingford, CT, U.S.A.) and optimized using the time-dependent density functional theory (TDDFT) methodology at the B3LYP/6-311++G(d,p) level for ECD and the GIAO-DFT at the mpw1pw91/6-311+g-(2d,p) level for NMR in the solvent phase. During the computation of Gaussian 09 software, the calculated ECD and NMR spectra were generated by GaussSum 2.2.5 and GaussView 5.0.8, respectively. TMS was calculated at the same level of theory (δ_{ref} = 183.143 ppm) as the reference compound for the calculated NMR spectra. For conformational searches, the calculated ECD and NMR spectra of compounds were averaged by the proportion of each conformer.[38]

Anti-inflammatory Activity Assay. The assay on superoxide anion generation and elastase release in response to fMLP stimulation of neutrophils was evaluated by the methods published by a co-author of this study, T.-L.H.[39]

Cytotoxicity Assay. The method for cytotoxicity assay was performed as previously described.[40,41] Briefly, three human cancer cell lines, HepG2 (1 × 10^4 cells), A549 (5 × 10^3 cells), and MDA-MB-231 (1 × 10^4 cells), were inoculated onto 96-well plates and treated with the samples (20 μg/mL). The medium was removed after 72 h of incubation then the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (100 μL, 0.5 mg/mL) was added into each well. Then, the plates were incubated at 37 °C for 1 h. The MTT dye was detected by the addition of 100 μL of dimethyl sulfoxide. The absorbance was estimated at 550 nm. The positive control was doxorubicin.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.0c02489.

1D and 2D NMR for compounds **1**−**5**, conformational search results of **1**−**5**, calculated and experimental ECD spectra of **1**−**7**, and calculated ^{13}C chemical shifts against the experimental data of **1**−**5** (PDF)

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https://dx.doi.org/10.1021/acsomega.0c02489
ACS Omega 2020, 5, 20991−20999

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Author Contributions

[+]H.-C.H. and C.-Y.L. contributed equally to this work. H.-C.H. and Y.-H.T. contributed to writing the manuscript, *in silico* calculations, and design of the Table of Contents image. C.-Y.L. and D.-Y.Y. contributed to collecting the physical data, optimizing fungal cultivation, and natural product purification and identification. C.-Y.L., D.-Y.Y., Y.-C.W., and A.H. contributed to the data analysis. T.-L.H., S.-L.C., and C.-H.Y. contributed to the bioassays. The experiment was designed, and the manuscript was revised by Y.-B.C., F.F., and F.-R.C.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This research was funded by the Ministry of Science and Technology, Taiwan, awarded to F.-R.C., grant nos. MOST 105-2628-B-037-001-MY3, MOST 106-2320-B-037-008-MY2, MOST 106-2911-I-037-504, MOST 108-2320-B-037-022-MY3, 108-2811-B-037-511, and 109-2927-I-037-502. In addition, this research was partially funded by the Drug Development and Value Creation Research Center of Kaohsiung Medical University & Department of Medical Research of Kaohsiung Medical University Hospital awarded to F.-R.C. (grant no. KMU-TC108A03-11). We appreciate the Dr. Cecilia Koo Botanic Conservation Center (KBCC) for providing plant material *A. brownii*. We also thank Prof. Chia-Wei Li who is the CEO of KBCC. The plant material and fungal strain were obtained from a project supported by the Ministry of Science and Technology, Taiwan, awarded to Prof. Yi-Ming Arthur Chen, aimed to build an herbal medicine database with KBCC.

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https://dx.doi.org/10.1021/acsomega.0c02489
ACS Omega 2020, 5, 20991−20999