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Sumalactones A–D, four new curvularin-type macrolides from a marine deep sea fungus *Penicillium Sumatrense*†

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Sumalactones A–D (1–4), four new curvularin-type macrolides, together with two known analogues, curvularin (5) and dehydrocurvularin (6), were isolated from *Penicillium Sumatrense*, a marine fungus isolated from deep-sea sediments. Sumalactones C (3) and D (4) are unprecedented curvularin-type macrolides bearing a rare 11-membered macrolide skeleton. Their structures were elucidated on the basis of intensive spectroscopic analysis. The absolute configurations of compounds 1–4 were determined by CD spectra and modified Mosher's method. Compound 6 showed significant inhibition activity towards LPS-induced nitric oxide production in RAW 264.7 macrophages with IC_{50} value of $0.91\ \mu\text{M}$.

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Introduction

Among fungal macrolides, resorcinylic acid lactones (RALs) and dihydroxyphenylacetic acid lactones (DALs) belong to a unique family of naturally occurring homologous macrolides, which are characterized by possessing a macrolide core structure fused to a resorcinol aromatic ring.¹ Curvularins, featuring a substituted resorcinol fragment fused to the β , γ -positions of the macrocyclic lactone ring, are produced by a number of fungal species mainly from the genera *Aspergillus*,² *Alternaria*,³ *Astragalus*,⁴ *Curvularia*,⁵ *Cochliobolus*,⁶ and *Penicillium*⁷ with diverse biological activities. They are biogenetically derived from the polyketide synthase pathways in bacterial and fungi, and have brought great interest and challenges for total synthesis and biosynthesis studies.⁸

In the course of our ongoing research on new bioactive secondary metabolites from marine fungi,⁹ *Penicillium sumatrense* was isolated from a deep-sea sediment sample (-2500 m depth) of the Indian Ocean, which resulted in the isolation of four new curvularin derivatives, sumalactones A–D (1–4) with 10- or 11- or 12-membered macrolide skeletons, as well as two

known compounds curvularin (5)¹⁰ and dehydrocurvularin (6).¹¹ Compounds 3 and 4 with 11-membered macrolides skeleton are considered rare in nature and haven't been reported before. All the compounds were evaluated for their inhibitory effect on the production of nitric oxide (NO) induced by lipopolysaccharide (LPS) in RAW 264.7 macrophages. Herein we report the isolation, structure elucidation, and biological activities of these compounds (Fig. 1).

Results and discussion

Sumalactone A (1) gave an HRESIMS ion peak at m/z 307.1197 $[\text{M} - \text{H}]^-$, corresponding to the molecular formula $\text{C}_{16}\text{H}_{20}\text{O}_6$, which required seven degrees of unsaturation. The UV spectrum showed absorption maxima at 204, 218, 269, and 297 nm. The ^1H signals (Table 1) suggested a pair of meta-coupled aromatic protons at δ_{H} 6.12 (d, $J = 1.8\ \text{Hz}$, H-4), and 6.24 (d, $J = 2.0\ \text{Hz}$, H-6) and one methyl group at δ_{H} 1.12 (3H, d, $J = 6.2\ \text{Hz}$, CH_3 -16).

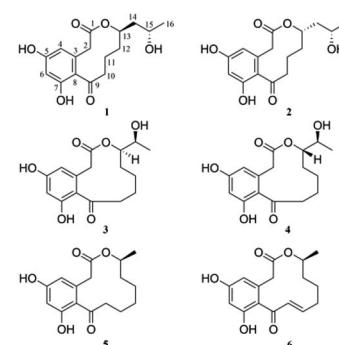


Fig. 1 Chemical structures of 1–6.

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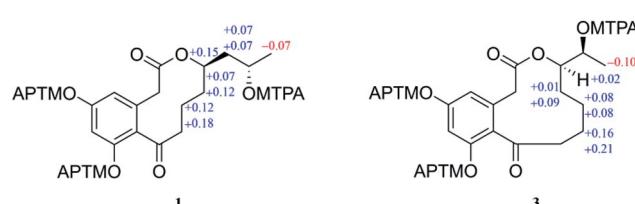
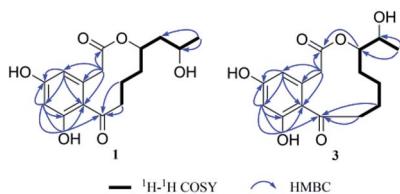
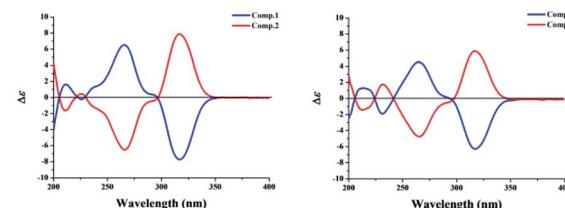
Table 1 ^1H NMR (500 MHz) and ^{13}C NMR (125 MHz) spectroscopic data of **1–4** in CD_3OD (δ in ppm, J in Hz)

1		2		3		4		
No.	δ_{C}	δ_{H}	δ_{C}	δ_{H}	δ_{C}	δ_{H}	δ_{C}	δ_{H}
1	171.8		172.7		172.4		172.5	
2	41.3	3.44, d (18.3) 3.96, d (18.4)	41.3	3.49, d (18.5) 3.98, d (18.4)	41.1	3.46, d (17.2) 4.14, d (17.1)	41.1	3.50, d (17.1) 4.11, d (17.2)
3	136.3		136.2		136.3		136.3	
4	110.6	6.12, d (1.8)	110.6	6.13, d (1.6)	111.5	6.16, d (2.2)	111.7	6.17, d (2.2)
5	161.0		161.0		160.9		160.9	
6	102.4	6.24, d (2.0)	102.4	6.24, d (2.0)	102.6	6.25, d (2.2)	102.6	6.24, d (2.2)
7	158.8		158.9		158.8		158.7	
8	122.6		122.6		122.0		122.0	
9	211.6		211.5		209.8		209.9	
10	46.7	2.70, m 3.06, m	46.6	2.70, ddd (2.1, 7.7, 15.9) 3.06, ddd (2.2, 10.5, 15.4)	41.7	2.87, ddd (3.4, 6.1, 17.5) 3.17, ddd (3.1, 11.6, 17.4)	41.8	2.86, ddd (3.3, 6.0, 17.3) 3.19, ddd (3.2, 11.6, 17.3)
11	23.4	1.87, 2H, m	23.4	1.87, 2H, overlapped	23.9	1.47, overlapped 1.89, m	23.9	1.47, overlapped 1.89, m
12	35.4	1.47, overlapped 1.95, m	36.3	1.47, overlapped 1.87, overlapped	22.0	1.23, m 1.66, m	22.1	1.23, m 1.67, overlapped
13	76.3	4.91, m	76.3	4.90, m	26.1	1.47, overlapped 1.75, m	26.3	1.47, overlapped 1.67, overlapped
14	45.3	1.47, overlapped 1.70, ddd (6.6, 7.5, 14.1)	45.9	1.47, overlapped 1.56, ddd (3.8, 7.9, 14.2)	79.8	4.75, ddd (3.3, 5.1, 10.5)	79.9	4.78, ddd (2.7, 6.0, 10.2)
15	65.5	3.69, m	65.1	3.65, m	69.4	3.63, dq (5.3, 6.3)	69.2	3.62, p (6.4)
16	23.9	1.12, d (6.2)	23.6	1.09, d (6.2)	18.7	0.98, d (6.4)	18.9	1.02, d (6.5)

Analysis of ^{13}C NMR and DEPT spectra data (Table 1) together with the HSQC data indicated the presence of six sp^2 quaternary carbons including one ketone carbonyl carbon (δ_{C} 211.6) and one ester carbonyl carbon (δ_{C} 171.8), two sp^2 methine carbons (δ_{C} 110.6 and 102.4), two sp^3 oxygenated methine carbons (δ_{C} 76.3 and 65.5), five sp^3 methylene carbons, and one methyl carbon (δ_{C} 23.9). The ^1H and ^{13}C NMR data were nearly identical to curvularin (5),¹⁰ except that C-13 methylene carbon in 5 was replaced by an oxygenated methine carbon (δ_{C} 76.3) in 1, which was confirmed by the ^1H – ^1H COSY correlations between H-13 (δ_{H} 4.91) and H₂-12 (δ_{H} 1.47 and 1.95). In addition, the key HMBC correlation from H-13 (δ_{H} 4.91) to ester carbonyl carbon C-1 (δ_{C} 171.8) indicated the location of the 10-membered lactone between C-1 and C-13. Thus, the planar structure of **1** was established (Fig. 2). In order to determine the absolute configuration of OH group at C-15, the modified Mosher's method was applied.¹² When reacted with (*R*)- and (*S*)-MTPA chloride, **1** gave the corresponding (*S*)- and (*R*)-MTPA esters, respectively. The observed chemical shift differences $\Delta\delta_{\text{H}(\text{S}-\text{R})}$ (Fig. 3) clearly defined the *S* configuration at C-15. The absolute configuration of C-13 was determined by CD spectrum (Fig. 4). The positive Cotton effect at approximately 265 nm and negative

Cotton effect at 316 nm of **1** suggested the *R* configuration at C-13, comparing to that of xestodecalactone A with opposite sign.^{7c,13} Therefore, the absolute configuration of **1** was established as 13*R*, 15*S*.

Sumalactone B (**2**) was isolated as a yellow oil and it was determined to be $\text{C}_{16}\text{H}_{20}\text{O}_6$ on the basis of negative HRESIMS, indicating that **2** is isomeric to **1**. Indeed, analysis of the NMR data indicated that compound **2** had the same planar structure as **1**. Considering that the only difference observed between **2** and **1** was the completely opposite CD curves (Fig. 4), we suggest that **2** is epimeric at C-13 relative to compound **1**. Consequently, the absolute configuration of **2** was assigned as 13*S*, 15*S*.

Fig. 3 $\Delta\delta(\delta_{\text{S}} - \delta_{\text{R}})$ values (in ppm) for the MTPA esters of **1** and **3**.Fig. 2 Key ^1H – ^1H COSY and HMBC correlations of **1** and **3**.Fig. 4 CD spectra of **1**–**4**.

Sumalactone C (**3**) was obtained as a yellow oil, for which the molecular formula was assigned as $C_{16}H_{20}O_6$ by HRESIMS, from a $[M - H]^-$ ion at 307.1191 (calc. 307.1182). The UV absorptions together with 1H and ^{13}C NMR data indicated the curvularin-type macrolide skeleton similar to that of compound **1**. The 1H - 1H COSY correlations between $H_{2-13}/H_{14}/H_{15}/H_{3-16}$, and HMBC correlation from H_{14} (δ_H 4.75) to $C-1$ (δ_C 172.4), confirmed the 11-membered macrolide in compound **3**, which was rare in nature and was first example of oxygenation at C-14 in curvularin skeleton. The absolute configuration at C-14 was assigned to be *R* because of the almost identical CD spectrum to that of **1** (Fig. 4). The OH group at C-15 was determined as *S* by the modified Mosher's method (Fig. 3). Therefore, the absolute configuration of **3** was established as 14*R*, 15*S*.

Sumalactone D (**4**) was attributed the molecular formula $C_{16}H_{20}O_6$ from its HRESIMS at m/z 307.1014 ($[M - H]^-$ 307.1014, calc. 307.1182). On analysis of its 1H and ^{13}C NMR spectra, similar features to that of **3** were evident, but with an opposite CD curve (Fig. 4). Therefore, the absolute configuration at C-14 was assigned as *S*. Thus, the absolute configuration of **4** was assigned as 14*S*, 15*S*.

In addition to compounds **1–4**, the known curvularin (**5**)¹⁰ and dehydrocurvularin (**6**)¹¹ were also isolated and identified from the fungal strain *P. sumatrense* MCCC 3A00612. All isolated compounds were tested for inhibitory activities against LPS-induced NO production in RAW 264.7 macrophages. As the results, only compound **6** showed significant NO production inhibitory activity with IC_{50} of $0.91 \pm 0.03 \mu\text{M}$, which was comparable to that of the positive control L-NMMA (IC_{50} of $41.91 \pm 1.27 \mu\text{M}$). The cell viability measured by the MTS assay showed that compound **6** had no significant cytotoxicity to the RAW 264.7 cells at the effective concentration for the inhibition of NO production.

Experimental section

General experimental procedures

Optical rotations were measured on an MCP 300 polarimeter, and UV spectra were measured on a U-2910 spectrometer. IR spectra were measured on an Affinity-1 FT-IR spectrometer. The CD spectra were recorded in MeOH using a Chiralscan spectropolarimeter at room temperature. HRESIMS spectra were obtained on Waters Synapt G2 TOF mass spectrometer. The NMR data were acquired with a Bruker AV 500 NMR spectrometer using solvent signals (CD_3OD : δ_H 3.30/ δ_C 49.0) as standards. Column chromatography (CC) was carried out on Sephadex LH-20 (Pharmacia, USA), and ODS (60–80 μm , YMC). TLC was performed on silica gel plate (SGF254, 0.2 mm, Merck, Germany). Analytical and semi-preparative HPLC were performed on an Agilent HPLC system equipped with a G1311B pump, a G1329B automated sample injector, a G1316A column compartment, and a G1315D diode array detector using a Phenomenex Kinetex C18 column (4.6 \times 250 mm, 5 μm), a Waters T3 C18 column (4.6 \times 250 mm, 5 μm) and a Phenomenex Kinetex C18 column (10.0 \times 250 mm, 5 μm).

Fungus material

The fungus *P. sumatrense* MCCC 3A00612 was isolated from deep-sea sediments collected from the Indian Ocean. The strain was identified by Dr Zongze Shao, and a voucher specimen (*P. sumatrense* MCCC 3A00612) has been deposited in the Marine Culture Collection of China.

Extraction and isolation

The fresh mycelia of *P. sumatrense* were grown on PDA medium at 28 °C for 4 days. Agar plugs were cut into small pieces and were selected to inoculate 10 Erlenmeyer flasks (500 mL) each containing 200 mL of PDB. The seed cultures were incubated at 28 °C on a rotary shaker (150 rpm) for 5 days and were then inoculated into 60 \times 500 mL conical flasks on rice solid medium (80 g rice, 0.36 g sea salt, and 120 mL filtered water) for 30 days at 28 °C. The fermented cultures were extracted with 70% acetone/water, and evaporated under reduced pressure to afford an aqueous solution, which was then extracted three times with EtOAc and afforded the EtOAc extract (10.4 g). The EtOAc extract (10.4 g) was fractionated by silica gel column chromatography (CC) eluting with $CHCl_3$ -MeOH (100 : 0, 95 : 5, 98 : 2, 9 : 1, 8 : 2, 1 : 1, and 0 : 100, v/v) to afford seven fractions (J1–J7). Fraction J5 (877.8 mg) was further subjected to Sephadex LH-20 CC using $CHCl_3$ -MeOH (1 : 1, v/v) to afford four subfractions (J5-1 to J5-4). Subfraction J5-3 (374.0 mg) was separated on a ODS column with a gradient of MeOH-H₂O (10 : 90, 30 : 70, 50 : 50, 70 : 30, and 100 : 0, v/v) to give three portions (J5-3-1 to J5-3-3). J5-3-1 (167.2 mg) was separated on semi-preparative HPLC using CH_3CN -H₂O (15 : 85, v/v) to give five portions (J5-3-1-1 to J5-3-1-5). J5-3-1-1 (9.4 mg) was purified on semi-preparative HPLC by using MeOH-H₂O (30 : 70, v/v) to yield **1** (7.3 mg). J5-3-1-2 (9.6 mg) was purified on semi-preparative HPLC by using CH_3CN -H₂O (22 : 78, v/v) to yield **2** (4.3 mg). J5-3-1-4 (7.4 mg) was purified on semi-preparative HPLC by using MeOH-H₂O (30 : 70, v/v) to yield **3** (2.9 mg). J5-3-1-5 (10.6 mg) was purified on semi-preparative HPLC by using MeOH-H₂O (30 : 70, v/v) to yield **4** (2.3 mg). J5-3-2 (112.0 mg) was purified on semi-preparative HPLC by using MeOH-H₂O (55 : 45, v/v) to yield **5** (8.5 mg). Fraction J3 (4.0 g) was subjected to a ODS column with a gradient of MeOH-H₂O (30 : 70, 50 : 50, 70 : 30, and 100 : 0, v/v) to give four subfractions (J3-1 to J3-4). Subfraction J3-2 (519.6 mg) was further separated by ODS CC eluting with MeOH-H₂O (50 : 50, v/v) to afford four portions (J3-2-1 to J3-2-4). J3-2-2 (21.0 mg) was purified on semi-preparative HPLC by using MeOH-H₂O (50 : 50, v/v) to yield **6** (6.8 mg).

Sumalactone A (1). Yellow oil (MeOH); $[\alpha]_D^{25} -124.0$ (*c* 0.1, MeOH); UV (MeOH) λ_{max} ($\log \varepsilon$) 204 (4.13), 218 (4.08), 269 (3.76), 297 (3.68) nm; CD (0.81 mM, MeOH) λ_{max} ($\Delta\varepsilon$) 211 (1.61), 226 (−0.22), 266 (6.52), 317 (−7.72) nm; IR (MeOH) ν_{max} 3194, 2968, 2938, 1715, 1663, 1607, 1589, 1472, 1339, 1267, 1161, 1136, 1024, 1007, 845, 669 cm^{-1} ; HRESIMS m/z 307.1197 [$M - H]^-$ (calcd for $C_{16}H_{19}O_6$, 307.1182); the 1H and ^{13}C NMR data, see Table 1.

Sumalactone B (2). Yellow oil (MeOH); $[\alpha]_D^{25} +86.1$ (*c* 0.1, MeOH); UV (MeOH) λ_{max} ($\log \varepsilon$) 203 (4.02), 218 (3.94), 268 (3.62),

298 (3.55) nm; CD (0.81 mM, MeOH) λ_{\max} ($\Delta\epsilon$) 210 (−1.61), 226 (0.45), 266 (−6.54), 317 (7.89) nm; IR (MeOH) ν_{\max} 3167, 2967, 2932, 1705, 1661, 1605, 1589, 1404, 1339, 1252, 1161, 1089, 1024, 989, 845 cm^{-1} ; HRESIMS m/z 307.1193 [M − H][−] (calcd for $\text{C}_{16}\text{H}_{19}\text{O}_6$, 307.1182); the ¹H and ¹³C NMR data, see Table 1.

Sumalactone C (3). Yellow oil (MeOH); $[\alpha]_D^{25}$ −41.6 (c 0.1, MeOH); UV (MeOH) λ_{\max} ($\log \epsilon$) 203 (4.04), 219 (3.95), 268 (3.61), 297 (3.54) nm; CD (0.81 mM, MeOH) λ_{\max} ($\Delta\epsilon$) 214 (1.30), 231 (−1.90), 265 (4.53), 318 (−6.30) nm; IR (MeOH) ν_{\max} 3256, 2934, 1703, 1651, 1607, 1591, 1462, 1335, 1261, 1161, 1088, 1042, 1013, 845, 665 cm^{-1} ; HRESIMS m/z 307.1191 [M − H][−] (calcd for $\text{C}_{16}\text{H}_{19}\text{O}_6$, 307.1182); the ¹H and ¹³C NMR data, see Table 1.

Sumalactone D (4). Yellow oil (MeOH); $[\alpha]_D^{25}$ +35.8 (c 0.1, MeOH); UV (MeOH) λ_{\max} ($\log \epsilon$) 203 (4.04), 219 (3.96), 268 (3.63), 298 (3.54) nm; CD (0.81 mM, MeOH) λ_{\max} ($\Delta\epsilon$) 213 (−1.45), 231 (1.76), 265 (−4.75), 317 (5.87) nm; IR (MeOH) ν_{\max} 3304, 2943, 1703, 1651, 1607, 1462, 1337, 1265, 1161, 1040, 993, 847 cm^{-1} ; HRESIMS m/z 307.1014 [M − H][−] (calcd for $\text{C}_{16}\text{H}_{19}\text{O}_6$, 307.1182); the ¹H and ¹³C NMR data, see Table 1.

Curvularin (5). $[\alpha]_D^{25}$ −22.5 (c 0.1, MeOH); literature value $[\alpha]_D^{25}$ −28.6 (c 0.4, EtOH).

Dehydrocurvularin (6). $[\alpha]_D^{25}$ −45.6 (c 0.1, MeOH); literature value $[\alpha]_D^{25}$ −65.9 (c 1.8, EtOH).

NO production bioassay

The murine macrophage cell line RAW 264.7 was obtained from Cell Bank of Chinese Academy of Sciences. RAW 264.7 cells were seeded in 96-well cell culture plates (1.5×10^5 cells per well) and treated with serial dilutions of the compounds with a maximum concentration of 25 μM in triplicate, followed by stimulation with 1 $\mu\text{g mL}^{-1}$ LPS (Sigma, St. Louis, MO, USA) for 18 h. NO production in the supernatant was assessed by Griess reagents (Reagent A & Reagent B, respectively, Sigma). The absorbance at 570 nm was measured with a microplate reader (Thermo, Waltham, MA, USA). N^G-methyl-L-arginine acetate salt (L-NMMA, Sigma), a well-known nitric oxide synthase (NOS) inhibitor, was used as a positive control.¹⁴ The viability of RAW 264.7 cells was evaluated by the MTS assay simultaneously to exclude the interference of the cytotoxicity of the test compounds.

Conclusions

In summary, six curvularin-type macrolides belonging to DALs group¹⁵ were isolated from *P. sumatrense* MCCC 3A00612, including four new ones. The curvularins are octaketides composed of a 12-membered macrolide skeleton attached to a 3,5-dihydroxyphenylacetic acid. Compounds 1–4 are the new curvularin-type macrolides with 10- or 11-membered macrolide skeleton, among them, compounds 3 and 4 are the first macrolides with 11-membered macrolide skeleton. Interestingly, compounds 1–4 were isolated as epimers at the lactone carbon. Although the configuration at the lactone carbon is obviously variable among the DALs including the (−)-(15S)-curvularin (compound 5) and (+)-(15R)-curvularin series⁵ but they were isolated from different sources. However, epimers at the non-

lactone carbon including (+)-(11S,15R)-11-hydroxycurvularin and (+)-(11R,15R)-11-hydroxycurvularin,⁵ as well as (−)-(11R,15S)-11-hydroxycurvularin and (−)-(11S,15S)-11-hydroxycurvularin¹⁶ were isolated from same sources. Therefore, the lactone formation does not change the absolute configuration of the lactone carbon, which is established upon generating the secondary hydroxyl group. Different positions of lactone cyclization would produce different ring sizes, which was certainly observed. In addition, compound 6 exhibited potent inhibitory effect on NO production when tested *in vitro*. High levels of NO are markers at the treatment of inflammatory disorders. Our results suggested that dehydrocurvularin (6) may be a potential candidate for further evaluation on the molecular mechanism of action on specific inflammatory disorders.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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