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Cytosporones W and X: Two Mutually Converting Epimers from a Mangrove Endophytic Fungus *Diaporthe* sp. ZJHJYZ-1

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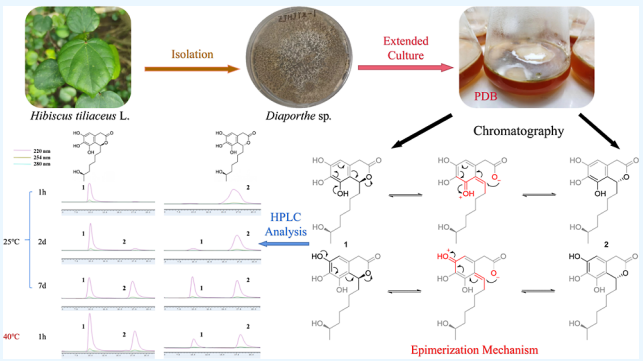
ABSTRACT: Two new octaketides, cytosporones W (**1**) and X (**2**), along with eight known cytosporone derivatives [(±)-**3**−**9**], were isolated from mangrove endophytic fungus *Diaporthe* sp. ZJHJYZ-1. Compounds **1** and **2** were a pair of epimers, whose configuration of C-1 could mutually convert, causing racemization of the lactone ring. The planar structures of compounds were elucidated through detailed 1D, 2D NMR, and HR-ESI-MS analysis. ECD spectra comparison and modified Mosher ester method were applied to determine the absolute configuration of **1** and **2**. In bioassays, (±)-**3** exhibited promising inhibitory activities against *Bacillus subtilis*, *Pseudomonas aeruginosa*, and *Penicillium italicum* with MIC, respectively, for 12.5, 12.5, and 3.13 μM.



1. INTRODUCTION

Tautomeric and stereoisomerized compounds are common in the field of natural products research, especially for ketone−enol,[1−4] enamine−imino,[5] hemiketal−aldol,[6,7] and (retro-)oxa-Micheal addition[8] tautomerism. Convincing hypotheses were proposed to clarify the converting mechanism, and NMR calculation, acid or alkali treatment, chiral-phase high-performance liquid chromatography (HPLC) analysis, and methylation reactions were reported to verify some of those hypotheses.[4−6]

Cytosporones are a series of polyketide derivatives, which were first isolated from *Cytospora* sp.[9] Dothiorelones[10] and pestalotiopsones[11] were subsequently reported, which were actually cytosporone derivatives with similar biosynthetic pathways, possessing different ring-forming approaches. Extensive biological activities were reported for cytosporone derivatives, including antibacterial,[9,12−14] cytotoxic,[10−13,15] antifungal,[16] and anti-inflammatory.[17] Interestingly, except the racemic single-crystal of cytosporone C,[9] there was no study on the stereoisomerization for C-1 of lactone cytosporones.

In this study, two new octaketides, cytosporones W (**1**) and X (**2**), along with eight known cytosporone derivatives, cytosporones B (**6**), C (**4**), (±)-D [(±)-**3**],[9] N (**7**),[18] dothiorelone A (**5**),[10] secocurvularin (**8**),[19] and pestalotiopsone B (**9**),[11] were isolated from ethyl acetate (EA) extracts of the secondary metabolites produced by a mangrove endophytic fungus *Diaporthe* sp. ZJHJYZ-1. Accidentally, the epimerization of **1** and **2** was detected during HPLC analysis, and (±)-**3** were discovered with the enantiomerization equilibrium appearance, but **4** ($[\alpha]^{25}_{D}$ = 0, *c* 0.26, MeOH) was found to be inseparable using multiple chiral columns. The stereoisomerization mechanism for C-1 of **1** and **2** and several methylation reactions aiming at those phenolic hydroxyl groups of **1** and **2** to verify the mechanism were discussed.

Herein, we report the isolation, structural identification, stereoisomerization mechanism, and methylation reactions for cytosporones, W (**1**) and X (**2**), and antimicrobial assays for **1**−**9** (Figure 1).

2. RESULTS AND DISCUSSION

2.1. Structure Identification. Cytosporone W (**1**) was isolated as a light-yellow oil. Its molecular formula $C_{16}H_{22}O_6$ (six degrees of unsaturation) was deduced by the ion peak of HR-ESI-MS *m*/*z* 333.1302 $[M + Na]^+$ (calcd for $C_{16}H_{22}O_6Na^+$, 333.1309). Analysis of its NMR data in CD_3OD (Table 1) showed the presence of one methyl, six methylenes including one adjacent to a carbonyl, two oxymethines, an ester carbonyl carbon, six olefinic carbons with three being oxygenated and one protonated. The $^1H-^1H$ correlation spectroscopy (COSY) signals from H-1 along to H_3-7′ and HMBC correlations from H_3-7′ to C-5′ and C-6′ of **1** revealed the presence of an aliphatic side chain with C-6′ oxygenated. The HMBC correlations from H_2-4 to C-3, C-5, C-4a, C-8a; H-5 to C-4, C-6, C-7, C-4a; and H-1 to C-3, C-4a, C-8a, C-2′ constructed the 6,7,8-trihydrox-

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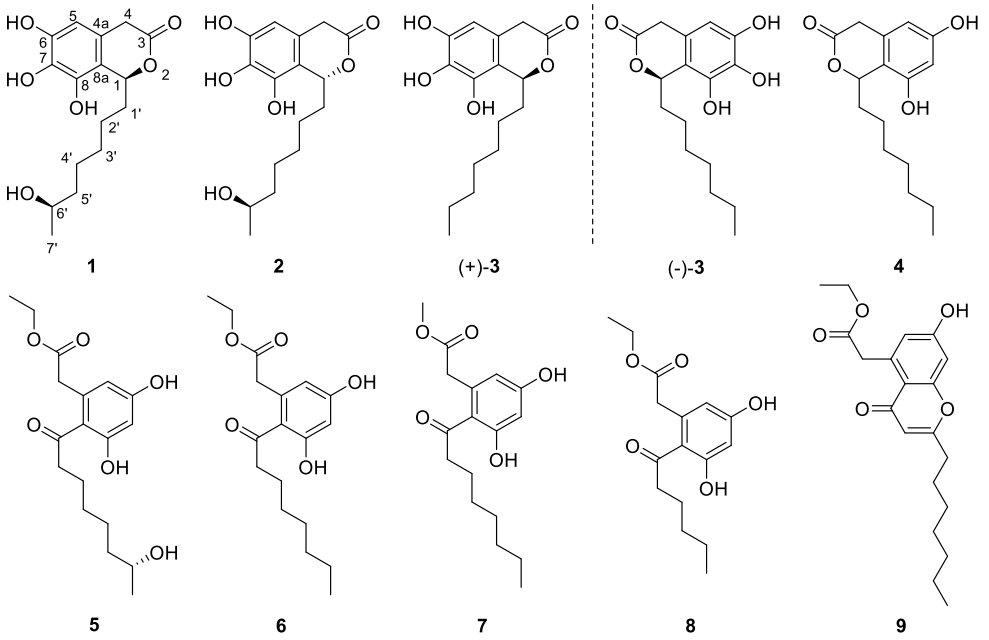

Figure 1. Structures of compounds **1**−**9**.

Table 1. ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR Data of Compounds 1−2

position	1[a]		2[a]	
	δ_C, type	δ_H (J in Hz)	δ_C, type	δ_H (J in Hz)
1	80.2, CH	5.63, dd (8.3, 5.4)	80.2, CH	5.63, dd (8.3, 5.5)
3	174.4, C		174.4, C	
4	35.0, CH_2	3.43, d (19.7)	35.0, CH_2	3.43, d (19.6)
		3.72, d (19.7)		3.72, d (19.6)
4a	114.5, C		114.5, C	
5	106.2, CH	6.19, d (1.1)	106.2, CH	6.19, d (1.1)
6	147.4, C		147.4, C	
7	133.1, C		133.1, C	
8	143.4, C		143.4, C	
8a	121.5, C		121.5, C	
1′	36.8, CH_2	1.84, m	36.8, CH_2	1.83, dtd (14.3, 8.5, 5.5)
2′	26.6, CH_2	1.53, m	26.6, CH_2	1.54, m
3′	26.7, CH_2	1.43, m	26.7, CH_2	1.43, m
4′	30.3, CH_2	1.36, m	30.3, CH_2	1.36, m
5′	40.1, CH_2	1.41, m	40.1, CH_2	1.41, m
6′	68.5, CH	3.71, overlap	68.5, CH	3.71, overlap
7′	23.5, CH_3	1.14, d (6.2)	23.5, CH_3	1.14, d (6.2)

[a]Recorded in CD_3OD within 1 h after separation.

yisochroman-3-one moiety with the aliphatic side chain connected to C-1 (Figure 2).

Cytosporone X (**2**) was also isolated as a light-yellow oil. Its molecular formula $C_{16}H_{22}O_6$ (six degrees of unsaturation) was deduced by the ion peak of HR-ESI-MS m/z 333.1300 $[M + Na]^+$ (calcd for $C_{16}H_{22}O_6Na^+$, 333.1309). Its NMR data were almost the same as **1**, which helped determine the planar structure of **2** as 6,7,8-trihydroxy-1-(6-hydroxyheptyl)-isochroman-3-one.

However, **1** and **2** were isolated from the prior crude fraction by using HPLC with a chiral-ND column, which showed excellent separation capability on this fraction. With the chiral-ND column, **1** and **2** were isolated, respectively, at t_R = 10.0 and

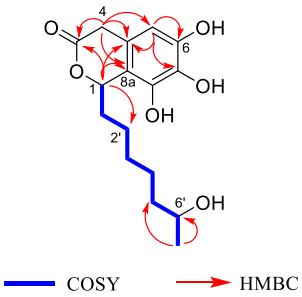

Figure 2. Key ^{1}H−^{1}H COSY and HMBC of compounds **1** and **2**.

17.5 min (the gradient was *n*-hexane/2-propanol v/v, 76:24, flow rate: 1 mL/min), which revealed **1** and **2** might possess different stereo configurations. Interestingly, **1** would slowly turn to nearly equivalent **1** and **2** after separation, and **2** shared the same behavior (Figure 3).

In the meanwhile, (±)-**3** were also isolated by using HPLC with the same chiral-ND column, respectively, at t_R = 7.5 and 10.0 min (the gradient was *n*-hexane/2-propanol v/v, 80:20, flow rate: 1 mL/min), and the structures of (±)-**3** were determined by comparing their NMR data to cytosporone D.[9] Similarly, (±)-**3** showed the enantiomerization equilibrium appearance with HPLC-DAD analysis.[5]

C-1 and C-6′ were two chiral centers of **1** and **2**, and C-1 was the unique chiral center of (±)-**3**. Sharing the similar converting behavior indicated that C-1 was the key to the converting mechanism, and configuration of C-6′ of **1** is the same as **2**, because it is unlikely for a chiral carbon's configuration converting on an aliphatic chain in this very situation, which could be further supported by their chemical shifts (δ_H-1′ 1.84 in **1** while at δ_H-1′ 1.83 in **2**).

To verify the above inferences, the absolute configuration of C-6′ of **1** and **2** were determined by using the modified Mosher ester method.[20,21] The treatment of the mixture of **1** and **2**, respectively, with (*R*)- and (*S*)-MTPA chlorides led to the esterification of 6′-OH, 6-OH, 7-OH, and 8-OH, obtaining (*S*)- and (*R*)-MTPA derivatives. The ^{1}H NMR chemical shift

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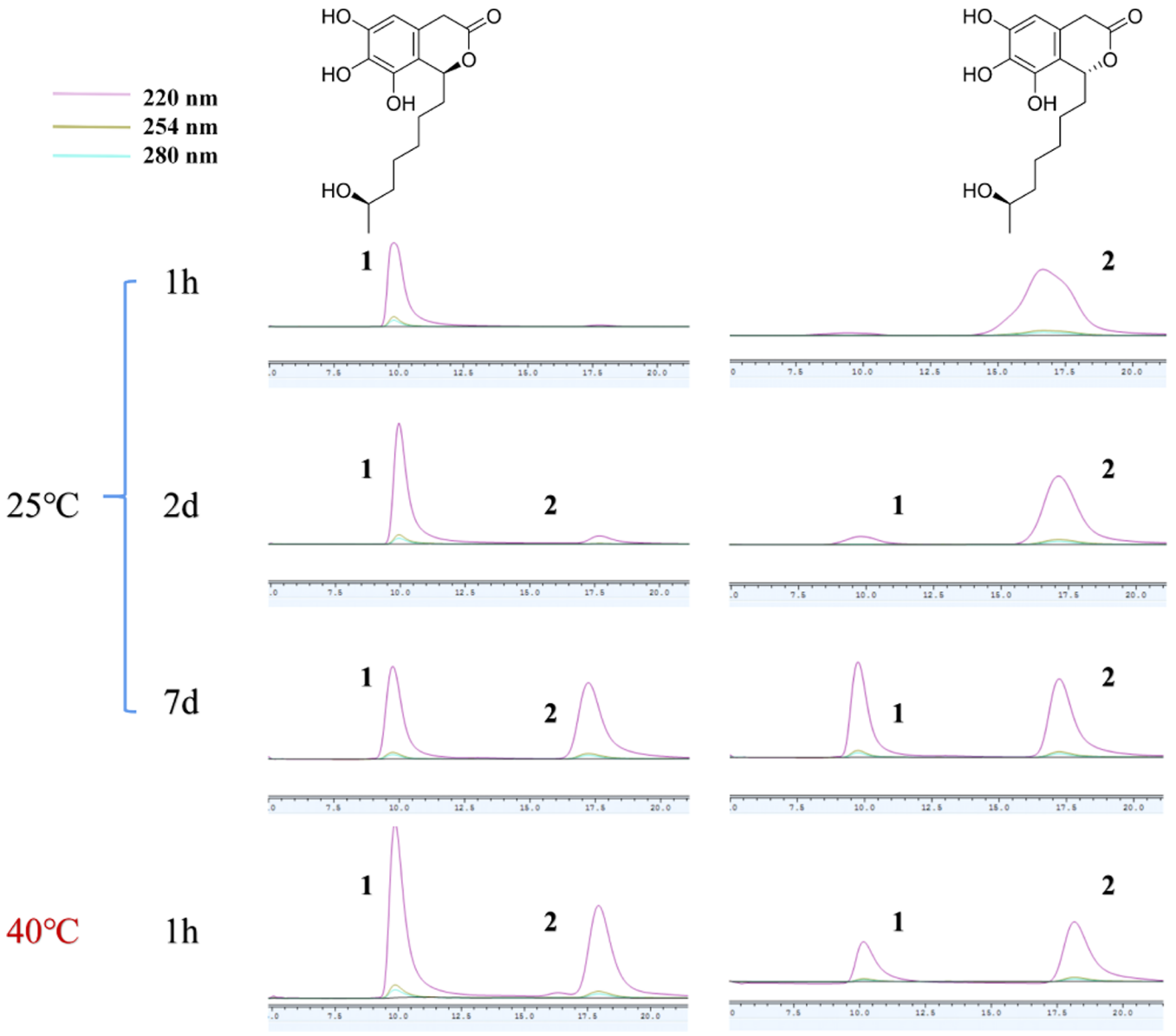

Figure 3. HPLC analysis of **1** and **2** within 7 days at different temperatures.

differences ($\Delta\delta_{S-R}$) were observed (Figure 4). Consequently, the configuration of C-6′ of **1** and **2** was determined as *R*.

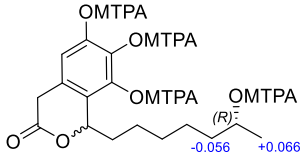

Figure 4. ^{1}H NMR $\Delta\delta_{S-R}$ values of MTPA ester of mixture of **1** and **2**.

Besides, electronic circular dichroism (ECD) spectra of **1** and **2** were acquired within 1 h after separation, which exhibited a good mirror symmetry (Figure 5) and confirmed the stereo configuration of C-1 was indeed converting. The absolute configuration of C-1 of **1** and **2** were deduced by comparison of the experimental and calculated ECD curves. To reduce the molecular flexibility, the aliphatic side chains were simplified as ethyl groups in the calculation.[22] Moreover, C-1 of **1** and **2** were, respectively, assigned to be *S* and *R*.

To sum up, the structures of **1** and **2** were determined as (1*S*,6′*R*)-6,7,8-trihydroxy-1-(6-hydroxyheptyl)isochroman-3-one and (1*R*,6′*R*)-6,7,8-trihydroxy-1-(6-hydroxyheptyl)-isochroman-3-one, which were named as cytosporones W and X.

2.2. Epimerization Mechanism. The plausible mechanism of the epimerization equilibrium between **1** and **2** was proposed (Figure 6).[3−5] The transferring of electrons caused the opening of lactone ring and sp^2 planarization of C-1. Then the oxygen anion could attack C-1 of the intermediate evenly from both directions of the plane, which finally led to the racemization for the lactone ring.

To verify hypotheses that is similar to the above plausible mechanism, methylation methods were performed in previously reported articles.[4,5] Likewise, methylation reactions of the hydroxyl groups of **1** and **2** with different reagents were applied: (i) sodium hydride and iodomethane, (ii) and (iii) thionyl chloride ($SOCl_2$) in methanol (MeOH) in different addition sequences, and (iv) (trimethylsilyl)diazomethane (TMS-CHN_2). The conditions and the results are listed in Table 2.

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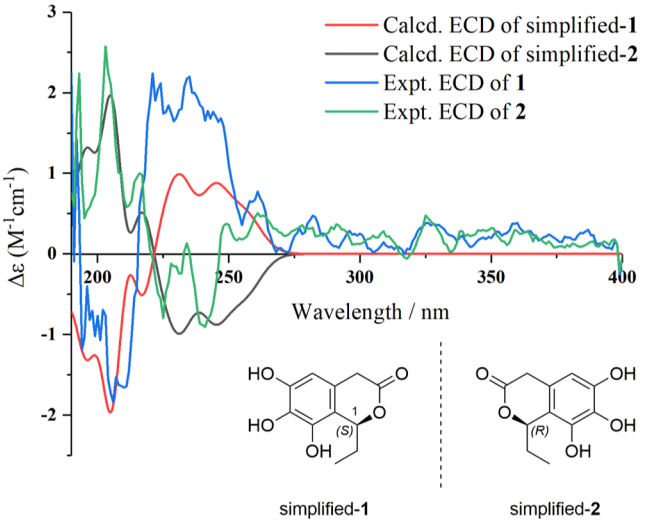

Figure 5. Experimental ECD spectra of **1**, **2**, and calculated ECD spectra of simplified-**1**, **2**.

The crude products of those reactions were analyzed by using HR-ESI-MS. (i) showed no ion peak of any methylation products. (ii) and (iii) showed only ion peak of monomethylation product at *m/z* 347.1470 $[M + Na]^+$ and 347.1464 $[M + Na]^+$ (calcd for $C_{17}H_{24}O_6Na^+$, 347.1465), respectively. (iv) showed ion peak of monomethylation, dimethylation, trimethylation, and tetramethylation products, respectively, at *m/z* 347.1469 $[M + Na]^+$ (calcd for $C_{17}H_{24}O_6Na^+$, 347.1465), *m/z* 361.1624 $[M + Na]^+$ (calcd for $C_{18}H_{26}O_6Na^+$, 361.1622), *m/z* 375.1781 $[M + Na]^+$ (calcd for $C_{19}H_{28}O_6Na^+$, 375.1778), and *m/z* 389.1937 $[M + Na]^+$ (calcd for $C_{20}H_{30}O_6Na^+$, 389.1935).

However, the products were not successfully prepared to verify the above converting mechanism, because of the lack of **1**, **2**, (±)-**3**, and low yield of the fourth reaction.

2.3. Antimicrobial Assays. The isolated compounds **1**−**9** were evaluated for antibacterial activities against methicillin-resistant *Staphylococcus aureus*, *Bacillus subtilis*, *Salmonella typhimurium*, and *Pseudomonas aeruginosa*, and antifungal activities against two agricultural plant pathogenic fungi *Colletotrichum gloeosporioides* and *Penicillium italicum*. (±)-**3** exhibited promising inhibitory activity against *B. subtilis*, *P. aeruginosa*, and *P. italicum* with MIC, respectively, for 12.5, 12.5, and 3.13 μM (Table 3). Comparing lactone-type cytosporones **1**&**2**, (±)-**3** and **4**, (±)-**3** showed promising antimicrobial activities, which would be significantly weakened with either the aliphatic side chain oxygenated (**1**&**2**) or the 7-OH deficiency (**4**). As for phenyl-type cytosporones **5**, **6**, **7**, and **8**, **6** showed better antimicrobial activities, which would also be attenuated with the octanoyl oxygenated (**5**), shortened to be a hexanoyl (**8**), or the acetylethoxyl replaced by an acetylmethoxyl (**7**).

In summary, two new octaketides, cytosporones W (**1**) and X (**2**), along with eight known cytosporone derivatives [(±)-**3**−**9**] were isolated from mangrove endophytic fungus *Diaporthe* sp. ZJHJYZ-1. **1** and **2** were a pair of epimers, whose configuration of C-1 could mutually convert, causing racemization for the lactone ring. ECD spectra comparison and modified Mosher ester method were applied to determine the absolute configuration of **1** and **2**. In bioassays, (±)-**3** exhibited promising inhibitory activity against *B. subtilis*, *P. aeruginosa*, and *P. italicum* with MIC, respectively, for 12.5, 12.5, and 3.13 μM.

3. EXPERIMENTAL SECTION

3.1. General Experimental Procedures. 1D and 2D NMR spectra were acquired on a Bruker Advance 400 MHz spectrometer at room temperature. HR-ESI-MS spectra were obtained from a Thermo Fisher LTQ-Orbitrap-LCMS spectrometer (Palo Alto, CA, USA). ECD and UV−vis data were recorded from an Applied Photophysics Chirascan spectropolarimeter (Surrey, UK). IR spectra were achieved on a PerkinElmer Frontier FT-IR spectrometer. Semi-preparative HPLC was applied on an Ultimate 3000 separation module combined with a DAD detector (detection wavelength 220, 254, 280 nm) manufactured by Thermo Fisher, and a chiral-ND column (5 μm, 4.6 × 250 mm) was utilized for separation at 22 °C. Organic solvent was evaporated by a Heidolph rotavapor with a vacuum pump.

3.2. Fungal Material. The fungus *Diaporthe* sp. ZJHJYZ-1 was isolated from a fresh leaf of the semi-mangrove plant *Hibiscus tiliaceus* L., which was collected in July 2019 from Zhanjiang Mangrove National Nature Reserve in Guangdong Province, China. The fungus stain was identified according to sequencing of the internal transcribed spacer, and the result of a BLAST search revealed it was most similar (99%) to the sequence of *Diaporthe* sp. (compared to MK299422.1). The

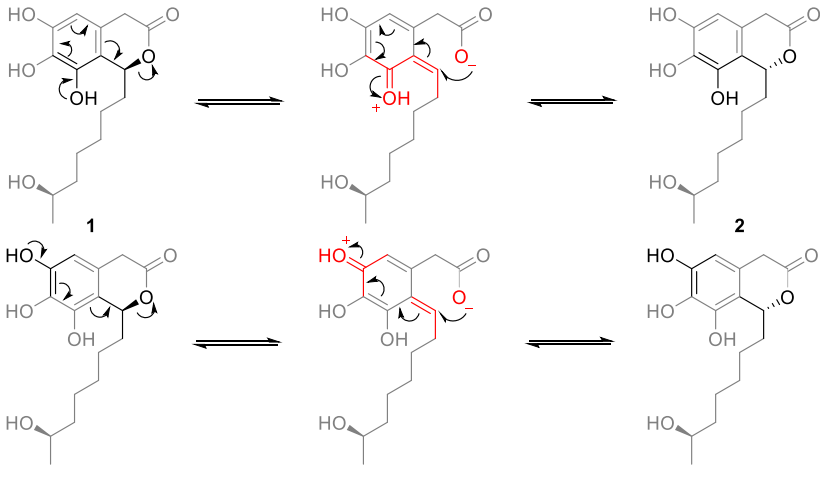

Figure 6. Plausible mechanism of the epimerization equilibrium between **1** and **2**.

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Table 2. Methylation Reaction (i), (ii), (iii), (iv) Methods and HR-ESI-MS Analysis

reaction	solvents	reactants and additional sequences			HR-ESI-MS analysis for the crude products			
		(1)	(2)	(3)	monomethylation	dimethylation	trimethylation	tetramethylation
i	CH_3CN	**1&2**	NaH	CH_3I				
ii	MeOH	**1&2**	$SOCl_2$		√			
iii	MeOH	$SOCl_2$	**1&2**		√			
iv	DCM[a]	**1&2**	TMS-CHN_2 in *n*-hexanes		√	√	√	√

[a]Dichloromethane.

Table 3. MIC for Antibacterial and Antifungal Activities of Compounds 1−9

	MIC of Compounds/μM									
	1&2	(±)-**3**	**4**	**5**	**6**	**7**	**8**	**9**	Ampicillin[a]	Ketoconazole[b]
MRSA	>100	100	>100	>100	50	>100	>100	>100	0.25	NT
B. subtilis	>100	12.5	>100	>100	50	50	>100	>100	0.25	NT
S. typhimurium	>100	100	>100	>100	50	100	>100	>100	0.25	NT
P. aeruginosa	>100	12.5	>100	>100	50	>100	>100	>100	0.13	NT
C. gloeosporioides	100	25	50	>100	25	100	>100	>100	NT	1.56
P. italicum	>100	3.13	50	>100	25	>100	>100	25	NT	1.56

[a]Positive control toward bacteria. [b]Positive control toward fungi.

sequence data have been deposited at GenBank with accession no. OQ881086. The fungus specimen was kept in our laboratory at −20 °C.

3.3. Fermentation, Extraction, and Isolation. The fungus was proliferated in potato dextrose broth (PDB) for 5 days and then cultured in autoclaved PDB in 300 Erlenmeyer flasks with a volume of 500 mL each, containing 150 mL PDB with 0.45 g NaCl. Then the culture medium was extracted with EA, and the mycothallus was soaked with MeOH and extracted with EA after concentration. The above two EA extracts were combined, and, finally, 24 g of crude extract was obtained. The crude extract was separated by a silica gel column utilizing a gradient of petroleum ether/EA from 1:0 to 0:1 to afford 9 fractions (Frs. 1−9).

Frs. 7 (3.5 g) was subjected to Sephadex LH-20 (DCM/MeOH v/v, 1:1) to yield four subfractions (SFrs. 7.1−7.4). SFrs. 7.4 (1.9 g) was applied to silica gel CC (DCM/MeOH, v/v, 50:1) to give mixture of **1** and **2** (19.4 mg), isolated by using HPLC with the chiral-ND column, respectively, at t_R = 10.0 and 17.5 min (the gradient was *n*-hexane/2-propanol v/v, 76:24, flow rate: 1 mL/min). Frs. 2 (6.9 g) was subjected to Sephadex LH-20 (DCM/MeOH v/v, 1:1) to yield six subfractions (SFrs. 2.1−2.6). SFrs. 2.4 (3.1 g) was applied to silica gel CC (DCM/MeOH, v/v, 400:1) to give **6** (10.5 mg), **7** (4.8 mg), **8** (3.5 mg), and **9** (2.3 mg). Frs. 5 (7.3 g) was subjected to Sephadex LH-20 (DCM/MeOH v/v, 1:1) to yield five subfractions (SFrs. 5.1−5.5). SFrs. 5.3 (2.7 g) was applied to silica gel CC (DCM/MeOH, v/v, 200:1) to give **5** (9.3 mg), and mixture of (±)-**3** (4.2 mg), isolated by using HPLC with the chiral-ND column, respectively, at t_R = 7.5 and 10.0 min (the gradient was *n*-hexane/2-propanol v/v, 80:20, flow rate: 1 mL/min). Frs. 3 (2.8 g) was subjected to Sephadex LH-20 (DCM/MeOH v/v, 1:1) to yield three subfractions (SFrs. 3.1−3.3). SFrs. 3.2 (1.1 g) was applied to silica gel CC (DCM/MeOH, v/v, 300:1) to give **4** (2.4 mg).

Cytosporone W (**1**). $C_{16}H_{22}O_6$; light-yellow oil; ($[\alpha]^{25}_D$ = +0.2, *c* 0.14, CH_3CN); UV (CH_3CN) λ_{max} (log ε) 205 (4.03) nm; IR (neat): ν_{max} 3336, 2925, 1703, 1606, 1457, 1270, 1151 cm^{-1}; HR-ESI-MS *m*/*z* 333.1302 $[M + Na]^+$ (calcd for $C_{16}H_{22}O_6Na^+$, 333.1309); ^1H and ^{13}C NMR data, see Table 1.

Cytosporone X (**2**). $C_{16}H_{22}O_6$; light-yellow oil; ($[\alpha]^{25}_D$ = −4.9, *c* 0.11, CH_3CN); UV (CH_3CN) λ_{max} (log ε) 205 (3.99) nm; IR (neat): ν_{max} 3336, 2925, 1704, 1606, 1457, 1270, 1151 cm^{-1}; HR-ESI-MS *m*/*z* 333.1300 $[M + Na]^+$ (calcd for $C_{16}H_{22}O_6Na^+$, 333.1309); ^1H and ^{13}C NMR data, see Table 1.

3.4. Preparation of MTPA Esters of 1 and 2 by the Modified Mosher Ester Method. The mixture of **1** and **2** (2.0 mg) was reacted with (*S*)-α-methoxy-α-(trifluoromethyl)-phenylacetyl chloride [(*S*)-MTPA-Cl, 50 μL] in pyridine-d_5 (600 μL) for 12 h at room temperature. Then the reaction mixture was concentrated to obtain a residue, which was purified by HPLC with the chiral-ND column at t_R = 15.0 min (the gradient was *n*-hexane/2-propanol v/v, 77:23, flow rate: 1 mL/min) to give (*R*)-MTPA ester (11.0 mg), and its molecular formula $C_{56}H_{50}F_{12}O_{14}$ was confirmed by HR-ESI-MS *m*/*z* 1197.2888 $[M + Na]^+$ (calcd for $C_{56}H_{50}F_{12}O_{14}Na^+$, 1197.2901). Through the same procedure, by using (*R*)-MTPA-Cl, (*S*)-MTPA ester (11.4 mg) was obtained and purified by HPLC with the chiral-ND column at t_R = 12.5 min (the gradient was *n*-hexane/2-propanol v/v, 77:23, flow rate: 1 mL/min), and its molecular formula $C_{56}H_{50}F_{12}O_{14}$ was confirmed by HR-ESI-MS *m*/*z* 1197.2894 $[M + Na]^+$ (calcd for $C_{56}H_{50}F_{12}O_{14}Na^+$, 1197.2901). The ^1H NMR spectra of the (*R*)- and (*S*)-MTPA esters were recorded, and the chemical shifts were assigned based on $^1H-^1H$ COSY spectra, in order to calculate the $\Delta\delta_{S-R}$ values.

3.5. ECD Computation Methods. Initial conformational analysis was performed by the Spartan 14′ software (Wavefunction Inc., Irvine, CA, USA) using the Merck molecular force field method. The conformation with a Boltzmann population greater than 1% was selected for optimization and calculation in acetonitrile at B3LYP/6-31+G(d,p) level with the density functional theory performed by Gaussian 09.[23] The ECD spectra were extracted and generated by the SpecDis 1.6 software (University of Würzburg, Würzburg, Germany) with σ = 0.30 eV.

3.6. Methylation Reaction Methods.

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(i) Cytosporones W and X (8.5 mg, 27.4 μmol) and NaH (3.2 mg, 133 μmol) in anhydrous acetonitrile (200 μL) were stirred at 20 °C for 30 min, and then iodomethane (13.7 μL, 220 μmol) was added slowly, and the reaction mixture was stirred at 20 °C for 12 h. After completion, the mixture was diluted with water and extracted with EA. The organic layer was washed with brine, dried over anhydrous Na_2SO_4, and concentrated to give the crude product.

(ii) Cytosporones W and X (2.0 mg, 6.5 μmol) in anhydrous MeOH (150 μL) were stirred at −10 °C for 15 min, and then $SOCl_2$ (12.0 μL, 202 μmol) was added slowly, and the reaction mixture was stirred at 20 °C for 1 h. After completion, the mixture was concentrated to give the crude product.

(iii) $SOCl_2$ (38.5 μL, 647 μmol) was added slowly to anhydrous MeOH (100 μL) at −10 °C and stirred for 15 min, and then cytosporones W and X (2.0 mg, 6.5 μmol) in anhydrous MeOH (50 μL) were added. The reaction mixture was stirred at 20 °C for 1 h. After completion, the mixture was concentrated to give the crude product.

(iv) Cytosporones W and X (2.9 mg, 9.4 μmol) were solved in DCM (200 μL) and the solution of TMS-CHN_2 in *n*-hexane (2.0 mol/L solution, 50 μL, 100 μmol) were added. The reaction mixture was stirred at 20 °C for 1 h. After completion, the mixture was concentrated to give the crude product.

3.7. Antimicrobial Assays. The isolated compounds were dissolved individually in dimethyl sulfoxide (DMSO), and the antimicrobial activities were assayed by a serial dilution test in the range of 0.1−100 μM in 96-well plates, according to the methods previously reported.[24,25] Ampicillin and ketoconazole were utilized as positive controls for antibacterial and antifungal tests, respectively, and DMSO was used as a blank control.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.3c03862.

HR-ESI-MS spectra, 1D and 2D NMR spectra, UV−vis and IR spectra, and ECD calculation details for simplified-**2** (PDF)

■ AUTHOR INFORMATION

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The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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