Microsphaerones A and B, Two Novel γ-Pyrone Derivatives from the Sponge-Derived Fungus *Microsphaeropsis* sp.

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Two new metabolites, microsphaerones A (**1**) and B (**2**), were identified from the EtOAc extract of the culture of an undescribed fungus of the genus *Microsphaeropsis*, isolated from the Mediterranean sponge *Aplysina aerophoba*. Compounds **1** and **2** represent the first examples of γ-pyrone derivatives of the fungal genus *Microsphaeropsis*. The structures of the compounds were elucidated on the basis of comprehensive spectral analysis (^{1}H, ^{13}C, ^{1}H−^{1}H COSY, HMQC, and HMBC NMR, as well as low- and high-resolution ESI and EIMS experiments). The (*S*)-2-methylsuccinic acid moiety present in **1** was established by GC−MS analysis of a hydrolysis product.

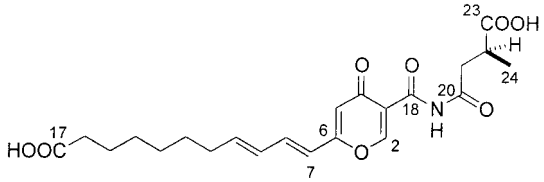
In recent years, increasing attention has been given to marine microorganisms in the search for new pharmaceutical or agrochemical lead structures.[1−3] They have been shown to produce a large number of bioactive substances[4−7] which might be due to their living conditions and functions in the ecosystem.[3,8] Many marine microorganisms are symbiotic to marine sponges and other invertebrates, and their secondary metabolites might contribute to protecting their hosts by chemically mediated defense mechanisms from dangers such as predation. In some cases, there is evidence that symbiotic or associated marine microorganisms might be the true sources of bioactive metabolites originally isolated from their macroorganism hosts.[9,10]

Sponge-derived fungi have previously been reported to produce a variety of secondary metabolites belonging to different classes of natural products, which include chlorinated sesquiterpenes,[11] alkaloids such as asperazine,[12] and hortein, featuring an unusual aromatic ring system.[13] From the sponge-derived fungus *Cladosporium herbarum*, macrolides and a furan carboxylic acid derivative have been isolated.[14] The fungus *Drechslera hawaiiensis*, isolated from the marine sponge *Callyspongia aerizusa*, yielded novel spiciferone derivatives.[15]

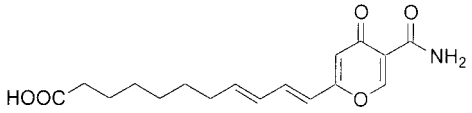
Isolates of the fungal genus *Microsphaeropsis*, all of them obtained from marine sponges, were reported to yield an unusual methyl-branched unsaturated fatty acid and its glyceride,[16] a cerebroside,[17] and a sesquiterpene of the eremophilane type, microsphaeropsisin.[18] Similarly, isolates of the genus *Microsphaeropsis* from terrestrial habitats have afforded a variety of bioactive secondary metabolites, which include a tetramic acid-related lactam epoxide that was isolated in the course of searching for new bradykinin binding inhibitors,[19] diketopiperazine antibiotics with inhibitory activity against DNA topoisomerase,[20] and a series of cell adhesion inhibiting polyketides, macrosphelides.[21−24]

In a previous report from our group, inhibitors of protein kinases, namely, betaenone derivatives and anthraquinone congeners, have been identified from an undescribed fungus of the genus *Microsphaeropsis*, isolated from the Mediterranean sponge *Aplysina aerophoba*.[4] As part of our continuing studies directed toward the discovery of novel natural products from sponge-derived fungi, we reinvestigated cultures of the same fungal strain.[25] The HPLC−UV profiles of the EtOAc extract of the culture of this fungus showed a prominent component together with a small amount of a related derivative, of which the UV spectra and retention times were different from the previously isolated compounds.[4] Further examination resulted in the isolation of two novel γ-pyrone derivatives, namely, microsphaerones A (**1**) and B (**2**). The structures including the absolute configuration of the compounds were established on the basis of NMR and mass spectrometric data as well as by GC−MS analysis.

Scheme 1. Structures of Microsphaerones A (**1**) and B (**2**)



1



2

The fungus *Microsphaeropsis* sp. (Deuteromycota, mitosporic fungi) was grown in liquid malt-extract medium, and the secondary metabolites were obtained from the EtOAc extract of the mycelia and broth. Isolation of the compounds was achieved by repeated chromatographic steps, including column chromatography on Sephadex LH-20, RP-18 Lobar, and silica gel, and finally by reversed-phase HPLC, yielding two novel compounds, microsphaerones A (**1**) and B (**2**) (Scheme 1).

Microsphaerone A (**1**), obtained as a yellow powder, showed a molecular ion peak in the low-resolution ESIMS

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Table 1. NMR Spectral Data of Microsphaerones A (**1**) and B (**2**)[a]

	1			**2**		
	δ_H (J Hz)	δ_C	HMBC	δ_H (J Hz)	δ_C	HMBC
1						
2	8.92 s	163.2 d	3, 4, 6, 18	8.74 s	161.4 d	3, 4, 6
3		118.1 s			119.1 s	
4		177.7 s			177.7 s	
5	6.63 s	113.5 d	3, 6, 7	6.51 s	113.7 d	3, 6, 7
6		163.5 s			162.7 s	
7	6.38 d (15.4)	119.6 d	5, 6, 9	6.36 d (15.4)	119.9 d	5, 6, 9
8	7.16 dd (15.4, 10.4)	138.9 d	6, 10	7.12 dd (15.4, 10.4)	137.8 d	6, 10
9	6.30 dd (15.1, 10.4)	129.2 d	7, 8, 11	6.33 dd (15.1, 10.4)	129.1 d	7, 11
10	6.25 dt (15.1, 6.9)	144.5 d	8, 11	6.23 dt (15.1, 6.9)	143.5 d	8, 11
11	2.16 m	32.5 t	9, 10, 13	2.15 m	32.3 t	9, 10, 13
12	1.38 m	28.1 t	10, 11, 14	1.38 m	28.1 t	14
13	1.27 m	28.4 t	11, 14, 15	1.27 m	28.3 t	14
14	1.27 m	28.3 t	13, 16	1.27 m	28.2 t	13
15	1.47 m	24.5 t	13, 16, 17	1.47 m	24.4 t	13, 16, 17
16	2.18 m	33.8 t	14, 15, 17	2.18 m	33.6 t	14, 15, 17
17		174.7 s			174.5 s	
18		161.3 s			163.2 s	
20		172.9 s				
21	A 3.09 m	41.3 t	20, 22, 23, 24			
	B 2.81 m		20, 22, 23, 24			
22	2.78 m	34.6 d	20, 21, 23, 24			
23		176.7 s				
24	1.13 d (7.3)	17.1 q	21, 22, 23			
NH	11.77 s (1H)		3, 21	A 8.61 d (3.1, 1H)		
				B 7.75 d (3.1, 1H)		
OH	12.05 br s (2H)			11.97 br s (1H)		

[a] Recorded in DMSO-d_6.

at m/z 434 $[M + H]^+$ and at m/z 432 $[M - H]^-$, which in conjunction with 1D NMR data (Table 1) suggested the empirical formula $C_{22}H_{27}NO_8$. This result was subsequently confirmed by high-resolution ESIMS. The ^{1}H NMR spectrum showed well-dispersed signals over a wide field range, disclosing the presence of one amide proton at δ 11.77, two conjugated double bonds, and one aliphatic methyl doublet. Furthermore, in the aromatic region two singlets at δ 8.92 and 6.63 were observed, while signals indicative of an aliphatic chain were present at high field. These findings were corroborated by the ^{13}C NMR and the DEPT spectra, which revealed six methine carbons in the aromatic region, while in the aliphatic region one methine, seven methylene, and one methyl group were discernible. Furthermore, out of the seven singlets present at low field, at least four were judged to be carbonyl groups on the basis of their chemical shifts (δ 172.9−177.7).

Detailed interpretation of one- and two-dimensional NMR spectral data resulted in the elucidation of three substructures for microsphaerone A (**1**) as follows. In the lower field of the ^{1}H NMR spectrum, the four proton signals appeared as one doublet (δ 6.38, J = 15.4 Hz), two doublets of doublets (δ 7.16, J = 15.4, 10.4 Hz, and δ 6.30, J = 15.1, 10.4 Hz), and one doublet of triplets (δ 6.25, J = 15.1, 6.9 Hz), indicative of two conjugated olefinic double bonds, both in *E*-configuration. The ^{1}H−^{1}H COSY spectrum revealed that this system was attached to a straight chain consisting of six methylene groups (δ 2.16, H_2-11; δ 1.38, H_2-12; δ 1.27, H_2-13−H_2-14; δ 1.47, H_2-15; δ 2.18, H_2-16). This chain was terminated by a carbonyl group (δ 174.7, C-17), as was evident by correlations from H_2-15 and H_2-16 to C-17 in the HMBC spectrum (see Table 1). Furthermore, the COSY spectrum also provided evidence of a second spin system, consisting of one methyl group (δ 1.13, J = 7.3 Hz, H_3-24) linked to an aliphatic methine group (δ 2.78, H-22), which in turn was connected to a methylene group (δ 3.09 and 2.81, H_2-21). Furthermore, HMBC correlations from H_3-24 to a carbon resonating at δ 176.7 (C-23) (see Figure 1) and from H_2-21 and H-22 to both C-23 and a carbon signal

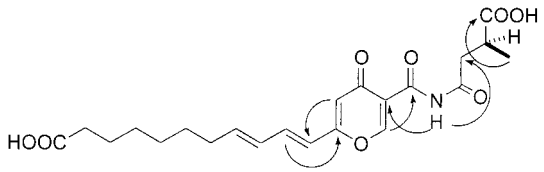

Figure 1. Selected HMBC correlations for microsphaerone A (**1**).

at δ 172.9 (C-20) (see Table 1) clearly indicated that both C-21 (δ 41.3) and C-22 (δ 34.6) were adjacent to carbonyl groups, C-20 and C-23, respectively. Finally, the two remaining proton singlets at δ 8.92 (H-2) and 6.63 (H-5) were assigned to the two protons of a *para*-disubstituted γ-pyrone, which was inferred by correlations from H-2 to C-3 (δ 118.1), C-4 (δ 177.7), and C-6 (δ 163.5), as well as from H-5 to C-3 and C-6, respectively, in the HMBC spectrum. This substructure was also in agreement with ^{1}H and ^{13}C NMR data reported for γ-pyrone derivatives in the literature.[26−28] The possibility of an α-pyrone moiety could be ruled out on the basis of ^{13}C chemical shifts, because the carbonyl group in such systems typically resonates at approximately δ 160−165.[29,30] The remaining aromatic carbon singlet at δ 161.3 (C-18) was attached at C-3, as was deduced by a ^3J correlation of H-2 to C-18 in the HMBC spectrum.

The connection of the substructures in **1** as mentioned above was established by key HMBC correlations from H-5 (δ 6.63) to C-7 (δ 119.6) (and vice versa), from H_2-8 (δ 7.16) to C-6 (δ 163.5), and from the amide proton at δ 11.77 to both C-3 (δ 118.1) and C-21 (δ 41.3) (see Figure 1). By comparison with the molecular formula, it was evident that both terminal carbonyl groups, C-17 (δ 174.7) and C-23 (δ 176.7), were present as free carboxylic acids, which was also in agreement with the observation of a broad signal at δ 12.05 in the ^{1}H NMR spectrum, integrating for two protons. Finally, the substituent at C-3 (δ 161.3, C-18) was inferred to be an amide carbonyl group, of which the unusual high-field chemical shift apparently was due to conjugation to the γ-pyrone ring.

The absolute stereochemistry of the chiral center at C-22 was determined by GC−MS analysis[31,32] of the (−)-menthyl ester of the hydrolysate of **1**, in comparison to authentic standards prepared from commercially available (*R*)-2-methylsuccinic acid and (*S*)-2-methylsuccinic acid. Specifically, compound **1** was hydrolyzed, yielding an optically pure enantiomer of 2-methylsuccinic acid, which was subsequently treated with (−)-menthol. Both the retention values and the online EI mass spectrum of the dimenthylated reaction product were virtually identical to the corresponding derivative as obtained from (*S*)-2-methylsuccinic acid. Thus, the absolute configuration at C-22 in compound **1** was assigned to be *S*.

Microsphaerone B (**2**), also obtained as a yellow powder, showed a molecular ion peak at *m/z* 319 in the low-resolution EIMS. Together with the consideration of ^{1}H and ^{13}C NMR data (Table 1), $C_{17}H_{21}NO_5$ was assigned as the molecular formula, which was confirmed by high-resolution EIMS. The ^{1}H and ^{13}C NMR data of compound **2** as well as the 2D NMR correlations were very similar to those of microsphaerone A (**1**). Detailed comparison of the NMR data revealed that **2** was lacking the 2-methylsuccinic acid partial structure as present in **1** since all the corresponding signals were missing in the 1D NMR spectra. Furthermore, in the ^{1}H NMR spectrum of **2** two signals attributable to the two amide protons (δ 8.61 and 7.75, $J = 3.1$ Hz) were observed, while only one carboxylic proton appeared at δ 11.97.

Due to the structure of **2**, one has to consider the possibility of **2** being an artifact of **1** produced during the extraction procedure. However, both compounds were clearly discernible in the HPLC/UV profiles of the crude extract of the culture broth. Furthermore, the hydrolysis of **1** leading to **2** in the course of determining the absolute stereochemistry of **1** required rather harsh conditions, indicating that **1** was stable at conditions present during the extraction procedure.[33] Thus, **2** should be regarded a true natural product rather than an artifact resulting from hydrolysis of **1**.

Both microsphaerones A (**1**) and B (**2**) exhibited no significant antiproliferative activity against the cell lines HL-60 and NB-4, respectively. When tested against larvae of the polyphageous pest insect *Spodoptera littoralis* or against brine shrimp, *Artemia salina*, both compounds displayed no or only moderate activity.

In the literature, there have been numerous reports of γ-pyrone derivatives from terrestrial fungi, which include rapicone from *Ramichloridium apiculatum*,[26] funicone from *Penicillium funiculosum*,[34] isofunicone[35] and deoxyfunicone,[27] both from *Penicillium* sp., and vermistatin from both *P. vermiculatum*[28] and *P. verruculosum*.[36] The latter compound, initially designated as fijiensin, was also isolated from a plant pathogenic fungus, *Mycosphaerella fijiensis*.[37,38] Derivatives **1** and **2** reported in this publication represent the first examples of γ-pyrones isolated from fungi obtained from the marine environment.

Experimental Section

General Experimental Procedures. Optical rotation was recorded on a Perkin-Elmer Model 341 LC polarimeter. UV spectra were obtained in methanol using a Beckman Model 25 spectrophotometer. Low- and high-resolution ESIMS analysis were performed on a Micromass Qtof 2 mass spectrometer for microsphaerone A (**1**). Low- and high-resolution EIMS were measured with a Finnigan MAT 95 mass spectrometer at 70 eV for microsphaerone B (**2**). NMR spectral data were acquired on a Bruker DRX-500 MHz NMR spectrometer. HPLC−UV analyses were conducted with a Dionex system coupled to a photodiode array detector using a 5 μm Eurospher-100 C18 column (4 mm i.d. × 150 mm; Knauer, Berlin, Germany). Routine detection was at 322 nm. Semipreparative HPLC was performed on a Merck-Hitachi instrument (pump L-7100, detector L-7400) using a 7 μm Eurospher-100 C18 column (8 mm i.d. × 300 mm; Knauer, Berlin, Germany). Column chromatography was performed on Sephadex LH-20 (Sigma-Aldrich, Steinheim, Germany), silica gel (0.040−0.063 mm; Merck, Darmstadt, Germany), or RP-18 Lobar columns (40−63 μm, 25 mm i.d. × 310 mm, Merck, Darmstadt, Germany), and TLC analyses were carried out using aluminum sheet precoated silica gel 60 F_{254} (Merck, Darmstadt, Germany). TLC plates were detected by UV absorption at 254 nm. All solvents used were distilled prior to use. Spectral grade solvents were utilized for chromatographic analysis. Standard chiral acids and reagents for derivatization were purchased from Sigma-Aldrich, Steinheim, Germany.

Microorganisms. The fungus *Microsphaeropsis* sp. was isolated from fresh samples of the Mediterranean sponge *Aplysina aerophoba*. Sponge samples were collected by scuba diving in the Mediterranean sea along the shores of Banyuls-sur-Mer in Southern France in the summer of 1996. Tissue samples were taken from inside the sponge body under sterile conditions and were reinoculated on malt agar slants that consisted of 15 g/L malt extract, 15 g/L agar, and 24.4 g/L artificial sea salt mixture (SERA, obtained from a local aquaristic supplier). The inoculated agar slants were then incubated at 27 °C. From the growing cultures a pure strain was isolated by repeated inoculation on malt agar plates. The fungal strain was identified as *Microsphaeropsis* sp. (Deuteromycota, mitosporic fungi)[39] by the Centraalbureau voor Schimmelcultures (CBS), Baarn, The Netherlands. The strain was deposited in the Kulturensammlung Marine Pilze Bremerhaven (KMPB) under the accession no. KMPB W-22. Its assignment was confirmed by the Marine Mycological Research Group of the Alfred-Wegener-Institute for Polar and Marine Research, Bremerhaven, Germany. More details were given in our previous communication.[4]

Extraction and Isolation. For large-scale fermentation (10 L), the fungus was grown in 1.5% malt-extract broth made up of distilled water supplemented with 2.44% artificial sea salt at 25 °C without shaking. After 41 days, the fungal biomass was separated from the culture broth, and both the mycelium and the broth were extracted with EtOAc. The combined extracts were evaporated under reduced pressure and taken to dryness (1.16 g). The residue was partitioned between EtOAc (200 mL × 4) and H_2O (200 mL). The organic fraction was again taken to dryness (0.68 g) and chromatographed over Sephadex LH-20 with MeOH−acetone (50:50) as solvent system. Eight fractions were obtained. Fractions 3 and 4 containing compounds **1** and **2** were subjected to column chromatography on RP-18 (MeOH−H_2O, 80:20) and silica gel 60 (CH_2Cl_2−MeOH, 92:8), and RP-HPLC with MeOH and H_2O (0.1% TFA) using the gradient 0−5 min, 58% MeOH; 10−20 min, 59% MeOH; 25−30 min, 100% MeOH, yielding microsphaerones A (**1**, 40.6 mg) and B (**2**, 8.3 mg).

Microsphaerone A (1): yellow powder (MeOH); $[\alpha]_D^{20}$ −8.9° (*c* 0.56, MeOH); UV (MeOH) λ_{max} (log ϵ) 229 (3.77), 312 (3.63) nm; ^{1}H and ^{13}C NMR data, see Table 1; ESIMS pos *m/z* 456 $[M + Na]^+$ (25), 434 $[M + H]^+$ (100), 320 (45); ESIMS neg *m/z* 432 $[M - H]^-$ (60), 318 (35); HRESIMS neg *m/z* 432.1647 $[M - H]^-$ (calcd for $C_{22}H_{26}NO_8$, 432.1658) (100), 318.1360 (calcd for $C_{17}H_{20}NO_5$, 318.1341) (55).

Microsphaerone B (2): yellow powder (MeOH); UV (MeOH) λ_{max} (log ϵ) 211 (3.65), 231 (3.63), 313 (3.71) nm; ^{1}H and ^{13}C NMR data, see Table 1; EIMS *m/z* 319 $[M]^+$ (100), 218 (55); HREIMS *m/z* 319.1417 $[M]^+$ (calcd for $C_{17}H_{21}NO_5$, 319.1420).

Hydrolysis of Compound 1. Saturated aqueous $NaHCO_3$ (400 μL) was added to a solution of **1** (2.0 mg in 400 μL MeOH), and the mixture was stirred at 50 °C for 12 h. After adjusting to pH 7 with 2 N HCl, the mixture was taken to dryness under reduced pressure.

Derivatization Reactions. The residue of the hydrolyzed compound **1** was dissolved in 300 μL of acetonitrile, and 100 μL of (−)-menthol (200 μg/μL in ethyl acetate) was added. The solution was taken to dryness under a gentle stream of nitrogen at 40 °C. After adding 20 μL of toluene and 1 μL of acetyl chloride, the resulting mixture was heated at 100 °C for 1 h, taken to dryness, and dissolved in toluene (100 μL) for direct analysis by GC−MS.[31,32] Each of the standard chiral acids, (*R*)-2-methylsuccinic acid (300 μg), (*S*)-2-methylsuccinic acid (300 μg), and a mixture of both (150 μg each) were dissolved in MeCN at a concentration of 10 μg/μL and reacted with (−)-menthol as described above.

Gas Chromatography−Mass Spectrometry. GC−MS analysis was performed on a Hewlett-Packard Model 5890 series II gas chromatograph coupled to a Model 5972 series mass selective detector. The injector and detector temperatures were at 260 and 280 °C, respectively. Samples (0.5 μL) were injected in the splitless mode. A Hewlett-Packard HP-5 MS column (0.25 mm i.d. × 30 m × 0.5 μm) was used for chromatographic separation. The following temperature program was used: 0−2 min, 220 °C; 12−13 min, 230 °C; 28−29 min, 233 °C. t_R of (−)-menthylated analytes: dimenthyl ester of (*R*)-2-methylsuccinic acid, 21.70 min; dimenthyl ester of (*S*)-2-methylsuccinic acid, 22.30 min; dimenthyl ester of hydrolysate of **1**, 22.30 min. The resulting dimenthylated derivatives of 2-methylsuccinic acid gave a weak molecular ion peak at *m*/*z* 408, but the identity of the reaction product was confirmed by a strong ion peak at *m*/*z* 409 $[M + H]^+$ in the ESI mass spectrum (data not shown).

Evaluation of Biological Activity. Antiproliferative activity was examined using cell lines HL-60 and NB-4 as described previously.[40] Activity against brine shrimp, *Artemia salina*, and insecticidal activity against larvae of *Spodoptera littoralis* were determined as described previously.[41,42]

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