Tetranorlabdane Diterpenoids from the Deep Sea Sediment-Derived Fungus Aspergillus wentii SD-310

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Abstract
Two new tetranorlabdane diterpenoids, asperolides D (1) and E (2), along with six related known congeners (3–8), were isolated and identified from the culture extract of the deep sea sediment-derived fungus Aspergillus wentii SD-310. The structures of these compounds were established on the basis of spectroscopic interpretation, and the skel-eton and absolute configurations of asperolides D (1) and E (2) were determined by X-ray crystallographic analysis. Compounds 1 and 2 were evaluated for their cytotoxic activity against seven tumor cell lines and antibacterial activity against two human and eight aquatic pathogens.

Supporting information available online at http://www.thieme-connect.de/products

Introduction
Marine-derived microorganisms have been proven as a promising source for the discovery of pharmaceutically important metabolites [1–5]. As part of our research toward the chemical investigation of marine-derived fungi, a variety of structurally interesting and biologically active compounds have been isolated and identified [6–11]. Among them, a series of tetranorlabdane diterpenoids, such as asperolides A–C and wentilactones A and B, have been identified from the marine brown alga-derived endophytic fungus Aspergillus wentii SD-310. The structures of these compounds exhibited both in vitro and in vivo antitumor activities against several human tumor cell lines [11–13]. In the course of our chemical screening experiments, another isolate of the deep sea sediment-derived fungal species, A. wentii SD-310, displayed identical morphological characteristics to that of our previously reported fungus A. wentii EN-48 [11]. A chemical investigation of the strain A. wentii SD-310 led to the isolation of two new tetranorlabdane diterpenoids, asperolides D (1) and E (2), together with six related derivatives (3–8) (Fig. 1). The structures of these compounds were established by extensive analysis of spectroscopic methods, and the absolute configurations of compounds 1 and 2 were determined by X-ray crystallographic analysis. The cytotoxic activity against seven tumor cell lines and antibacterial activity against two human and eight aquatic pathogens were evaluated. This paper describes the isolation, structure elucidation, and bioactivity of compounds 1–8.

Results and Discussion
The combined extracts from the mycelium and culture broth of A. wentii SD-310 were purified by a combination of column chromatography (CC) including silica gel, Lobar LiChroprep RP-18, Sephadex LH-20, and semipreparative HPLC to yield compounds 1–8.

Asperolide D (1) was obtained as a colorless crystal. Its molecular formula was demonstrated as C13H18O6 by high-resolution electrospray ionization mass spectrometry (HRESIMS), with nine degrees of unsaturation. The 13C NMR data (Table 1) revealed the presence of sixteen carbon atoms and were clarified into seven non-protonated carbons, five methines (with three olefinic methines), two methylenes, and two methyls. Combined with the 1H NMR assignments (Table 1) along with a heteronuclear single quantum coherence (HSQC) experiment, two methyl singlets (H-18 and H-20), three doublets for olefinic protons (H-2, H-3, and H-7), and one singlet for an exchangeable proton (OH-9) were assigned for 1. Exhaustive analysis of the 1D NMR data indicated that compound 1 belongs to the tetranorlabdane di-
terpenoid class and possesses the same carbon skeleton as that of asperolide A (3) [11]. However, signals for the oxygenated CH at C-1 and the double bond at C-9/C-11 in the NMR spectra of 3 disappeared in that of 1. Instead, resonances corresponding to a carbon at C-1 (δC 198.9), an oxygenated quaternary carbon at C-9 (δC 70.9), and a CH2 unit at C-11 (δH 3.04 and 3.39, δC 38.2) were present in the NMR spectra of 1. The above evidence suggested that the structure of 1 had a conjugated carbonyl group at C-1 with the double bond at C-2/C-3, while the double bond at C-9/C-11 was reduced, combined with the hydroxylation at C-9. The observed heteronuclear multiple-bond correlations (HMBCs) from the proton of OH-9 to C-9, C-11, and C-17, from H-3 to C-1, C-5, and C-18, from H2-20 to C-1, C-5, C-9, and C-10, as well as other essential HMBCs as shown in Fig. 2 allowed for the elucidation of the planar structure of 1 as depicted in Fig. 1. The relative configuration of 1 was assigned by analysis of its nuclear Overhauser effect spectroscopy (NOESY) spectrum. The observed NOEs from H-5 to H-6, H-18, and the proton of 9-OH located them on the same face of the molecule (Fig. 3). The absolute configuration of 1 was determined by the X-ray diffraction using Cu/Kα radiation, and the final refinement of the crystallographic data resulted in a Flack parameter of 0.0(5), which allowed for the determination of the absolute configuration of 1 as 4S, 5R, 6R, 9R, and 10S (Fig. 4).

Asperolide E (2) was also obtained as a colorless crystal. The molecular formula, C16H18O5, was determined by positive HRESIMS, indicating eight degrees of unsaturation. Detailed analysis of the 1D NMR data (Table 1) indicated that compound 2 also belongs to the tetranorlabdane diterpenoid class and possesses the same carbon skeleton as asperolide A (3) [11]. The 1D NMR spectroscopic data (Table 1) differed from those of 3 mainly in the absence of the signals for the double bond at C-9/C-11. Instead, resonances corresponding to a CH2 unit at δH 2.90/3.01 and δC 31.3 (C-11) and to a CH unit at δH 2.60 and δC 42.3 (C-9) were present in the NMR spectra of 2. In addition, the carbonyl carbon signal at δC 163.4 (C-12) and the double bond carbon signal at δC 132.3 (C-8) in 3 shifted downfield at δC 173.8 (C-12) and δC 140.4 (C-8) in 2, respectively, while the double bond carbon signal at δC 121.8 (C-7) in 3 shifted upfield at δC 118.0 in 2. These data proved the absence of the conjugated system at C-7/C-8 and C-12 in 2. The observed correlation spectroscopy (COSY) correlation between H-9 and H-11 (Fig. 2) supported the above deduction. The planar structure of compound 2 was thus determined. The relative configuration of 2 was also deduced by the NOESY spectrum. Protons of H-1, H-5, H-6, H-9, and H-18 were placed on the same face by the observed NOESY correlations of H-9 with H-1, H-5, H-6, H-9, and H-18 (Fig. 3). The X-ray crystallographic experiment also proved the above deduction and the Cu/Kα radiation used for the diffraction, with the refined Flack parameter of 0.0(2), allowed for the assignment of the absolute configuration of the stereogenic centers in the new terpene-diterpenoids, asperolides A (3) and B (4) [11], wentilactones A (5) and B (6) [14], and additional tetranorlabdane diterpenoids (7) and (8) [15], were also isolated and identified.

Table 1 1H (500 MHz) and 13C (125 MHz) NMR data for compounds 1–3 (in DMSO-d6).

<table>
<thead>
<tr>
<th>Pos.</th>
<th>Asperolide D (1)</th>
<th>Asperolide E (2)</th>
<th>Asperolide A (3)</th>
</tr>
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<tr>
<td>δH (J in Hz)</td>
<td>δC type</td>
<td>δH (J in Hz)</td>
<td>δC type</td>
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<tr>
<td>1</td>
<td>198.9 s</td>
<td>4.12 br s</td>
<td>72.7 d</td>
</tr>
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<td>2</td>
<td>5.89 d (9.9)</td>
<td>128.9 d</td>
<td>5.52 br d (9.9)</td>
</tr>
<tr>
<td>3</td>
<td>6.79 d (9.9)</td>
<td>145.6 d</td>
<td>5.62 dd (9.9, 1.9)</td>
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<tr>
<td>4</td>
<td>45.2 s</td>
<td>44.3 s</td>
<td>44.1 s</td>
</tr>
<tr>
<td>5</td>
<td>3.30 d (5.0)</td>
<td>44.0 d</td>
<td>2.22 d (4.9)</td>
</tr>
<tr>
<td>6</td>
<td>5.26 td (5.0, 1.2)</td>
<td>71.3 d</td>
<td>5.09 td (4.9, 1.3)</td>
</tr>
<tr>
<td>7</td>
<td>6.09 d (1.2)</td>
<td>118.2 d</td>
<td>6.06 d (1.3)</td>
</tr>
<tr>
<td>8</td>
<td>140.5 s</td>
<td>140.4 s</td>
<td>132.4 s</td>
</tr>
<tr>
<td>9</td>
<td>70.9 s</td>
<td>2.60 t (7.4)</td>
<td>42.3 d</td>
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<td>47.7 s</td>
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<td>11a</td>
<td>3.04 d (15.2)</td>
<td>38.2 t</td>
<td>2.90 dd (9.9, 15.4)</td>
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<td>11b</td>
<td>3.39 d (15.2)</td>
<td>3.01 dd (6, 15.4)</td>
<td>173.8 s</td>
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<td>171.6 s</td>
<td>173.8 s</td>
<td>163.5 s</td>
</tr>
<tr>
<td>13</td>
<td>4.91 d (14.8)</td>
<td>68.2 t</td>
<td>4.81 d (14.7)</td>
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<tr>
<td>14</td>
<td>4.97 d (14.8)</td>
<td>4.84 d (14.7)</td>
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<td>15</td>
<td>1.51 s</td>
<td>23.3 q</td>
<td>23.4 q</td>
</tr>
<tr>
<td>16</td>
<td>176.5 s</td>
<td>178.3 s</td>
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<tr>
<td>17</td>
<td>1.04 s</td>
<td>20.0 q</td>
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<td>1-OH</td>
<td>5.19 br s</td>
<td>5.50 br s</td>
<td></td>
</tr>
<tr>
<td>9-OH</td>
<td>5.66 s</td>
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</table>
The new compounds 1 and 2 were assayed for their cytotoxic activities against seven tumor cell lines. Compound 2 displayed cytotoxic activities against the HeLa, MCF-7, and NCI-H446 cell lines, with IC50 values of 10.0, 11.0, and 16.0 µM, respectively, whereas compound 1 was found to exhibit no significant activities. In combination with our previously reported data [11–13], it is likely that the tetranorlabdane diterpenoids having two (five- and six-membered) lactone moieties usually exhibit good cytotoxic activities, but the keto substitution at C-1 decreases the activity (1 vs. 2).

All of the isolated compounds were examined for their antibacterial activity against two pathogenic bacteria (Escherichia coli and Staphylococcus aureus) and eight aquatic bacteria (Aeromonas hydrophila, Edwardsiella tarda, Micrococcus luteus, Pseudomonas aeruginosa, Vibrio alginolyticus, Vibrio anguillarum, Vibrio harveyi, and Vibrio paraheamolyticus). Compounds 1 and 3 showed moderate activity against E. tarda, each with an MIC value of 16 µg/mL.

Materials and Methods

General
Melting points (m.p.) were measured on an SGW X-4 micromelting-point apparatus. Optical rotations were determined on a Jasco P-1020 digital polarimeter. UV spectra were measured on a PuXi TU-1810 UV-visible spectrophotometer. 1D and 2D NMR spectra were recorded at 500 MHz and 125 MHz for 1H and 13C, respectively, on a Bruker Avance 500 spectrometer with TMS as the internal standard. Mass spectra were determined on a VG Autospec 3000 or an API QSTAR Pulsar 1 mass spectrometer. CC was performed with silica gel (200–300 mesh, Qingdao Haiyang Chemical Co.), Lobar LiChroprep RP-18 (40–63 µm, Merck), and Sephadex LH-20 (18–110 µm, Merck). Semipreparative HPLC was performed using a Dionex HPLC system equipped with a P680 pump, an ASI-100 automated sample injector, and a UVD340U multiple wavelength detector controlled using Chromelon software, version 6.80. All solvents used were distilled prior to use.

Fungal material
The fungus A. wentii SD-310 was isolated from a deep sea sediment sample collected in the South China Sea at a depth of 2038 m, in May 2012. The sediment sample was stored in a sterile plastic bag and was transported to the laboratory immediately where it was kept frozen until processed. The sample was diluted 10-, 100-, and 1000-fold using sterile seawater. One milliliter of each diluted sample (one milliliter of sterile seawater was used as the blank control) was processed utilizing the spread plate method in PDA medium (200 g of sliced potato, 20 g of dextrose, 20 g of agar, and 1 L of seawater) plates. The plates were incubated at 28°C for 7 days. After purifying the isolates several times, the final pure culture was selected and deposited at the Key Laboratory of Experimental Marine Biology, Institute of Oceanology, Chinese Academy of Sciences. The fungus was identified by classical microscopic analysis and using a molecular biological protocol by DNA amplification and sequencing of the ITS region, as described in our previous report [16]. The sequence data derived from the fungal strain have been deposited at Genbank (accession no. KM409566). A BLAST search result showed that the sequence was the same (100%) as that of the sequence of A. wentii (compared to no. HM014129.1 and KF921087.1).

Extraction and isolation
For chemical investigations, the fungal strain was statically fermented for 30 days at room temperature in liquid medium containing 50% (v/v) seawater, 20% potato juice, 2% glucose, 0.5% peptone, and 0.3% yeast extract (pH 6.0). The whole fermented cultures (10 L) were filtered to separate the broth from the mycelia. The former was extracted three times
with EtOAc (20 L), while the latter was extracted three times with a mixture of 80% acetone and 20% water (5 L). The acetone fraction was evaporated under reduced pressure to afford an aqueous fraction (1 L), which was then extracted three times with EtOAc (3 L). Since the TLC and HPLC profiles of the two EtOAc fractions were almost identical, they were combined and concentrated under reduced pressure to give an extract (7.5 g) for further separation.

The crude extract was fractionated by silica gel vacuum liquid chromatography (VLC) using different solvents of increasing polarity from petroleum ether (PE) to MeOH (1 L for each elution) to yield four fractions (Fr. 1–4), and the tetranorditerpenoid compounds were concentrated in Fr. 3 based on HPLC analysis. Fr. 3 (3.2 g), eluted with CHCl₃:MeOH (40:1) in the VLC procedure, was further purified by CC on reversed-phase silica gel C₁₈ eluted with a MeOH-H₂O gradient (10% to 20%) to produce three parts (P.1–P.3). P.1 was further separated by CC on silica gel eluting with a CHCl₃:acetone gradient (5:1) to afford compounds 1 (8.6 mg, purity > 99%) as single crystals and 5 (354.4 mg, purity > 95%). P.2 was also purified by CC on silica gel eluting with a CHCl₃:acetone gradient (5:1) to afford compound 6 (254.2 mg, purity > 95%) and a subfraction (505.4 mg), which was further purified by semipreparative HPLC (Agilent Prep RP-18 column, 21 × 250 mm, 10 µm, 30% MeOH-H₂O, 16 mL/min) to afford compounds 2 (8.3 mg, tR 17.8 min, purity > 99%) as single crystals and 7 (213.5 mg, tR 19.5 min, purity 99%). P.3 was further purified by CC on silica gel eluting with a CHCl₃:acetone gradient (10:1) and semipreparative HPLC (Agilent Prep RP-18 column, 21 × 250 mm, 10 µm, 25% MeCN-H₂O, 16 mL/min) to afford compounds 3 (145.1 mg, tR 17.3 min, purity > 99%), 4 (7.8 mg, tR 20.5 min, purity > 95%), and 8 (15.5 mg, tR 21.3 min, purity > 95%).

**Asperolide D** (1): Colorless single crystal (MeOH); m.p. 243–244 °C; [α]D²⁰ +4.0 (c 0.55, MeOH); UV (MeOH) λmax (log ε) 202 (6.33), 229 (4.72) nm; 1H (500 MHz) and 13C NMR (125 MHz) data, see Table 1; ESIMS m/z 327 [M + Na]+; HRESIMS m/z 327.0846 [M + Na]+ (calcd. for C₁₆H₁₈O₅Na, 327.0839).

**Asperolide E** (2): Colorless single crystal (MeOH); m.p. 264–266 °C; [α]D²⁰ +19.7 (c 0.55, MeOH); UV (MeOH) λmax (log ε) 202 (5.86), 258 (0.68) nm; 1H (500 MHz) and 13C NMR (125 MHz) data, see Table 1; ESIMS m/z 313 [M + Na]+; HRESIMS m/z 313.1051 [M + Na]+ (calcd. for C₁₆H₁₈O₅Na, 313.1046).

X-ray analysis of compounds 1 and 2

Colorless crystals were obtained from a solution of MeOH of compounds 1 and 2. All crystallographic data were collected on a Srigaku Mercury CCD/AFCR diffractometer equipped with graphite-monochromatized Cu/Kα radiation (λ = 1.54178 Å) at 293(2) K. The data were corrected for absorption by using the program SADABS [17]. The structures were solved by direct methods with the SHELXTL software package [18]. All non-hydrogen atoms were refined anisotropically. The H atoms were located by geometrical calculations, and their positions and thermal parameters were fixed during the structure refinement. The structures were refined by full-matrix least-squares techniques [19]. Crystallographic data of compounds 1 and 2 have been deposited in the Cambridge Crystallographic Data Centre as CCDC 1040480 for compound 1 and CCDC 1040479 for compound 2. These data can be obtained free of charge via http://www.ccdc.cam.ac.uk/data_request/cif (or from the CCDC, 12 Union Road, Cambridge CB21EZ, U.K.; fax: +44-1223-336-033; e-mail: deposit@ccdc.cam.ac.uk).

**Crystal data for compound 1**

C₁₆H₁₈O₅, fw = 290.30, monoclinic space group P1(1), unit cell dimensions a = 6.1153(2) Å, b = 14.2007(5) Å, c = 8.5139(4) Å, β = 90°, V = 1707.50(14) Å³, α = γ = 90°, β = 106.89(2)°, Z = 2, dcalc = 1.363 mg/m³, crystal dimensions 0.4 × 0.2 × 0.1 mm, µ = 0.840 mm⁻¹, F(000) = 308. The 2231 measurements yielded 1788 independent reflections after equivalent data were averaged, and Lorentz and polarization corrections were applied. The final refinement gave R₁ = 0.0391 and wR₂ = 0.1036 [I > 2σ(I)]. The Flack parameter was 0.0(2) in the final refinement for all 2229 reflections with 1503 Friedel pairs.

**Crystal data for compound 2**

C₁₆H₁₈O₅, fw = 304.29, monoclinic space group P2(1), unit cell dimensions a = 9.4895(12) Å, b = 7.8239(6) Å, c = 10.0412(12) Å, β = 715.50(14)°, α = γ = 90°, β = 106.311(13)°, Z = 2, dcalc = 1.412 mg/m³, crystal dimensions 0.6 × 0.28 × 0.10 mm, µ = 0.915 mm⁻¹, F(000) = 320. The 2229 measurements yielded 1503 independent reflections after equivalent data were averaged, and Lorentz and polarization corrections were applied. The final refinement gave R₁ = 0.0394 and wR₂ = 0.0892 [I > 2σ(I)]. The Flack parameter was 0.0(5) in the final refinement for all 2229 reflections with 1503 Friedel pairs.

**Cytotoxicity assays**

The cytotoxic activities of the new compounds 1 and 2 against seven tumor cell lines including A549 (human lung cancer cell line), H446 (human small cell lung cancer cell line), HeLa (human cervix carcinoma cell line), HuH7 (human hepatocarcinoma cell line), LO2 (human hepatic cell line), MCF-7 (human breast carcinoma cell line), and SW1990 (human pancreatic carcinoma cell line) were determined according to previously reported methods [20]. Paclitaxel and adriamycin were used as positive controls. Paclitaxel (Sigma-Aldrich Co., purity 98.5%) displayed cytotoxic activities against the HeLa and MCF-7 cell lines with IC₅₀ values of 1.8 and 4.9 µM, respectively. Adriamycin (Sigma-Aldrich Co., purity 98%) displayed cytotoxic activities against the NCI-H446 cell line with an IC₅₀ value of 4.0 µM.

**Antibacterial assays**

Antibacterial assays against two pathogenic bacteria (E. coli QDIO-1 and S. aureus QDIO-2) and eight marine bacterial strains (A. hydrophila QDIO-3, E. tarda QDIO-4, M. luteus QDIO-5, P. aeruginosa QDIO-6, V. alginolyticus QDIO-7, V. anguillarum QDIO-8, V. harveyi QDIO-9, and V. parahaemolyticus QDIO-10) were carried out by the microplate assay [21]. The pathogenic bacteria and aquatic pathogen strains were provided by the Institute of Oceanology, Chinese Academy of Sciences, while the plant pathogenic fungi strains were provided by Qingdao Agricultural University. All of the microbial strains used in the bioassays are preserved at the Key Laboratory of Experimental Marine Biology, Institute of Oceanology, Chinese Academy of Sciences. Chloramphenicol (Sinopharm Chemical Reagent Co., purity 98%) and ampicillin (Sinopharm Chemical Reagent Co., purities 98%) were used as positive controls. Chloramphenicol shows MIC values of 4.0, 0.5, 4.0, 8.0, 8.0, 0.5, 1.0, 4.0, and 0.5 µg/mL against the bacterial strains QDIO-1 to QDIO-10, respectively, while ampicillin shows MIC values of 8.0, 4.0, 4.0, 2.0, 4.0, 2.0, 1.0, 2.0, 4.0, and 8.0 µg/mL against the bacterial strains QDIO-1 to QDIO-10, respectively.
Supporting information
The HRESIMS and 1D and 2D NMR spectra for compounds 1 and 2 are available as Supporting Information.

Acknowledgements
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Conflict of Interest
The authors declare no conflicts of interest.

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