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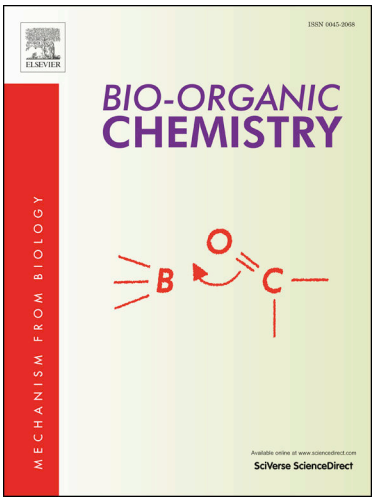

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Three new bioactive natural products from the fungus *Talaromyces assiutensis* JTY2

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Abstract:

A novel cyclopentenone derivative, talarocyclopenta A (**1**), a new phenolicethers derivative, talarocyclopenta B (**2**) and a new itaconic acid derivative, talarocyclopenta C (**3**) together with four known itaconic acid derivatives (**4-7**) were isolated from the *Talaromyces assiutensis* JTY2. Their structures were elucidated by the detailed analysis of comprehensive spectroscopic data. Among them, talarocyclopent (**1**) is the first represent an unusual type of cyclopentenone derivative, possessing a cyclopentenone unit, a 2-butanone unit and a 3-hydroxybutyric acid unit. All isolated compounds were evaluated for their anti-inflammatory and antibacterial activities. Compounds **1-4** showed inhibitory activities against the nitric oxide (NO) production induced by lipopolysaccharide in mouse macrophage RAW 264.7 cells *in vitro*. Compound **2** showed broad spectrum antibacterial against six terrestrial pathogenic bacteria.

Keywords: *Talaromyces assiutensis*; cyclopentenone derivative; phenolicethers derivative; itaconic acid; anti-inflammatory activity; antibacterial activity

1. Introduction

Marine microorganisms, especially marine fungi are well known as rich sources of new natural products with promising biological and pharmacological activities [1-3]. Chemical investigations of mangrove-derived endophytic fungi, especially those from the subtropical island of Hainan, P. R. China, have shown a sharp increase in recent years [4-6]. As a result of adaptation to some special environments mangrove endophytic fungus have formed unique genetic backgrounds and metabolic pathways [7-9]. Fungi in the genus *Talaromyces* produce various bioactive metabolites, such as cyclic peptides, azaphilones, meroterpenoids and butenolides [10-13]. In our investigation on natural antibacterial and anti-inflammatory products from mangrove fungi in the South China Sea, a fungus *Talaromyces assiutensis* JTY2 obtained from the leaves of *Ceriops tagal* attracted our attention. The EtOAc extract of a solid rice fermentation of the fungus exhibited antimicrobial activities and anti-inflammatory activities. Bioassay-guided fractionation of the bioactive extract led to the isolation of novel cyclopentenone derivative, talarocyclopenta A (**1**), a new phenolicethers derivative, talarocyclopenta B (**2**) and a new itaconic acid derivative, talarocyclopenta C (**3**) together with four known itaconic acid derivatives asperitaconic B (**4**) [14], (3*S*)-9-hydroxy-3-carboxy-2-methylenenonanoic acid (**5**) [15], (3*S*,8*R*)-8-hydroxy-3-carboxy-2-methylenenonanoic acid (**6**) [15] and (2*S*)-Butylitaconic acid (**7**) [16] (**Fig. 1**). Compounds **1-4** showed inhibitory activities against the nitric oxide (NO) production induced by lipopolysaccharide in mouse macrophage RAW 264.7 cells *in vitro*. Compound **2** showed broad spectrum antibacterial against six terrestrial pathogenic bacteria. Herein, we report the isolation, structure elucidation and biological activities of these compounds.

2. Experimental

2.1 General Experimental Procedures

IR spectra were recorded on a Nicolet 6700 spectrophotometer. Optical rotations were measured on a JASCO P-1020 digital polarimeter. NMR spectra were recorded on a Bruker AV spectrometer (400 MHz for ^{1}H and 100 MHz for ^{13}C) and a JNM-ECZS spectrometer (600 MHz for ^{1}H and 150 MHz for ^{13}C). TMS was used as an internal standard. HRESIMS spectra were measured on a Q-TOF Ultima Global GAA076 LC mass spectrometer.Semi-preparative HPLC was performed on an Agilent 1260 LC series with a DAD detector using an Agilent Eclipse XDB-C_{18} column (9.4 × 250 mm, 5 μm), Silica gel (Qing Dao Hai Yang Chemical Group Co.; 200–300 mesh), octadecylsilyl silica gel (YMC; 12 nm–50 μm) and Sephadex LH-20 (GE) were used for column chromatography (CC). Precoated silica gel plates (Yan Tai Zi Fu Chemical Group Co.; G60, F-254) were used for thin layer chromatography (TLC).

2.2 Fungal Materials

The fungus JTY2 was isolated from the leaves of *Ceriops tagal*, which were collected in the South China Sea in August, 2016. The fungal strain was deposited in the Key Laboratory of Tropical Medicinal Plant Chemistry of Ministry of Education, College of Chemistry and Chemical Engineering, Hainan Normal University of P. R. China, Hainan with an accession number JN899320.1.

The fungus was identified according to its morphological characteristics and a molecular biological protocol by 18S rRNA amplification and sequencing of the ITS region. The sequence data have been submitted to GenBank, with an accession number JN899320.1, and the fungal strain was identified as *Talaromyces assiutensis*.

2.3 Extraction and isolation

The fungal strain was cultivated in 16 Kg grown on solid rice cultures in 1 L Erlenmeyer (amount: 200; to 80 g commercially available rice was added 80 mL of

brine water and kept overnight prior to autoclaving) at 25 °C without shaking for 30 days. The fermentation was extracted three times with an equal volume of EtOAc. The combined EtOAc layers were evaporated to dryness under reduced pressure to give an EtOAc extract (254 g), which was subjected to silica gel column chromatography (CC) (petroleum ether, EtOAc, MeOH v/v, gradient) to generate nine fractions (Frs. 1−8). Fr. 3 was isolated by CC on silica gel eluted with petroleum ether−EtOAc (from 50:1 to 1:1) to afford four subfractions (3a–3d). Subfraction 3b was further purified by using octadecylsilyl silica gel (45% MeOH/H_2O) to obtain **1** (11 mg). Subfractions 3c was further separated by Semi-Preparative HPLC (CH_3CN/H_2O, 40: 60 *v*/*v*) to obtain **3** (13 mg), **4** (15 mg), **5** (8 mg), **6** (7 mg) and **7** (4 mg), respectively. Fr. 4 was isolated by CC on silica gel eluted with petroleum ether−EtOAc (from 50:1 to 1:1) and then subjected to repeated Sephadex LH-20 CC eluting with mixtures of $CHCl_3$−MeOH (1:1) to afford three subfractions (4a–4c). Subfractions 4c was further separated by Semi-Preparative HPLC (CH_3CN/H_2O, 30: 70 *v*/*v*) to obtain **2** (6 mg).

2.4 Physio-chemical properties of compounds 1-3

Talarocyclopenta A (**1**): colorless gum; $[\alpha]^{25}_D$ +32.8 (*c* 2.0, MeOH); UV (MeOH) λ_{max} (log ε) 243 (2.5) nm; IR (KBr) ν_{max} 3365, 2928, 1713 and 1634 cm^{-1}; ^1H and ^{13}C NMR see Table 1 and 2; HR-ESI-MS *m/z* 319.1163 $[M + Na]^+$ calcd for $C_{15}H_{20}O_6Na$, 319.1158.

3-Methyl-2-oxo-5-(2-oxobutyl)cyclopent-3-enecarboxylic acid (**1a**): colorless gum; ^1H and ^{13}C NMR see Table 1 and 2; HR-ESI-MS *m/z* 209.0815 $[M - H]^-$ calcd for $C_{11}H_{13}O_4$, 209.0819.

Talarocyclopenta B (**2**): colorless gum; $[\alpha]^{25}_D$ +28.6 (*c* 2.0, MeOH); UV (MeOH) λ_{max} (log ε) 356 (1.2), 272 (2.3), 210 (3.8) nm; IR (KBr) ν_{max} 3418, 1766, 1452 and

1384 cm^{-1}; ^{1}H and ^{13}C NMR see Table 1 and 2; HR-ESI-MS *m/z* 225.0731 $[M - H]^-$ calcd for $C_{11}H_{13}O_5$, 225.0736.

Talarocyclopenta C (**3**): colorless gum; $[\alpha]^{25}_D$ +7.4 (*c* 2.0, MeOH); ECD (*c* 2.5×10^{-4} mol/L, MeOH) λ_{max} ($\Delta\varepsilon$) 216 (2.3), 261 (0.2); UV (MeOH) λ_{max} (log ε) 215 (3.2) nm; IR (KBr) ν_{max} 3421, 2925, 1644, and 1118; ^{1}H and ^{13}C NMR see Table 1 and 2; HR-ESI-MS *m/z* 287.1497 $[M + H]^+$ calcd for $C_{14}H_{23}O_6$, 287.1495.

2.6 Antibacterial activity assay

Compounds **1**–**7** was tested for their antibacterial activity against six terrestrial pathogenic bacteria *Micrococcus tetragenus* (ATCC 13623), *Staphylococcus aureus* (ATCC 8799), *Staphylococcus albus* (ATCC 8799), *Bacillus cereus* (ATCC 14579), *Bacillus subtilis* (ATCC 6633), *Escherichia coli* (ATCC 25922) using a 96-well plate-based method [17], and ciprofloxacin was used as the positive control.

2.7 Anti-inflammatory activity

The RAW 264.7 cells were incubated in RPMI 1640 medium containing 10% fetal bovine serum, 2 mmol/L glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. Cell concentration was adjusted to 5×10^5 cells/mL, and 200 μL of cell suspension was seeded in each well of a 96-well plate. After 1 h incubation, cells were treated with LPS (1 μg/mL) and test samples were dissolved in DMSO at concentrations of 0.0625, 0.32, 1.6, 8 and 40 μM (final DMSO concentration 0.2%, *v*/*v*) for 24 h at 37 °C. A 100 μL sample of the culture supernatant was determined by the Griess reaction [18] The Griess reagent (50 μL of 1% sulfanilamine in 5% H_3PO_4, and 50 μL of 0.1% *N*-1-naphthylethylenediamine dihydrochloride) was added to each well. After 10 min, the reaction products were colorimetrically quantitated at 540 nm using a microplate reader. The experiments were performed in triplicate. Hydrocortisone was used as a positive control; The cytotoxicity assay was performed using the MTT method in 96-well microplates [19]. An MTT solution (200 μg/mL)

was added after the 24 h treatment and then incubated for another 4 h at 37 °C. The reduced MTT-formazan was solubilized with 150 μL of DMSO, and the absorbance of the MTT-formazan solution at 570 nm was measured by an immunoreader. The percentage of suppression was calculated by comparing the absorbance of sample treated cells with that of nontreated cells.

2.8 Statistical analysis

All experiments were carried out in triplicate, and each experiment was repeated three times. Data analysis was carried out using SPSS statistical package version 22.0 (SPSS, Inc.). Differences between the test and control groups were analyzed by *t*-tests. The IC_{50} values (the concentration of drug necessary to induce 50% inhibition) were measured for all of the tested drugs using the Probit test in SPSS software.

2.9 hydrolysis of talarocyclopent (1)

Acid hydrolysis of talarocyclopenta A (**1**): A solution of **1** (6 mg) in 5% H_2SO_4 (2 mL) was heated under reflux for 3h. The mixture was separated by the semi-preparative HPLC to obtain a 3-hydroxybutyric acid (1.5 mg) and 3-methyl-2-oxo-5-(2-oxobutyl)cyclopent-3-enecarboxylic acid (**1a**) (2.8 mg).

3. Results and discussion

3.1 Structure elucidation

Compound **1** was isolated as colorless gum. Its molecular formula of $C_{15}H_{20}O_6$ was determined by HR-ESI-MS (calcd for $[M + Na]^+$ 319.1158, found 319.1163). The ^{1}H-NMR data revealed one olefinic proton δ_H 7.27 (br s), three sp^3 methine signals at δ_H 5.34 (m), 3.55 (m) and 2.96 (d, J = 2.8 Hz), three sp^3 methylene signals at δ_H 2.72/2.55, 2.68/2.61 and 2.43 (q, J = 7.2 Hz) and three methyl groups δ_H 1.05 (t, J = 7.2 Hz), δ_H 1.35 (d, J = 6.4 Hz) and δ_H 1.75 (s). The ^{13}C NMR/135DEPT data showed 15 resonances, including two carbonyls (δ_C 209.1, 202.3), two carboxyls (δ_C 174.5,

168.0), two olefinic carbons (δ_C 160.8, 140.4), three sp^3 methine carbons (δ_C 68.7, 58.4 and 38.8), three sp^3 methylene carbons (δ_C 45.8, 40.8 and 36.2) and three methyl carbons (δ_C 19.8, 10.3 and 7.8). The ^1H-^1H COSY correlation of H-4'/H-3'/H-2' combined with the HMBC correlations from H-2' to C-1' indicated the presence of a 3-hydroxybutyric acid unit in **1** (**Fig. 2**). The ^1H-^1H COSY correlation of H-1/H-2, H-4/H-5/H-6 and H-5/H-9 combined with the HMBC correlations from H-10 to C-7/8/9, from H-2/4 to C-3 and from H-6 to C-7/11 indicated the presence of a 3-methyl-2-oxo-5-(2-oxobutyl)cyclopent-3-enecarboxylic acid unit in **1**. The location of the 3-hydroxybutyric acid unit at C-11 was confirmed by the HMBC correlation from H-3' to C-11. The relative configuration of **1** was assigned on the basis of NOESY correlation. The NOESY correlation of H-6/H-4 indicated that H-6 and C-4 were on the same face (**Fig. 3**). The acidic hydrolysis of **1** afforded a (+)-(*S*)-3-hydroxybutyric acid (**1b**). It was confirmed by the NMR data and $[\alpha]^{25}_D$ +24.8, *c* 2.0, H_2O (authentic sample: $[\alpha]^{20}_D$ +25.1, *c* 1.5, H_2O). In addition, a 3-methyl-2-oxo-5-(2-oxobutyl)cyclopent-3-enecarboxylic acid (**1a**) (The NMR data of **1a** see tables 1 and 2) was also obtained. Thus, compound **1** was identified as a novel cyclopentenone derivative, which is the first represent an unusual type of cyclopentenone derivative, possessing a cyclopentenone unit, a 2-butanone unit and a 3-hydroxybutyric acid unit. We named compound **1** talarocyclopenta A.

Compound **2** was also isolated as colorless gum. The HR-ESI-MS showed a molecular ion at *m/z* 225.0731 $[M - H]^-$ calcd for $C_{11}H_{13}O_5$, 225.0736, which agreed with molecular formula $C_{11}H_{14}O_5$. The ^1H and ^{13}C NMR spectra also showed a 3-hydroxybutyric acid signal δ_H: 1.32 (d, J = 6.0 Hz), 4.69 (m), 2.63/2.80 and δ_C: 20.0, 42.0, 74.1, 173.9. In addition, there are a 1,2,3-trisubstituted benzene and a methoxy signals δ_H: 6.60 (dd, J = 8.4, 8.0 Hz), 6.50 (dd, J = 8.4, 1.2 Hz), 6.48 (dd, J = 8.0, 1.2 Hz), 3.69 (s) and δ_C: 147.2, 146.8, 137.6, 119.8, 111.0, 110.2, 52.3. These data closely resembled those of 3-(2,3-dihydroxyphenoxy)-butanoic acid [20] except

for the presence of one methoxy signal (δ_C 52.3 and δ_H 3.69, s) in **2**. The location of the methoxy group at C-1' was further confirmed by the HMBC correlation between H-5' and C-1'. The stereo configuration of 3'-OH was assigned by comparison of the optical rotation with the (+)-(*S*)-3-hydroxybutyric acid ($[\alpha]^{25}_D$ +28.6 *vs* $[\alpha]^{20}_D$ +25.1), and the same biosynthetic pathway with those of **1**. Thus the stereo configuration of 3'-OH was assigned *S*-form. Hence, the structure of **2** was determined, and named as talarocyclopenta B.

Compound **3** was also obtained as colorless gum, a molecular formula of $C_{14}H_{22}O_6$, was determined by HRESIMS signals at *m/z* 287.1497 $[M + H]^+$ (cald for $C_{14}H_{23}O_6$, 287.1495). The ^{1}H-NMR data revealed two olefinic proton δ_H 6.14 (s) and 5.51 (s), one sp^3 methine signal at δ_H 3.52 (t, J = 7.2 Hz), six sp^3 methylene signals at δ_H 1.30-1.82 (10H), 4.04 (2H, t, J = 6.4 Hz) and one methyl group δ_H 2.02 (s). The ^{13}C NMR/135DEPT data showed 14 resonances, including three carboxyls (δ_C 176.4, 174.0 and 173.1), two olefinic carbons (δ_C 144.5 and 123.4), one sp^3 methine carbon (δ_C 48.6), six sp^3 methylene carbons (δ_C 26.8-32.4 and 65.7) and one methyl carbon (δ_C 20.8). These data including optical rotation ($[\alpha]^{25}_D$ +7.4) closely resembled those of known compound **4** ($[\alpha]^{25}_D$ +7.70, *c* 2.50 MeOH) [14] except for the presence of one methoxy signal (δ_C 52.2 and δ_H 3.64, s) in **3**. The location of the methoxy group at C-1 was further confirmed by the HMBC correlation between H-14 and C-1.Thus the stereo configuration of **3** was assigned *S*-form. Further more, the experimental ECD spectrum of **3** (**Fig. 4**) showed two positive (216 nm) and (261 nm) Cotton effects. The calculated ECD spectrum (the ECD calculation details see supporting information) for the (*S*) enantiomer showed a similar ECD curve. The above information allowed the determination of the absolute configuration of **3** as *S*. Herein the structure of **3** was established, and named as talarocyclopenta C.

The structures of known compounds **4**−**7** were identified by comparison of their spectroscopic data with those in the literature.

3.2 Antibacterial activity

Compounds **1**–**7** were evaluated for their antibacterial activities against six pathogenic bacteria including *M. tetragenus*, *S. aureus*, *S. albus*, *B. cereus*, *B.subtilis*, *E. coli.* Compound **2** show broad spectrum antibacterial against six terrestrial pathogenic bacteria, and **1** exhibited weak antibacterial activities (table 3), The MIC values of other compounds higher than 40 μg/mL were regarded as inactive.

3.2 Anti-inflammatory activity

Compounds **1**–**7** were evaluated their anti-inflammatory activities *via* testing the inhibitory activities against the nitric oxide (NO) production induced by lipopolysaccharide in mouse macrophage RAW 264.7 cells *in vitro*. As a result, compounds **1-4** showed significant inhibitory activities with the IC_{50} value of 3.38 ± 0.12 μM, 6.26 ± 0.10 μM, 12.56 ± 0.08 μM and 15.63 ± 0.12 μM, respectively. While the positive control, hydrocortisone, showed a inhibitory activity with the IC_{50} value of at 3.68 ± 0.10 μM. No cytotoxicity was observed in compounds 1-4 treated cells (cell viability > 85%). The IC_{50} values of other compounds higher than 40 μM were regarded as inactive.

4. Conclusions

In this study, A novel cyclopentenone derivative, talarocyclopenta A (**1**), a new phenolicethers derivative, talarocyclopenta B (**2**) and a new itaconic acid derivative, talarocyclopenta C (**3**) together with four known itaconic acid derivatives (**4-7**) were isolated from the *Talaromyces assiutensis* JTY2. talarocyclopenta A (**1**) is the first represent an unusual type of cyclopentenone derivative, possessing a cyclopentenone unit, a 2-butanone unit and a 3-hydroxybutyric acid unit. It presence as characteristic marker may be helpful in chemotaxonomical classifications. The significant inhibitory activities on NO production of compounds **1-4** and antibacterial activities of compound **2** could be used for the development of new anti-inflammatory and

antibacterial agents.

Conflict of interest

The authors have declared that there is no conflict of interest.

Acknowledgements

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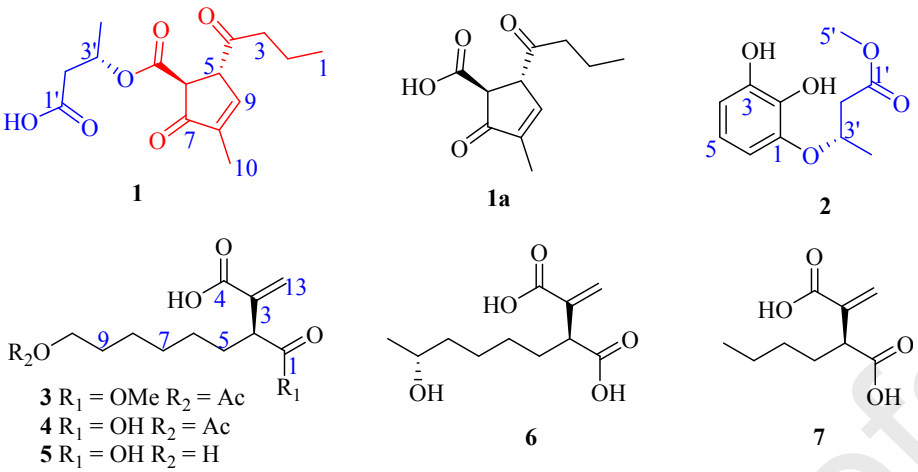


Fig. 1 The Structure of compounds **1**–**7** and **1a**

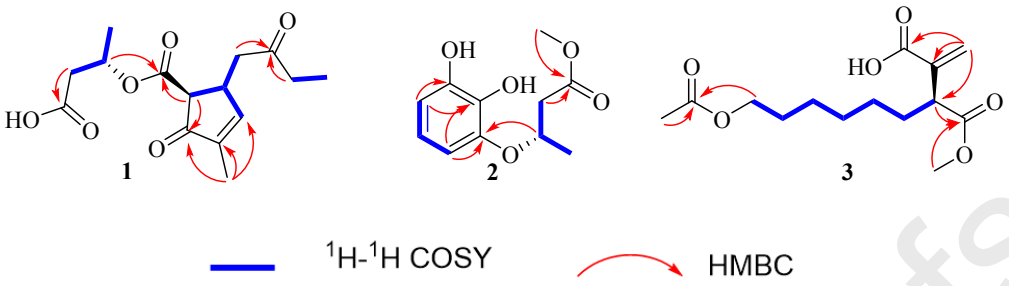


Fig. 2 Key ^{1}H-^{1}H COSY and HMBC correlations for Compounds **1**–**3**

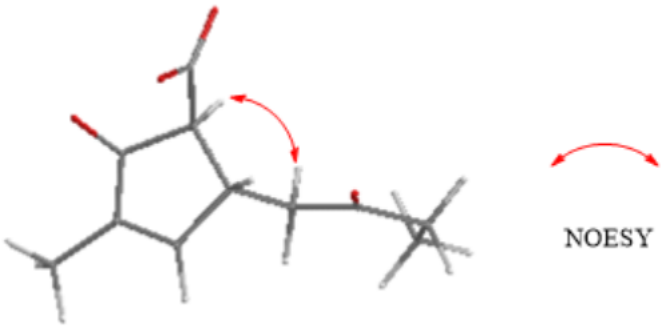


Fig. 3 Key NOESY correlations for Compound **1**

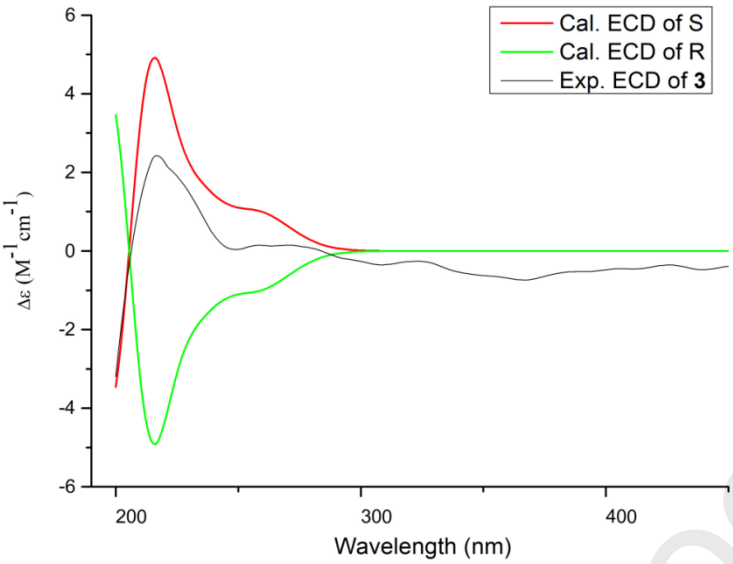


Fig. 4 Experimental ECD spectra (200–400 nm) of JTY in MeOH and the calculated ECD spectra of the model molecules of **3** at the B3LYP/6-311+G(d, p) level..

Table 1. ^{1}H NMR Data (δ in ppm, J in Hz) of compounds 1–3 (400 MHz) and 1a/1b (600 MHz)

position	**1**[a]	**1a** (1-11)/**1b** (1'-5')[a]	**2**[b]	**3**[b]
	δ_H (J in Hz)		δ_H (J in Hz)	δ_H (J in Hz)
1	1.05 (t, 7.2)	1.07 (t, 7.2)	-	-
2	2.43 (q, 7.2)	2.44 (q, 7.2)	-	3.52 (t, 7.2)
3	-		-	-
4	2.68 (dd, 8.0, 4.0) 2.61 (d, 8.0)	2.71 (dd, 17.4, 7.2) 2,60 (dd, 17.4, 8.4)	6.50 (dd, 8.4, 1.2)	-
5	3.55 (m)	3.59 (m)	6.60 (dd, 8.4, 8.0)	1.82/1.70 (m)
6	2.96 (d, 2.8)	3.01 (d, 3.0)	6.48 (dd, 8.0, 1.2)	1.34 (m)
7	-	-	-	1.34 (m)
8	-	-	-	1.34 (m)
9	7.27 (br s)	7.29 (m)	-	1.61 (m)
10	1.75 (s)	1.78 (t, 1.2)	-	4.04 (t, 6.4)
11	-	-	-	-
1'/12	-	-	-	2.02 (s)
2'/13	2.72 (dd, 10.0, 6.0) 2.55 (dd, 10.0, 2.4)	2.59 (dd, 18.0, 6.6) 2.49 (dd, 18.0, 8.4)	2.80 (dd, 12.0, 8.0) 2.63 (dd, 12.0, 4.8)	6.14/5.51 (s)
3'/14	5.34 (m)	4.54 (m)	4.69 (m)	3.64 (s)
4'	1.35 (d, 6.4)	1.32 (d, 6.0)	1.32 (d, 6.0)	-
5'	-	-	3.69 (s)	-

[a] Measured in $CDCl_3$.

[b] Measured in CD_3OD.

Table 2. ^{13}C NMR Data (δ in ppm, J in Hz) of compounds 1–3 (100 MHz) and 1a/1b (150 MHz)

position	**1**[a]	**1a** (1-11)/**1b** (1'-5')[a]	**2**[b]	**3**[b]
	δ_C, type		δ_C, type	δ_C, type
1	7.8, CH_3	7.8, CH_3	147.2, C	176.4
2	36.2, CH_2	36.2, CH_2	137.6, C	48.6, CH
3	209.1, C	208.8, C	146.8, C	144.5, C
4	45.8, CH_2	46.0, CH_2	111.0, CH	173.1, C
5	38.8, CH	38.9, CH	119.8, CH	32.4, CH_2
6	58.4, CH	58.2, CH	110.2, CH	29.6, CH_2
7	202.3, C	201.9, C	-	28.7, CH_2
8	160.8, C	160.3, C	-	26.8, CH_2
9	140.4, CH	140.5, CH	-	30.1, CH_2
10	10.3, CH_3	10.4, CH_3	-	65.7, CH_2
11	168.0, C	169.3, C	-	174.0, C
1'/12	174.5, C	174.9, C	173.9, C	20.8, CH_3
2'/13	40.8, CH_2	45.6, CH_2	42.0, CH_2	123.4, CH
3'/14	68.7, CH	65.1, CH	74.1, CH	52.2, CH_3
4'	19.8 CH_3	22.2 CH_3	20.0, CH_3	-
5'	-	-	52.3, CH_3	-

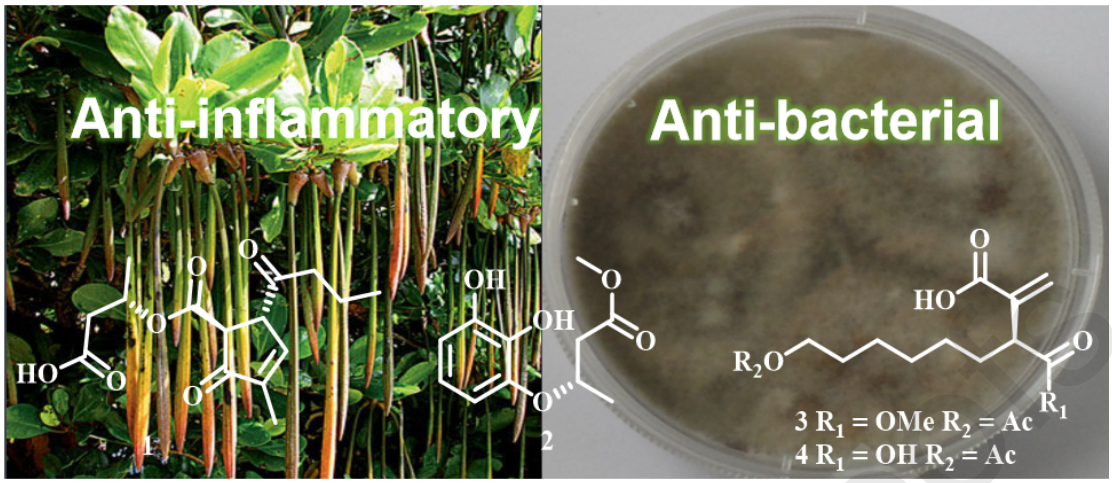
[a] Measured in $CDCl_3$.

[b] Measured in CD_3OD.

Table 3 Antibacterial activity of 1–2

Compounds	MIC values (μg/mL)					
	M. tetragenus	*S. aureus*	*S. albus*	*B. cereus*	*B. subtilis*	*E. coli*
1	>40	20	>40	>40	>40	40
2	5	2.5	5	2.5	2.5	1.25
Ciprofloxacin[a]	0.313	0.313	0.625	0.625	0.625	0.625

[a] Ciprofloxacin was used as a positive control.



Highlights

Three new Bioactive natural products from the Fungus *Talaromyces assiutensis* sp. JTY2.

Compound **1** is the first represent an unusual type of cyclopent derivative.

The anti-inflammatory activities and antibacterial activities of all isolated compounds were evaluated in vitro.

Compounds **1-4** exhibited significant inhibitory effects against the nitric oxide (NO) production induced by lipopolysaccharide.

Compound **2** showed broad spectrum antibacterial against six terrestrial pathogenic bacteria.