New Secondary Metabolites from the Marine Endophytic Fungus *Apiospora montagnei*†

Christine Klemke, Stefan Kehraus, Anthony D. Wright, and Gabriele M. König*

Institute for Pharmaceutical Biology, University of Bonn, Nussallee 6, D-53115 Bonn, Germany

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The marine fungus *Apiospora montagnei* was isolated from the inner tissue of the North Sea alga *Polysiphonia violacea*. Cultivation of this fungal strain led to the isolation of several new secondary metabolites, including the diterpene myrocin A (**1**) and the polyketide apiosporic acid (**2**). Furthermore the new monomethyl ester of 9-hydroxyhexylitaconic acid (**3**) and the (−)-enantiomer (**4**) of the known (+)-hexylitaconic acid were found together with the known (+)-epiepoxydon (**5**), (+)-epoxydon monoacetate, *R*-mellein, *R*-8-methoxymellein, 5-hydroxymethylfuran-2-carboxylic acid, and the xanthone derivative anomalin A. The structures were elucidated mainly by 1D and 2D NMR, MS, UV, and IR spectral data. Compound **5** exhibited significant cytoxicity against human cancer cell lines.

Marine microorganisms are an interesting source of many structurally diverse and pharmacologically active natural products.[1−3] Our research on marine fungi from different marine sources, e.g., sponges, jelly fish, and algae, has yielded intriguing new structures with prominent biological activity.[4−10] Focusing on marine fungi associated with algae, the endophytic fungus *Apiospora montagnei* Saccardo (Apiosporaceae, Ascomycetes) was obtained from the inner tissue of the North Sea alga *Polysiphonia violacea* (Roth) Spreng. (Rhodomelaceae, Rhodophyta).

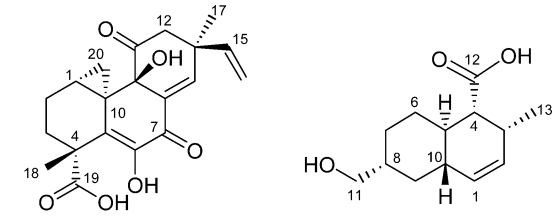
Our isolation method[6,11] allows a distinction to be made between merely associated and endophytic algicolous fungi and thus assured that *A. montagnei* was endophytic. Endophytic fungi, especially from higher plants, have been shown to be a rich source of biologically active metabolites.[12] However, in contrast to terrestrial endophytic fungi,[13−15] marine endophytes are, as yet, rarely investigated for their secondary metabolite content.

Prior investigations of terrestrial strains of *A. montagnei* revealed the natural products apiosporamide[16] with antifungal activity and the cyclic peptides TMC-95A−D[17] acting as proteasome inhibitors. The genus *Arthrinium*, which is the anamorphic state of *Apiospora*, is also known for interesting secondary metabolites such as arthrinone,[18] arthrichitin,[19] terpestacin,[20] CAF-603,[21] and the xanthone derivative norlichexanthone.[18,22]

A. montagnei was selected for chemical investigations on the basis of ^{1}H NMR spectra of the crude extract. The fungus was mass cultivated on a solid biomalt medium. Successive fractionation of the EtOAc extract with normal phase (NP) vacuum liquid chromatography (VLC), followed by normal and reversed (RP-C_{18}) phase HPLC separations yielded new compounds **1**−**4** and six known compounds.

Compound **5** exhibited significant cytotoxic activity both in the brine shrimp assay[23] and toward human cancer cell lines. At a concentration of 3.6 μg/mL it reduced the number of viable human breast cancer cells by 50% (LC_{50}).[24]

The molecular formula of compound **1** was deduced by accurate mass measurement (HREIMS) to be $C_{20}H_{22}O_6$, implying 10 degrees of unsaturation. The ^{13}C NMR spectrum showed 20 carbon signals, attributable to 2 × CH_3, 5 × CH_2, 3 × CH, and 10 × C in the DEPT spectrum. Considering the molecular formula and IR data (ν_{OH} 3426) for **1** the three remaining protons had to be present as hydroxyl groups. ^{1}H and ^{13}C NMR chemical shifts (Table 1) as well as IR data showed the presence of three double bonds and three carbonyl groups (δ_C 209.2, 183.5, 180.6; $\nu_{C=O}$ 1700−1715), and thus the resultant carbon skeleton had to be tetracyclic. In the ^{1}H NMR spectrum three upfield proton signals (δ_H 0.83/1.13, H_2-20, 1.84, H-1) correlated by HSQC to ^{13}C shifts at δ_C 12.4 and 17.3 and a ^{13}C signal for a quarternary carbon (C-10) at δ_C 30.7, indicating a cyclopropyl moiety. Also evident from the ^{1}H and ^{13}C NMR spectra of **1** was an isolated olefinic proton conjugated with a ketone (δ_H 6.90, CH-14) and an isolated vinyl group (δ_H 5.05, H_2-16, 5.74, H-15).



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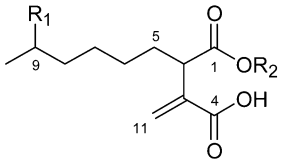
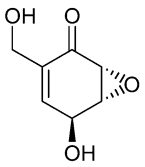


3 R_1 = OH, R_2 = CH_3
4 R_1 = H, R_2 = H



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Two partial structures followed from ^{1}H−^{1}H COSY and HMBC data (**a** and **b,** Figure 1). In fragment **a** the ^{1}H−^{1}H COSY correlations for the H_2-20 to H_2-3 spin system were difficult to interpret, due to the overlap of ^{1}H NMR signals in the 1.83−1.88 ppm region (H-1, H_2-2, H_2-3). However, correlations were visible from both H_2-20 (δ_H 0.83, 1.13) to H-1 (δ_H 1.84) and from H-2b (δ_H 2.11) to H-3b (δ_H 1.48).

† Dedicated to Professor A. Zeeck on the occasion of his 65th birthday.

* To whom correspondence should be addressed. Tel: +49228733747. Fax: +49228733250. E-mail: g.koenig@uni-bonn.de. Internet: http://www.uni-bonn.de/pharmbio/queen/GAWK.html.

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Table 1. 1D and 2D NMR Spectral Data for Compound **1**

atom no.	$^{13}C^{a,c}$ (δ in ppm)	$^{1}H^{a,c}$ (δ ppm, mult., J in Hz)	COSY[a,c]	NOESY[b]	HMBC[b,d]
1	17.3 (CH)	1.84 (m)	2b, 20a/b	2b, 20b	2[e], 10[e], 20
2	18.4 (CH_2)	2.11 (Hb, m)	2a, 3b	1, 18	1[e], 4, 19[e], 20
		1.88 (Ha, m)	2b	20a[e]	
3	30.2 (CH_2)	1.83 (Ha, m)	3b	3b, 20a	2[e], 4, 18[e], 19[e]
		1.48 (Hb, m)	2b, 3a	3a	
4	45.7 (C)				
5	133.9 (C)				
6	145.7 (C)				
7	183.5 (C)				
8	137.8 (C)				
9	77.1 (C)				
10	30.7 (C)				
11	209.2 (C)				
12	54.1 (CH_2)	2.85 (Ha, d, 13.7)	12b	12b, 14, 17, 20b	9, 11, 13, 14, 15[e], 17
		2.54 (Hb, dd, 1.5, 13.7)	12a, 14	12a, 15, 17	9, 11, 13, 15
13	43.5 (C)				
14	144.6 (CH)	6.90 (d, 1.5)	12b	12a, 15, 16a/b, 17	7, 8[e], 9, 12, 13, 15[e], 17
15	142.8 (CH)	5.74 (dd, 10.4, 17.4)	16a/b	12b, 14, 16a/b, 17	12, 13, 16, 17
16	115.3 (CH_2)	5.05 (Ha, dd, 0.6, 10.4)	15	12b, 14, 15, 17	13, 15, 17[e]
		5.05 (Hb, dd, 0.6, 17.4)	15	12b, 14, 15, 17	13, 15
17	27.7 (CH_3)	1.40 (s)		12a/b, 14, 15, 16a/b	8, 11, 12, 13, 14, 15[e]
18	20.1 (CH_3)	1.44 (s)		2b	4, 5, 19
19	180.6 (C)				
20	12.4 (CH_2)	1.13 (Ha, t, 6.4)	1	2a[e], 3a, 20b	1, 5, 9, 10
		0.83 (Hb, dd, 6.4, 9.2)	1	1, 12a, 20a	1, 5[e], 9, 10

[a] CD_3OD, 500/125.7 MHz. [b] Acetone-d_6, 300/75.5 MHz. [c] Assignments are based on extensive 1D and 2D NMR measurements (HMBC, HSQC, COSY). [d] Numbers refer to carbon resonances. [e] Weak signal.

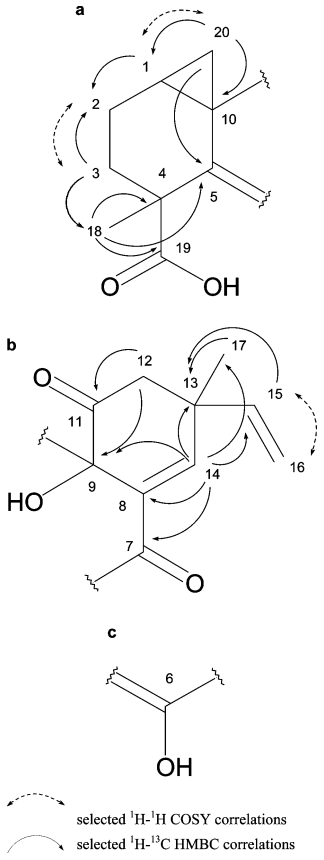

Figure 1. Partial structures **a**, **b**, and **c** of myrocin A (**1**).

According to HMBC correlations from H_2-3 to C-18 and from H_3-18 to the quarternary carbon C-4, the methyl group (CH_3-18) must be located at C-4, and C-3 had to be adjacent to C-4. H_3-18 also correlated with the sp^2-hybridized carbon C-5, and thus C-5 had to be connected to C-4. An HMBC correlation from H_2-20 to both C-5 and C-10 completed the cyclohexane ring. Due to these 2D NMR data (Table 1) the direct connection of the cyclopropyl moiety (CH-1, CH_2-20, C-10) with the cyclohexane ring was confirmed. One of the three carbonyl groups (C-19) showed HMBC correlations from both H_2-3 and H_3-18 and was thus connected to quaternary carbon C-4 as a carboxylic acid moiety (δ_C 180.6). In fragment **b** the $^{1}H-^{1}H$ COSY correlation observed between H-15 and H_2-16 established the vinyl group. Together with a singlet methyl group (CH_3-17) it was bonded to C-13, as deduced from HMBC correlations from H-15 to C-13 and from H_3-17 to C-13. Two further HMBC correlations from H_2-12 and H-14 to C-13 (δ_C 43.5) were evident, thus placing the quaternary C-13 between C-12 and C-14. A strong $^{1}H-^{13}C$ HMBC correlation connected the methylene group (CH_2-12) to the ketone (C-11, δ_C 209.2). An oxygenated quaternary carbon (C-9, δ_C 77.1) also showed an HMBC correlation from H_2-12, and following from these data, it had to be connected to C-11. Olefinic proton H-14 (δ_H 6.90) showed HMBC correlations to C-9 and the sp^2-hybridized carbon C-8, which completed the cyclohexene ring. An HMBC correlation from olefinic H-14 to the carbonyl C-7 confirmed the α,β-unsaturated ketone, inferred from the characteristic ^{13}C NMR shift (δ_C 183.5). Further, HMBC data revealed the connection of fragments **a** and **b** at C-9 and C-10, due to an HMBC correlation from H_2-20 to C-9. The deshielded sp^2-hybridized carbon C-6 (δ_C 145.7 structure **c**, Figure 1) had to bear the third −OH evident from MS and IR data. Additionally, no HMBC correlations to C-6 were evident; thus, to close the tetracyclic ring system, C-6 had to be located between C-5 and C-7. From a 2D NOESY experiment we deduced the relative configuration of **1** to be 1*R**, 4*R**, 9*R**, 10*R**, 13*S**. Diagnostic $^{1}H-^{1}H$ NOESY correlations were seen from H-12a to H-20b and H_3-17 and from H-12b to H-15. This clearly positioned CH_3-17 on the same side of the molecule as the cyclopropane ring. NOESY correlations from H-20a to H-3a and to H-2a, from H-1 to H-2b, and from H-2b to H_3-18 (Figure 2) showed CH_3-18 to be on the opposite side of the cyclopropane ring, i.e., the β-side of the molecule. The NOESY correlation between H-12a and H-20b also clarified the relative configuration at C-9, since this NOE is possible only if the hydroxyl group is β-oriented. This relative configuration for compound **1** is in agreement with that previously published for the related

Table 2. 1D and 2D NMR Spectral Data for Compound **2**

atom no.	$^{13}C^{b,c}$ (δ in ppm)	$^{1}H^{a,c}$ (δ ppm, mult., J in Hz)	COSY[a]	NOESY[a]	HMBC[a,d]
1	131.6 (CH)	5.40 (d, 9.9)	2, 3, 10	2, 9a/9b, 10	2, 3, 5, 9[e], 10
2	131.8 (CH)	5.57 (ddd, 2.8, 4.1, 9.9)	1, 3, 10	3, 13	1, 3, 4, 10
3	32.9 (CH)	2.56 (m)	2, 4, 13	2	2, 4[e]
4	49.7 (CH)	2.52 (dd, 6.0, 11.2)	5, 13		3, 5, 10, 12, 13
5	37.4 (CH)	1.30 (qd, 11.2, 2.8)	4, 6a/b, 10	9a, 13[e]	4, 6[e]
6	30.1 (CH_2)	0.94 (Ha, qd, 12.4, 2.8)	5, 6b, 7b	6b, 8, 10	5
		2.09 (Hb, brdd, 2.8, 12.4)	5, 6a, 7b	6a	7, 8, 10
7	30.4 (CH_2)	1.03 (Ha, qd, 12.4, 3.4)	6a/b, 7b, 8	7b, 9a, 11	6, 8, 11[e]
		1.84 (Hb, m)	6b, 7a, 8	7a, 8, 11	5, 6, 8
8	41.9 (CH)	1.57 (m)	7a/b, 9a/b, 11	6a, 9b, 10, 11	7, 9, 11
9	36.9 (CH_2)	0.79 (Ha, q, 12.4)	8, 9b, 10	5, 7a, 9b, 11	2, 5, 7, 8, 10, 11
		1.85 (Hb, m)		1, 8, 9a, 10	5, 8, 10
10	42.1 (CH)	1.74 (ddd, 2.8, 11.2, 12.4)	1, 2, 5, 9a/b	1, 6a, 8, 9b	2, 9
11	68.1 (CH_2)	3.37 (dd, 1.8, 6.4)	8	7b, 8, 9a/b	7, 8, 9
12	174.7 (C)				
13	18.0 (CH_3)	0.92 (d, 6.9)	3, 4	2	1, 2, 3, 4

[a] Acetone-d_6, 500/125.7 MHz. [b] Acetone-d_6, 300/75.5 MHz. [c] Assignments are based on extensive 1D and 2D NMR measurements (HMBC, HSQC, COSY). [d] Numbers refer to carbon resonances. [e] Weak signal.

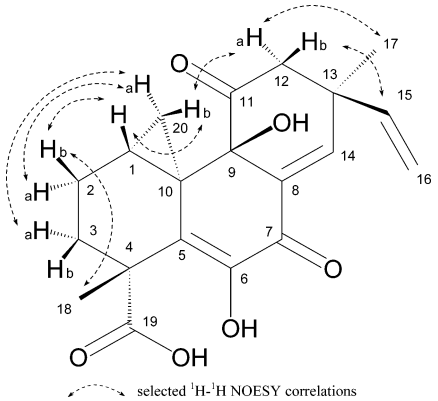

Figure 2. Relative configuration of myrocin A (**1**).

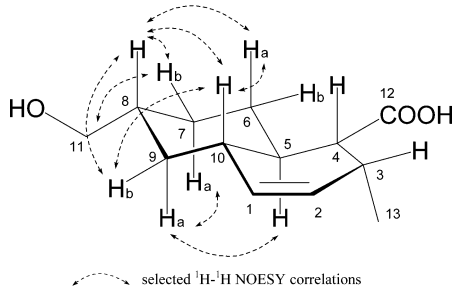

Figure 3. Relative configuration of apiosporic acid (**2**).

pimarane diterpenes myrocin B[25] and C,[26] and we thus propose the trivial name myrocin A for compound **1**.

Compound **2** had the molecular formula $C_{13}H_{20}O_3$, with four degrees of unsaturation according to NMR, IR, and HREIMS. The ^{13}C NMR spectrum (Table 2) showed 13 signals, for 1 × CH_3, 4 × CH_2, 7 × CH, and 1 × C according to the DEPT135 spectrum. Due to ^{1}H and ^{13}C NMR chemical shifts and IR data ($\nu_{C=O}$ 1705) the presence of one carbonyl group and one double bond was certain, implying a bicylic ring system. From the molecular formula and IR data (ν_{OH} 3426), two hydroxyl groups were evident. Starting from a methyl doublet (δ_H 0.92, CH_3-13) the carbon skeleton for **2** could be deduced almost exclusively from ^{1}H−^{1}H COSY data (Table 2), which delineated a continuous spin system. The CH_3-13 methyl group showed ^{1}H−^{1}H COSY correlations to H-3 and H-4, while H-3 had ^{1}H−^{1}H COSY correlations to H-4 and H-2. Olefinic H-2 coupled to H-1 and allylic H-10, and additional ^{1}H−^{1}H COSY correlations between H-5 and both H-4 and H-10 closed the cyclohexene ring. Further ^{1}H−^{1}H COSY correlations connected the three methylene groups CH_2-6, CH_2-7, and CH_2-9 to methine group CH-8, and correlations between H_2-9 and H-10, and H_2-6 and H-5, connected this partial structure to the cyclohexene ring to establish an unsaturated decalin ring system. Oxygenated CH_2-11 (δ_H 3.37, δ_C 68.1) was COSY correlated to H-8. Thus C-8 had to be substituted with a hydroxy-methylene group. HMBC data confirmed this part of the structure. Finally an HMBC correlation between H-4 and the carboxylic C-12 (δ_C 174.7) established the position of the carboxylic acid moiety.

To solve the relative configuration of compound **2**, several correlations in the ^{1}H−^{1}H NOESY spectrum were useful (Figure 3); however ^{1}H−^{1}H coupling constants were especially informative. A ^{1}H−^{1}H coupling constant of 11.2 Hz between H-4 and H-5 (Table 2) placed both of these protons axial, while from $J_{3/4}$ = 6.0 Hz H-3 was assigned as equatorial. From these data it followed that CH_3-13 at C-3 must be pseudoaxial. A *trans*-decalin ring system was indicated by $J_{5/10}$ = 11.2 Hz with H-5 and H-10 axial. Using homonuclear decoupling experiments, the coupling constants between H-8 and H-9a and between H-9a and H-10 both proved to be 12.4 Hz. H-8 thus had to be axial. From these data it was concluded that CH_3-13, the carboxyl group at C-4, H-5, and the hydroxymethylene group at C-8 had to be on the same side of the molecule (i.e., α-oriented). These findings revealed the relative configuration of compound **2** (Figure 3) to be 3*R**, 4*R**, 5*R**, 8*R**, 10*R**. Compound **2** is structurally related to apiosporamide,[16] isolated from a terrestrial *A. montagnei* strain, to solanapyrones,[27] and to ascosalipyrrolidinones A and B.[6] The trivial name apiosporic acid is proposed for compound **2**.

Accurate mass measurement (HREIMS) of compound **3** revealed the molecular formula to be $C_{12}H_{20}O_5$ with three degrees of unsaturation. Twelve carbon signals were evident in the ^{13}C NMR spectrum (Table 3) and were deduced to be 2 × CH_3, 5 × CH_2, 2 × CH, and 3 × C from the DEPT135 spectrum. According to ^{1}H and ^{13}C NMR spectra two carbonyl groups and one exo-methylene group (δ_H 5.76/6.31, δ_C 126.5, CH_2-11, δ_C 140.3, C-3) were evident, and therefore compound **3** had to be acyclic. Other structural elements obvious from 1D NMR data were a methoxyl group (δ_H 3.61, δ_C 51.9, OCH_3-12) and an oxygen-bonded methine carbon (δ_H 3.71, δ_C 67.3, CH-9). Considering the molecular formula and ^{13}C NMR and IR data (ν_{OH} 3453), the two remaining protons had to be present as hydroxyl groups. From ^{1}H−^{1}H COSY and HMBC spectra the structure of compound **3** (Figure 4) was deduced. The methyl group CH_3-10 was directly connected to the hydroxylated

Table 3. 1D and 2D NMR Spectral Data for Compound **3**

atom no.	$^{13}C^{b,c}$ (δ in ppm)	$^{1}H^{a,c}$ (δ ppm, mult., J in Hz)	COSY[a]	NOESY[a]	HMBC[a,d]
1	174.0 (C)				
2	47.3 (CH)	3.50 (t, 7.6)	5, 11	5a/b, 6, 11b	1, 3, 4, 5, 11
3	140.3 (C)				
4	167.7 (C)				
5	32.1 (CH_2)	1.84 (Ha, m)	2, 6	2, 6, 11b	1, 2, 3, 11
		1.67 (Hb, m)	2, 6	2, 6, 11b	1, 3
6	28.3 (CH_2)	1.31 (m)	5	2, 5a/b, 7	2, 5
7	26.3 (CH_2)	1.38 (m)		6	9
8	40.0 (CH_2)	1.38 (m)	9	9, 10	10
9	67.3 (CH)	3.71 (dd, 6.2, 11.5)	8, 10	8, 10	7
10	24.0 (CH_3)	1.10 (d, 6.2)	9	8, 9	8, 9
11	126.5 (CH_2)	6.31 (Ha, s)	11b	11b	2, 3, 4
		5.76 (Hb, s)	2, 11a	2, 5a/b, 11a	
12	51.9 (CH_3)	3.61 (s)		2	1

[a] Acetone-d_6, 500/125.7 MHz. [b] Acetone-d_6, 300/75.5 MHz. [c] Assignments are based on extensive 1D and 2D NMR measurements (HMBC, HSQC, COSY). [d] Numbers refer to carbon resonances.

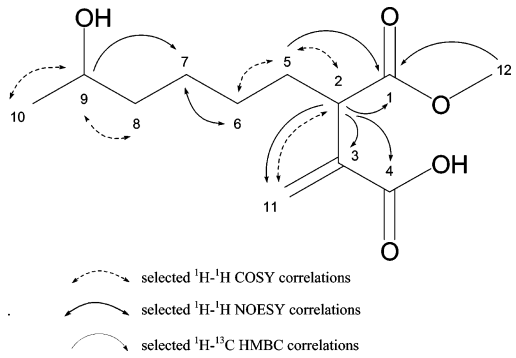

Figure 4. Structure of 9-hydroxyhexylitaconic acid-1-methyl ester (**3**).

methine carbon CH-9 (δ_H 3.71, δ_C 67.3), according to a $^{1}H-^{1}H$ COSY correlation from H_3-10 to H-9. Although $^{1}H-^{1}H$ COSY correlations placed H-9 adjacent to H_2-8, $^{1}H-^{1}H$ COSY correlations from H_2-8 to H_2-7 and from H_2-7 to H_2-6 were difficult to interpret, due to overlapping ^{1}H NMR signals between 1.31 and 1.38 ppm. The connectivity of CH_2-8, CH_2-7, and CH_2-6 was thus proven from HMBC correlations between H-9 and C-7 and NOESY correlation between H_2-7 and H_2-6. Further $^{1}H-^{1}H$ COSY correlations from H_2-6 to H_2-5 and from H_2-5 to H-2 were observed and completed the aliphatic chain. The partial structure was extended using HMBC correlations. H_2-5 correlated with carbonyl group C-1, linking C-1 to methine group CH-2. The methoxyl group (OCH_3-12) had to be bonded to the carbonyl group (C-1), according to a diagnostic HMBC correlation from OCH_3-12 to C-1. HMBC correlations from H-2 to the quaternary carbons C-3 and C-11 placed the exo-methylene group (C-3/CH_2-11) adjacent to CH-2. According to HMBC correlations from H-2 to carbonyl C-4, the latter carbon was also bonded to the exo-methylene group. The remaining hydroxyl group was assigned to the carboxylic acid moiety (C-4). Compound **3**, for which the name 9-hydroxyhexylitaconic acid-1-methyl ester is suggested, is closely related to the known fungal metabolite hexylitaconic acid[28] (**4**), also isolated from this fungal extract. The determination of the chirality at the hydroxylated C-9 was attempted using Mosher's method with Ba(II),[29] but was unsuccessful due to identical chemical shift differences in the ^{1}H NMR resonances of H_3-10 and H_2-5 to H_2-8.

For compound **4** spectral data were identical to literature data for hexylitaconic acid, except for the value of the optical rotation.[28] This is thus the first report of the (−)-enantiomer of hexylitaconic acid, with an optical rotation of $[\alpha]^{23}_D$ −17.9° (*c* 0.5 MeOH) compared to $[\alpha]^{20}_D$ +15.3° (*c* 2.0 MeOH) for the known compound.[28]

In addition to these new secondary metabolites the algicolous strain of *A. montagnei* produced several known compounds: the ubiquitous fungal metabolites *R*-mellein,[36,37] *R*-8-methoxymellein,[38] and 5-hydroxymethylfuran-2-carboxylic acid,[39] (+)-epiepoxydon (**5**),[31,33,34] (+)-epoxydon monoacetate,[35] as well as the recently discovered xanthone derivative anomalin A.[10]

In antibacterial, antifungal, and antialgal assays[30] the crude extract, compounds **1**−**5**, and (+)-epoxydon monoacetate were all found to be inactive at the 50 μg/disk level. In the brine shrimp assay[23] the crude extract showed weak activity and compound **5** was strongly cytotoxic. Thus the cytotoxic effects of compounds **1**, **5**, and (+)-epoxydon monoacetate against HM02, HepG2, and MCF7 human cancer cell lines[24] were investigated, and compound **5** was found to be active. Compound **5** showed 50% growth inhibition (GI_{50}) at concentrations of 0.7 μg/mL (HM02), 0.75 μg/mL (HepG2), and 0.8 μg/mL (MCF7). Additionally, total growth inhibition (TGI) was determined and found to be 1.0 μg/mL (HM02), 4.6 μg/mL (HepG2), and 1.5 μg/mL (MCF7). Most significant was the 50% reduction of the initial cell number (LC_{50}) at a concentration of 3.6 μg/mL for the breast adenocarcinoma cell line (MCF7). In the case of HM02 and HepG2 cells the LC_{50} of compound **5** was >10 μg/mL. The cytotoxicity of **5** is remarkable but not unexpected, since this compound was already described as being active toward P388 lymphocytic leukemia cell lines with an ED_{50} of 0.2 μg/mL.[31] Surprisingly however, (+)-epoxydon monoacetate was not cytotoxic despite its close structural similarity to compound **5**. Furthermore compound **1** was expected to have some cytotoxicity due to the activity described for related structures.[25,26] In all three human cancer cell lines (see above) compound **1** showed no effect at the 10 μg/mL level.

Experimental Section

General Experimental Procedures. Optical rotations were measured on a Jasco DIP 140 polarimeter. UV and IR spectra were obtained employing Perkin-Elmer Lambda 40 and Perkin-Elmer Spectrum BX instruments, respectively. ^{1}H (1D, 2D COSY) and ^{13}C (1D, DEPT 135, 2D HSQC, 2D HMBC) NMR spectra were recorded on Bruker Avance 500 DRX and Bruker Avance 300 DPX spectrometers in acetone-d_6, methanol-d_4, and $CDCl_3$. Spectra were referenced to residual solvent signals with resonances at $\delta_{H/C}$ 2.04/28.9 (acetone-d_6), $\delta_{H/C}$ 3.35/49.0 (methanol-d_4), and $\delta_{H/C}$ 7.26/77.0 ($CDCl_3$). HREIMS were recorded on a Finnigan MAT 95 spectrometer. HPLC-MS measurements were recorded employing an Agilent 1100 Series HPLC including DAD (250 nm), with a reversed phase C_{18} column (Macherey-Nagel Nucleodur 100, 125 mm × 2 mm, 5 μm) and gradient elution (from MeOH/H_2O 10/90 in 20 min to MeOH/H_2O 100/0, MeOH 100% for 10 min) coupled with an API 2000, Triple Quadrupole, LC/MS/MS, Applied Biosystems/MDS Sciex and ESI source. HPLC was carried out using a Waters system consisting of a 717 plus autosampler, 600 controller pump, and a 996 photodiode array detector or a Waters system with 515 HPLC pump and a Knauer K-2300 differential refractometer as detector. All other experimental details were as previously reported.[32]

Origin of the Algal Sample. The fresh algal sample was collected in July 2000 in the North Sea near Helgoland. During transport the sample was stored in sterile artificial seawater [(g/L): KBr (0.1), NaCl (23.48), $MgCl_2 \times H_2O$ (10.61), $CaCl_2 \times 2\ H_2O$ (1.47), KCl (0.66), $SrCl_2 \times 6\ H_2O$ (0.04), Na_2SO_4 (3.92), $NaHCO_3$ (0.19), H_3BO_3 (0.03)] with added antibiotics [benzyl penicillin (250 mg/L), streptomycin sulfate (250 mg/L)]. The

alga was identified as *Polysiphonia violacea* (Roth) Spreng. by Prof. A. Flores-Moya, University of Malaga, Spain.

Isolation and Taxonomy of the Fungus. The isolation of the fungus was carried out using an indirect isolation method. After surface sterilization with 70% EtOH for 15 s the alga was rinsed in sterile water. To distinguish remaining epiphytic fungi from endophytic fungi, an imprint of the algal surface on biomalt agar was done. The alga was aseptically cut into small pieces and placed on agar plates containing isolation medium: 15 g/L agar (Fluka Chemie AG, Germany), artificial seawater, benzyl penicillin (250 mg/L), and streptomycin sulfate (250 mg/L). The endophytic fungus found to grow out of the algal tissue was separated on the following medium: 15 g/L agar, 20 g of sterile mashed alga, 1 L of artificial seawater. For sporulation the fungus was inoculated on potato/carrot medium: 15 g/L agar, 20 g/L potato mash, 20 g/L carrot mash. The fungal strain was identified as *Apiospora montagnei* Saccardo by the Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands.

Cultivation. The fungal strain (strain number 581/H2 15E, culture collection Institute for Pharmaceutical Biology, University of Bonn, Germany) was cultivated on 9.5 L (38 Fernbach flasks) solid biomalt medium containing 20 g/L of Biomalt (Villa Natura Gesundheitsprodukte GmbH, Germany) and 10 g/L agar at room temperature for 3 months. Each Fernbach flask was inoculated with a piece of fungal biomass (1 cm^2) dissolved in 10 mL of sterile water.

Extraction and Isolation. The fungal biomass, including the medium, was diluted with water (100 mL/L) and homogenized using an Ultra-Turrax. Exhaustive extraction with 31.5 L of ethyl acetate (EtOAc) in three steps yielded 3.1 g of brown oily extract. This was fractionated by NP VLC (silica gel 60, 0.063−0.200 mm) using a gradient elution from petroleum ether (PE), EtOAc, acetone, to MeOH, to yield 12 fractions. Fractions 2, 3, 5, and 6 had interesting ^{1}H NMR spectra and were chosen for further investigations. In the case of fractions 5 and 6 further NP VLC was carried out in the same manner. Fractions 2 and 3 were first fractionated by reversed phase solid-phase extraction (Bakerbond SPE C_{18}). All further fractions were subjected to either NP HPLC (column A: Knauer Si LiChrospher-60, 250 × 8 mm, 5 μm; column B: Knauer Si Eurospher-100, 250 × 8 mm, 5 μm; column C: Merck Si LiChrospher-60, 125 × 4 mm, 5 μm) or reversed phase (RP) HPLC (column: Waters X-Terra C_{18}, 250 × 4.6 mm, 5 μm). Fraction 2 gave 9.3 mg of *R*-mellein after RP SPE with gradient elution from MeOH to H_2O and NP HPLC (column A, PE/acetone, 8.5:1.5, 2 mL/min). After C18 RP SPE, NP (column A, PE/EtOAc/acetone, 4:1:1, 2 mL/min) and RP HPLC (gradient MeOH/H_2O, 1 mL/min) of fraction 3 yielded compound **4** (9.2 mg). Fraction 5 was fractionated by NP VLC to yield six fractions. After separation on NP HPLC (column C, PE/acetone, 1:7, 1 mL/min) fraction 5-6 yielded 5-hydroxymethylfuran-2-carboxylic acid (1.5 mg). Fraction 5-2 was fractionated with NP HPLC (column B, PE/EtOAc/acetone, 4:0.5:0.5, 2 mL/min) to give *R*-8-methoxymellein (14.4 mg) and another five fractions. Fraction 5-2-1 was first separated by NP HPLC (column B, PE/EtOAc/acetone, 3:1:1, 2 mL/min), then on RP SPE, and finally purified by RP HPLC (gradient MeOH/H_2O, 1 mL/min) to yield compound **3** (2.9 mg). Fraction 5-2-4 afforded 7.9 mg of (+)-epoxydonmonoacetate after NP HPLC (column B, PE/EtOAc/acetone, 2:0.5:0.5, 2 mL/min). NP HPLC separation (column B, PE/EtOAc/acetone, 2.5:0.75:0.75, 2 mL/min) of fraction 5-2-5 yielded 7.7 mg of compound **2** and another 2.5 mg of compound **4**. Fraction 5-2-6 was first fractionated on NP HPLC (column B, PE/EtOAc/acetone, 1.5:0.5:0.5, 2 mL/min) and then on RP HPLC (gradient MeOH/H_2O, 1 mL/min) to give 4.4 mg of compound **1** and 3.3 mg of anomalin A. Further fractionation of fraction 6 with NP VLC, as described above, gave another 18.4 mg of 5-hydroxymethylfuran-2-carboxylic acid. Subsequent NP HPLC (column B, PE/EtOAc/acetone, 3:1:1, 2 mL/min) yielded the major compound (**5**, 169 mg) of this extract.

Myrocin A, 4,9a-dihydroxy-3,7-dimethyl-5,9-dioxo-7-vinyl-1,2,3,5,7,8,9,9a,10,10a-decahydrocyclopropa[*d*]-phenanthrene-3-carboxylic acid (1): yellowish solid (4.4 mg, 0.46 mg/L); $[\alpha]^{22}_D$ −418.6° (*c* 0.36 MeOH); UV (MeOH) λ_{max} (log ϵ) 204 nm (4.10), 254 nm (3.76); IR (ATR) γ_{max} 3426, 2926, 1715, 1700, 1616, 1398, 768, 630, 528 cm^{-1}; ^{1}H and ^{13}C NMR data (see Table 1); EIMS *m*/*z* 358 (25), 341 (50), 314 (100), 297 (90); HREIMS *m*/*z* 358.1415 (calcd for $C_{20}H_{22}O_6$ 358.1416).

Apiosporic acid, 6-(hydroxymethyl)-2-methyl-1,2,-4a,5,6,7,8,8a-octahydro-1-naphthalenecarboxylic acid (2): colorless oil (7.7 mg, 0.81 mg/L); $[\alpha]^{28}_D$ −103.6° (*c* 0.50 MeOH); UV (MeOH) λ_{max} (log ϵ) 202 nm (3.30), 242 nm (3.03), 248 nm (3.03); IR (ATR) γ_{max} 3426, 2914, 1706, 1450, 1190, 1036, 633, 535 cm^{-1}; ^{1}H and ^{13}C NMR data (see Table 2); ESIMS *m*/*z* 223 [M − H^+], EIMS *m*/*z* 224 (0.8), 206 (8), 178 (100); HREIMS *m*/*z* 206.1311 (calcd for [M − H_2O] $C_{13}H_{18}O_2$ 206.1307).

9-Hydroxyhexylitaconic acid-1-methyl ester, 2-[6-hydroxy-1-(methoxycarbonyl)heptyl]acrylic acid (3): colorless oil (2.9 mg, 0.31 mg/L); $[\alpha]^{22}_D$ −4.8° (*c* 0.24 MeOH); UV (MeOH) λ_{max} (log ϵ) 205 nm (4.00); IR (ATR) γ_{max} 3453, 2929, 1734, 1719, 1702, 1453, 1212, 667, 500 cm^{-1}; ^{1}H and ^{13}C NMR data (see Table 3); EIMS *m*/*z* 244 (2), 229 (5), 226 (10), 211 (10), 199 (70), 168 (70), 140 (100); HREIMS *m*/*z* 244.1310 (calcd for $C_{12}H_{20}O_5$ 244.1311).

(−)-Hexylitaconic acid, 2-hexyl-3-methylensuccinic acid (4): colorless solid (9.2 mg, 0.97 mg/L); $[\alpha]^{23}_D$ −17.9° (*c* 0.5 MeOH) cp. $[\alpha]^{20}_D$ +15.3° (*c* 2.0 MeOH);[28] UV (MeOH) λ_{max} (log ϵ) 205 nm (3.68); IR (ATR) γ_{max} 2927, 1701, 1626, 1418, 1283, 960, 668 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 0.87 (3H, t, J = 6.0 Hz, H_3-10), 1.29 (8H, m, H_2-6, H_2-7, H_2-8, H_2-9), 1.73 (1H, m, H-5b), 1.91 (1H, m, H-5a), 3.46 (1H, t, J = 7.4 Hz, H-2), 5.87 (1H, s, H-11b), 6.54 (1H, s, H-11a), 10.98 (2H, br s, 2 × COOH); ^{13}C NMR ($CDCl_3$, 75.5 MHz) δ 179.7 (C-1, C), 171.7 (C-4, C), 137.2 (C-3, C), 129.8 (C-11, CH_2), 46.6 (C-2, CH), 31.5 (C-8, CH_2), 30.3 (C-5, CH_2), 28.9 (C-7, CH_2), 27.3 (C-6, CH_2), 22.5 (C-9, CH), 14.0 (C-10, CH_3); EIMS *m*/*z* 214 (10), 196 (5), 178 (5), 169 (100), 129 (80), 112 (75).[28]

Biological Assays. Activities of the crude extract, (+)-epoxydon monoacetate, and compounds **1**−**5** were tested in agar diffusion assays against the bacteria *Bacillus megaterium* and *Escherichia coli*, the fungi *Microbotryum violaceum*, *Eurotium repens*, and *Mycotypha microspora*, and the green microalga *Chlorella fusca*.[30] Cytotoxicity of the crude extract and compound **5** was tested in the brine shrimp assay,[23] and additionally, cytotoxicity of compounds **1**, **5**, and epoxydon monoacetate was investigated using the human cancer cell lines HM02 (gastric adenocarcinoma), HepG2 (hepatocellular carcinoma), and MCF7 (breast adenocarcinoma) following the NCI standards.[24]

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Supporting Information Available: Spectroscopic data for the isolated known natural products are available free of charge via the Internet at http://pubs.acs.org.

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