New Macrolides and Furan Carboxylic Acid Derivative from the Sponge-Derived Fungus *Cladosporium herbarum*

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Bioassay-guided fractionation of organic extracts of *Cladosporium herbarum*, isolated from the marine sponge *Callyspongia aerizusa*, yielded two new macrolide metabolites: pandangolide 3 and 4 (**1** and **2**) and the known fungal metabolites pandangolide 2 **(3)**, cladospolide B (**4**), and iso-cladospolide B (**5**). Also isolated were the antimicrobially active (against *Bacillus subtilis* and *Staphylococcus aureus*) furan carboxylic acids: Sumiki's acid (**6**) and its new derivative, acetyl Sumiki's acid (**7**). All structures were elucidated by spectroscopic methods.

Growing interest in marine natural products has led to the discovery of an increasing number of bioactive secondary metabolites from marine fungi.[1−5] These include fungal metabolites containing 12-membered lactone rings such as recifeiolide isolated from *Cephalosporium recifei*,[6] cladospolide A, B, and C from *Cladosporium cladosporioides*[7] and *Cladosporium tenuissimum*,[8] and patulolides A, B, and C from *Penicillium urticae S11r59*.[9,10] Cladospolides A and B were shown to be phytotoxic,[8] whereas patulolides A and B were reported to have strong antifungal and slight antibacterial activities.[10]

A recent report described the isolation of the polyketide metabolites iso-cladospolide B (**5**), pandangolide 1, pandangolide 2 (**3**), and seco-patulolide 2 from an unidentified fungus obtained from an unidentified sponge collected in Indonesia.[11] In the present paper we describe the isolation and identification of the known metabolites cladospolide B (**4**), iso-cladospolide B (**5**), and pandangolide 2 (**3**), as well as of a new 12-membered macrolide, which we name pandangolide 3 (**1**), and a new macrolide dimer, which we name pandangolide 4 (**2**), from *Cladosporium herbarum* (Pers.) isolated from the marine sponge *Callyspongia aerizusa*. In addition we report on the isolation of furan carboxylic acid derivatives including 5-hydroxymethyl-2-furancarboxylic acid, also known as Sumiki's acid (**6**), and its new derivative, acetyl Sumiki's acid (**7**).

Fungal cultures of *C. herbarum* were isolated from a tissue sample of *C. aerizusa*, which was collected in Indonesia. It is interesting to note that the fungal culture in the study of Smith et al.,[11] which was described as septate and as having colony color of light gray to olive, agrees with the description of *C. herbarum* isolated by us. It would appear, however, that the unknown marine sponge from which their fungus was isolated,[11] described as bright orange and encrusting, differs from *C. aerizusa*.

The fungal isolate was fermented in malt extract medium for 28 days followed by extraction of the culture broth and mycelia with EtOAc. The crude extract, after complete removal of the solvent, was partitioned between petroleum ether, EtOAc, n-BuOH, and H_2O. Brine-shrimp assay of the resulting solvent phases found the EtOAc phase to be active. Both the EtOAc and the *n*-BuOH extracts were also active against *Bacillus subtilis* and *Staphylococcus aureus*. Comparison of the HPLC chromatograms of both extracts showed that the *n*-BuOH extract contained only one polar compound (**6**), which was also present in the EtOAc extract. It was inferred that this polar compound could be responsible for the antimicrobial activity of both extracts; hence isolation was concentrated on the EtOAc extract. The EtOAc phase afforded the metabolites pandangolide 3 (**1**), pandangolide 4 (**2**), pandangolide 2 (**3**), cladospolide B (**4**), iso-cladospolide B (**5**), and the antimicrobially active furan carboxylic acid derivatives obtained from the polar fractions, Sumiki's acid (**6**) and acetyl Sumiki's acid (**7**).

Pandangolide 3 (**1**) was obtained as an oil which showed a base peak in the positive ion ESIMS at *m*/*z* 363 ($[M + H]^+$) and a molecular ion peak at *m*/*z* 362 with EIMS. The exact mass of this ion, which was measured by HREIMS to be 362.1391, corresponds to the molecular formula $C_{16}H_{26}O_7S$. The ^{13}C NMR spectrum of **1** was similar to that of pandangolide 2 (**3**) with an additional low-field signal from a methine carbon (C-14) at 72.0 ppm, indicating an oxygen-bonded carbon (Table 1). Another low-field signal at 52.6 ppm suggested a methoxyl carbon (C-16) which was attached to C-15, based on an HMBC correlation of H_3-16 (δ 3.79) with C-15 (δ 174.5). Correlations between H_2-13 and H-14 in the COSY spectrum as well as between H_2-13 with C-14 and C-15, respectively, in the HMBC spectrum revealed the nature of the sulfur-containing side chain in **1**. The position of the sulfide group with respect to the macrocyclic lactone ring was evident from a long-range correlation between H-13 and C-3, and vide infra. As in **3**, the positions of C-1 to C-6 in **1** were established from correlations between H_2-2, H-3, H-5, and H_2-6 with C-4, and H_2-2 as well as H-3 with C-1, respectively.[11] The assignments of C-7 to C-10 were based on key HMBC correlations between H-5 with C-7, H-6B with C-8, and H_3-12 with C-10, respectively.

Pandangolide 4 (**2**) was obtained as an oil whose positive ion ESIMS spectrum showed an intense pseudo molecular ion peak at *m*/*z* 487 ($[M + H]^+$). EIMS measurement gave

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Table 1. ^{13}C NMR (CD_3OD) Data for **1** and **2**

position	**1**	**2**
1	172.5	174.5
2	42.2	41.9
3	43.7	43.6
4	215.0	214.5
5	77.8	77.7
6	32.4	34.0
7	21.7	21.6
8	29.0	29.0
9	23.7	23.6
10	34.1	32.4
11	74.4	74.5
12	20.0	19.9
13	36.6	
14	72.0	
15	174.5	
16	52.6	

a molecular ion peak at *m*/*z* 486. This ion had a molecular weight of 486.2276 as measured by HREIMS, corresponding to the molecular formula $C_{24}H_{38}O_8S$. The ^{13}C NMR showed only 12 signals, while integration of the proton signals in the ^1H NMR spectrum yielded only 19 protons, suggesting that **2** was a symmetrical dimer. The ^{13}C NMR spectrum was similar to that of pandangolide 2 (**3**) and 3 (**1**) but with the absence of the alkyl side chain. The similarity of the chemical shifts of C-2 and C-3 with those of **1** and **3** indicates that in **2** the sulfide was retained, but instead of being linked to an alkyl side chain, it bridged two identical macrocyclic lactone rings to form the dimer. This was corroborated by EIMS signals at *m*/*z* 228, which corresponds to the mass of a monomer, and at *m*/*z* 260, corresponding to a monomer with a thiol group. Furthermore, the dimeric structure of **2** was proved by an HMBC experiment where H-3, aside from showing a residual direct correlation signal with C-3, also gave a long-range correlation with the respective carbon C-3*.

The absolute configuration of the chiral centers of cladospolide B (**4**) was already established as 4*S*, 5*S*, and 11*R*.[8] Attempts at determining the relative stereochemistry of C-3 and C-5 in the new compounds **1** and **2**, however, proved unsuccessful when measurement of their NOE and ROESY spectra at two different magnetic field strengths exhibited no significant NOEs involving H-3 and H-5, as well as H-11 and H-12. This may have been due to the flexibility of the bonds in the macrolide structure. Mosher reaction, which could only have established the absolute stereochemistry of C-5 and C-11 (after hydrolysis) of both compounds, as well as of C-14 of **1**, was not performed due to the limited amounts isolated.Sumiki's acid (**6**), which we isolated as a brown oil, is a known fungal metabolite originally isolated from *Penicillium italicum* and *Pyricularia grisea*.[12] **6** and its new derivative, acetyl Sumiki's acid (**7**), showed similar fragmentation patterns in their EIMS spectra. **7**, which was also isolated as a brown oil, gave a molecular ion peak at *m*/*z* 184 and an ion peak at *m*/*z* 142 corresponding to $(M - CH_3CO)^+$, which is also the molecular ion peak of **6**. Both compounds gave ion peaks at *m*/*z* 125 and 97, corresponding to $(M - OH)^+$ and $(M - COOH)^+$ in **6**, respectively. Furthermore, the UV spectra of **6** (maximum absorption at 260 nm) and **7** (maximum absorption at 256 nm) were nearly identical. The ^1H NMR spectra of both compounds were also very similar. The differences include the downfield shift of H-6 from 4.61 to 5.14 in the acetylated derivative, together with an extra methyl group signal at 2.11 ppm. These and the MS data established the structure of acetyl Sumiki's acid (**7**).

Like previously reported for pandangolide 1 and 2 (**3**),[11] pandangolides 3 (**1**) and 4 (**2**) were inactive when tested against Gram-positive and Gram-negative bacteria. This appears to confirm previously drawn observations[10] that antimicrobial activity of these macrolides requires the presence of a double bond flanked by two carbonyl carbons, which are common in other antimicrobial agents such as pyrenophorin, pyrenolides, and vermiculine. This structural feature, however, is absent in the macrocyclic lactones isolated in this report. Consistent with findings from initial bioassays, the polar furan carboxylic acid metabolite Sumiki's acid (**6**) and its acetyl derivative (**7**) were active against *B. subtilis* and *S. aureus* (see Table 3), whereas they were inactive against *Escherichia coli* and *Candida albicans*.

Experimental Section

General Experimental Procedures. ^1H NMR and ^{13}C NMR spectra (chemical shifts in ppm) were recorded at 300 K on Bruker DPX 300, ARX 400, or AVANCE DMX 600 NMR spectrometers. Mass spectra, (EIMS and ESIMS) were measured on a Finnigan MAT 8430 mass spectrometer and Finnigan MAT TSQ-7000 mass spectrometer, respectively. Specific rotations were measured on a Perkin-Elmer Model 341 LC polarimeter. For HPLC analysis, samples were injected into an HPLC system coupled to a photodiode-array detector (Gynkotek, Munich, Germany). Routine detection was at 254 nm in aqueous MeOH. The separation column (125 × 4 mm, i.d.) was prefilled with Eurospher C_{18} (Knauer, Berlin, Germany).

Solvents were distilled prior to use, and spectral grade solvents were used for spectroscopic measurements. TLC was performed on TLC plates precoated with Si 60 F_{254} (Merck, Darmstadt, Germany). The compounds were detected from their UV absorbance at 254 nm and by spraying the TLC plates with anisaldehyde reagent.

Animal Material. Samples of the sponge *Callyspongia aerizusa* (Desqueyroux-Faúndez, 1984) were collected by scuba diving in Bali Bata National Park, Indonesia, in September of 1997. A voucher fragment is kept under the registration number ZMA POR. 13437 in the Zoological Museum, Amsterdam, Netherlands. Under sterile conditions, a piece of tissue from the inner part of the freshly collected sponge was cut and inoculated on malt agar slants consisting of 15 g/L agar and 24.4 g/L SERA artificial sea-salt mixture. The inoculated agar slants were incubated at 27 °C, and from these a pure fungal culture was isolated after repeated inoculation on fresh malt agar plates. The isolated fungus was identified as *Cladosporium herbarum* (Pers.) by the Centraalbureau voor Schimmelcultures, Baarn, The Netherlands. Mass cultivation of the fungus was carried out in Erlenmeyer flasks in malt extract broth consisting of 15 g/L malt extract in distilled water supplemented with 24.4 g/L artificial sea salt mixture. After 41 days incubation at room temperature without shaking, the mycelium and the broth were extracted with EtOAc.

Extraction and Isolation. The total dried extract (7.01 g) was partitioned between H_2O (50 mL) and the following solvents in succession: petroleum ether, EtOAc, and *n*-BuOH (50 mL × 4). Each of the solvent extracts was taken to dryness and screened for bioactivity. Brine-shrimp assay found the EtOAc extract to be active, whereas both EtOAc and *n*-BuOH extracts were active against *B. subtilis* and *S. aureus* in the antimicrobial assay. The EtOAc extract was then chromatographed over a C_{18} reversed-phase column using MeOH−H_2O (64:36) as eluent. From the TLC and HPLC chromatograms of the isolates, five fractions were obtained. Fraction 1 was purified using a C_{18} Lobar reversed-phase column with H_2O−MeOH (85:15) to give **6** (9.5 mg), which was also found in the *n*-BuOH extract. Fraction 2, upon purification using a Lobar silica column with CH_2Cl_2−*i*-PrOH (75:25) followed by gradient C18 preparative HPLC, yielded **5** (13.2 mg) and **7** (1.2 mg). From fraction 3 was obtained **1** (2.3 mg) and **4** (2.3 mg) after purification using a Lobar C18 reversed-phase column with MeOH−H_2O (6:4) and gradient C_{18} preparative HPLC. Fraction 4 gave **3** (3.15 mg) after purification using a Lobar diol column with EtOAc−hexane (7:3) followed by gradient C_{18}

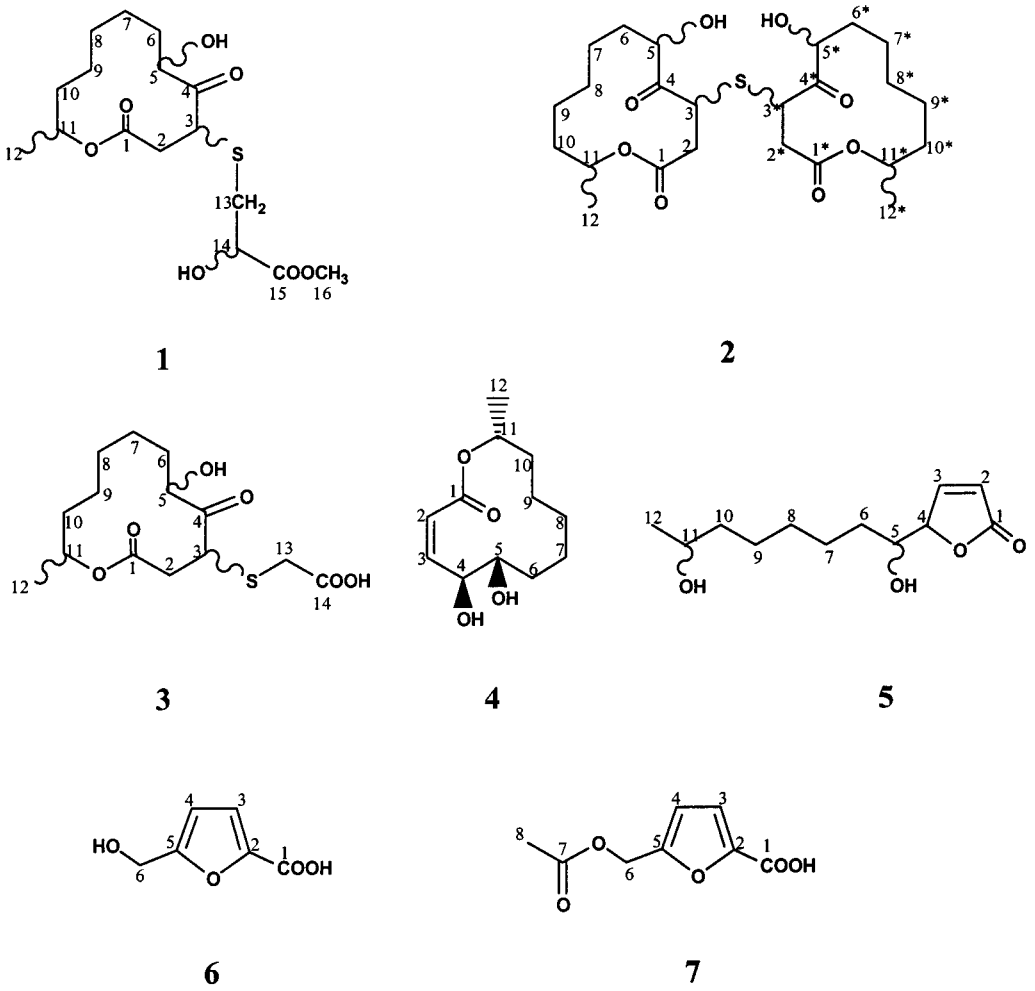

Figure 1. Compounds isolated from sponge-derived *C. herbarum* (Pers.).

Table 2. ^{1}H NMR (CD_3OD) Data[a] for **1** and **2**

position	1	2
2A	3.49 (dd, 19.4, 12.4)	3.46 (dd, 19.4, 12.7)
2B	3.00 (dd, 19.4, 3.8)	2.95 (dd, 19.4, 3.7)
3	3.72 (dd, 12.4, 3.8)	3.98 (dd, 12.6, 3.8)
5	4.05 (dd, 8.0, 3.6)	4.06 (dd, 7.9, 3.3)
6A	1.91 (m)	1.86 (m)
6B	1.85 (m)	1.86 (m)
7A	1.63 (m)	1.64 (m)
7B	1.26 (m)	1.24 (m)
8A	1.26 (m)	1.18−1.55 (m)
8B	1.26 (m)	1.18−1.55 (m)
9A	1.44 (m)	1.18−1.55 (m)
9B	1.26 (m)	1.18−1.55 (m)
10A	1.66 (m)	1.64 (m)
10B	1.50 (m)	1.50 (m)
11	4.90 (m)[b]	4.88 (m)[b]
12	1.23 (d, 6.2)	1.10 (d, 6.1)
13A	3.12 (dd, 13.9, 6.5)	
13B	3.06 (dd, 13.9, 5.5)	
14	4.41 (dd, 6.5, 5.5)	
16	3.79 (s)	

[a] Presented here as δ signal (multiplicity, J in Hz). [b] Obscured by the residual water signal.

preparative HPLC. Fraction 5 gave **2** (3.16 mg) after purification with gradient C_{18} HPLC.

Pandangolide 3 (1): $[\alpha]_D$ −57.3° (*c* 0.72, MeOH); ^{1}H NMR data, see Table 2; ^{13}C NMR data, see Table 1; ESIMS *m/z* 363 $[M + H]^+$; EIMS *m/z* 362 $[M]^+$ (100); 344 (36), 303 (11), 285 (22); HREIMS *m/z* 362.1391 (calcd for $C_{16}H_{26}O_7S$, 362.1399).

Pandangolide 4 (2): $[\alpha]_D$ −55.1° (*c* 0.53, MeOH); ^{1}H NMR data, see Table 2; ^{13}C NMR data, see Table 1; ESIMS *m/z* 487 $[M + H]^+$; EIMS *m/z* 486 $[M]^+$ (77), 468 (19), 458 (11.5), 361 (23), 319 (77), 260 (23), 228 (100); HREIMS *m/z* 486.2276 (calcd for $C_{24}H_{38}O_8S$, 486.2287).

Table 3. Zones of Inhibition (mm) of Compounds **6** and **7** in Bacterial Cultures

	B. subtilis	*S. aureus*	*E. coli*	*C. albicans*
EtOAc phase (1 mg)	9	13	n.a.	n.a.
n-BuOH phase (1 mg)	11	11	n.a.	n.a.
petroleum ether phase (1 mg)	n.a.	n.a.	n.a.	n.a.
6 (5 μg)	7	7	n.a.	n.a.
7 (5 μg))	7	7	n.a.	n.a.

Acetyl Sumiki's Acid (7): ^{1}H NMR (CD_3OD, 300 MHz) δ 7.16 (d, J = 3.4 Hz, 1H), 6.61 (d, J = 3.4 Hz, 1H), 5.14 (s, 2H), 2.11 (s, 3H); EIMS *m/z* 184 $[M]^+$ (31), 154 (38), 142 (100), 125 (57), 97 (30).

Agar Plate Diffusion Assays. Susceptibility disks (5.5 mm) were impregnated with 1 mg of extract or 5 μg of the pure isolated compound and placed on agar plates with the test bacterium: *B. subtilis* 168, *S. aureus* ATCC 25923, *E. coli* ATCC 25922, and *C. albicans*. The plates were checked for inhibition zones after 24 h of incubation at 37 °C.

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