Aromatic Polyketides from Marine Algicolous Fungi

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The investigation of the marine-derived fungi *Acremonium* sp. and *Nodulisporium* sp. led to the isolation of the new natural products acremonisol A (**1**) and (3*R*)-7-hydroxy-5-methylmellein (**2**). Both fungi are endophytes of marine algae. Compounds **1** and **2** are biosynthetically related by both being aromatic pentaketides belonging to the dihydroisocoumarins. All structures were elucidated by extensive spectroscopic measurements.

Organisms from the marine habitat, including marine microorganisms, represent an important source for structurally diverse natural products.[1] In the search for novel secondary metabolites from microbes, we have focused on fungi associated with marine algae.[2] Screening of fungal strains isolated from the inner tissue of algae directed our attention to fungi of the genera *Acremonium* and *Nodulisporium*.

Some of our previous work demonstrated the ability of *Acremonium* spp. to produce interesting natural compounds, such as acremonin A, which has a most unusual bicyclo[4.2.0]octa-1,3,5-triene nucleus.[3] The *Acremonium* sp. investigated herein is closely related to *A. alabamense* and was isolated from a red alga, *Plocamium* sp., collected at Helgoland, Germany. The ^{1}H NMR spectra of the crude fungal extract revealed the presence of a single main compound (**1**) in high yield (18 mg/L). Another new polyketide, (3*R*)-7-hydroxy-5-methylmellein, **2**, was isolated from *Nodulisporium* sp., a fungus located in the inner tissue of an algal species collected in Corfu, Greece. The endophyte was cultivated for 10 weeks on solid Czapek-Dox medium and then extracted with ethyl acetate. Through a multistep isolation procedure the new compound **2** could be obtained (5.5 mg/L medium). Melleins are well-known dihydroisocoumarins, which are widely distributed in fungi;[4–6] (−)-mellein was first described in 1933 from *Aspergillus melleus*.[7]

The molecular formula of compound **1** was deduced by accurate mass measurement (HREIMS) to be $C_{12}H_{15}ClO_4$, implying five degrees of unsaturation. The ^{13}C NMR spectrum contained 12 resonances resulting from one methyl, two methoxy, two methylene, one methine, and six quaternary carbons, including one carboxy group (δ_C 171.4). Due to the molecular formula and 14 protons evident from ^{1}H NMR, one proton had to be present as a hydroxyl group, most probably as part of a carboxylic acid function. UV-absorption maxima at λ_{max} 208 and 288 nm indicated an aromatic nucleus. One aromatic methine group and five quaternary, sp^2-hybridized carbons suggested a pentasubstituted aromatic system. With three elements of unsaturation attributed to carbon−carbon double bonds of the aromatic moiety and one carboxyl group, a monocyclic structure was in agreement with the five degrees of unsaturation. From ^{1}H and ^{13}C NMR spectra an *n*-propyl chain (CH_2-4 $\delta_{H/C}$ 2.74/34.6, CH_2-3 $\delta_{H/C}$ 1.64/24.0, CH_3-9 $\delta_{H/C}$ 1.01/14.6) was deduced and confirmed by ^{1}H−^{1}H COSY correlations. The ^{1}H NMR spectrum displayed one aromatic proton (CH-7 $\delta_{H/C}$ 6.68/95.6) and two methoxy groups (CH_3-12 $\delta_{H/C}$ 3.97/56.8, CH_3-11 $\delta_{H/C}$ 3.90/56.7). ^{1}H−^{13}C HMBC NMR measurements (Figure 2) clarified the substitution pattern. Both methoxy proton signals

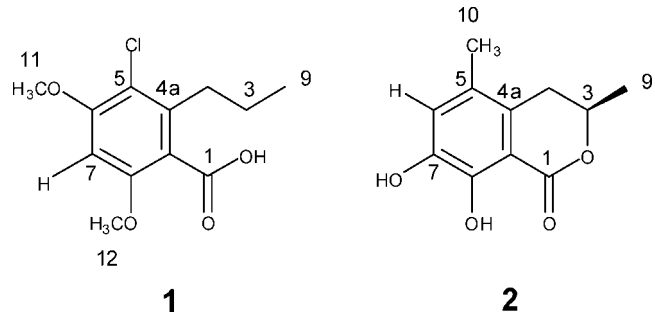

Figure 1. Chemical structures of compounds **1** and **2**.

possessed HMBC cross-peaks to C-7, so they had to be adjacent to this aromatic proton and thus *meta* positioned to each other. Additionally, CH_3-12 showed an HMBC correlation to δ_C 158.0 (C-8) and CH_3-11 to δ_C 157.0 (C-6), proving their positions on the aromatic ring. The methylene protons H_2-4 and H_2-3 showed HMBC correlations to δ_C 140.0 (C-4a); further correlation of H_2-4 to δ_C 119.0 (C-5), δ_C 115.1 (C-8a), and δ_C 171.4 (C-1) defined the position of all substituents on the nucleus. Based on the elemental composition, the remaining substituent at C-5 was a chlorine atom. The systematic name for compound **1** is 3-chloro-4,6-dimethoxy-2-propylbenzoic acid, and we propose the trivial name acremonisol A.

The molecular formula of **2** was determinated as $C_{11}H_{12}O_4$ by accurate mass measurement (HREIMS), implying six degrees of unsaturation. The ^{1}H NMR spectrum contained a signal typical for a chelated hydroxyl group (δ_H 11.0, OH-8) and the signals for a single aromatic proton, an oxygen-bearing methine group, a methylene group, as well as two methyl groups. ^{13}C NMR data revealed six resonances for sp^2 carbons being part of a benzene ring, two of them characterized by a downfield shift due to pendant hydroxyl groups (δ_C 144.8, C-7; δ_C 149.1, C-8). The ^{13}C NMR spectrum further revealed the presence of a carbonyl group at δ_C 171.5 (C-1) forming a phenylogous acid together with OH-8. This information taken together suggested a dihydroisocoumarin skeleton. The structural details of the lactone ring were elucidated using ^{1}H−^{1}H-COSY in which couplings between H_2-4 and H-3 as well as between H-3 and H_3-9 were visible. HMBC correlations (Figure 2) of CH_2-4 to δ_C 109.0 (C-8a), δ_C 125.8 (C-5), and δ_C 128.3 (C-4a) as well as of CH-3 to δ_C 171.5 (C-1) proved the connection of the two rings. The aromatic methyl group CH_3-10 was bonded to a carbon with a resonance at δ_C 125.8 (C-5), as evident by a cross-peak from CH_3-10 to CH_2-4 in the NOESY spectrum. HMBC and also NOESY correlations between CH_3-10 and CH-6 proved the vicinal position of C-5 and C-6. Additionally, the chelated proton of OH-8 showed no correlations in the HMBC and NOESY experiments to H-6 or CH_3-10; obviously the second hydroxyl group

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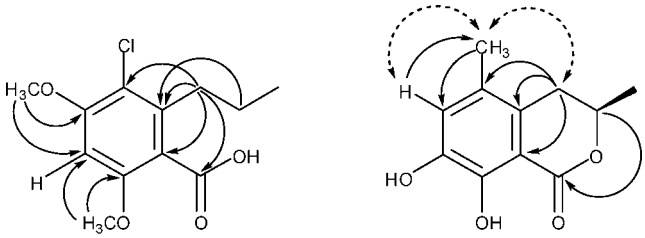

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Figure 2. Important HMBC (→) and NOESY (↔) correlations for **1** and **2**.

had to be located at C-7. The deduced structure of **2** is in good agreement with published results of similar 7,8-dihydroxymellein derivatives.[8,9]

Thus, compound **2** was identified as a new representative of the dihydroisocoumarins with the semisystematic name 7-hydroxy-5-methylmellein. The absolute configuration at C-3 was deduced from optical rotation data in comparison with similar naturally occurring melleins.[4,10] The observed value of $[\alpha]^{20}_D$ −57.4 ($CHCl_3$) for **2** is close to values of homochiral dihydroisocoumarins such as 5,6-dihydroxymellein with $[\alpha]^{20}_D$ −61.7 ($CHCl_3$) (Figure S1).[11] Hence, the (3*R*)-absolute configuration is indicated for compound **2**.

Compound **1** is structurally related to differanisole A,[12] a fungal metabolite of *Chaetomium* sp., and DIF-1 to DIF-3, mold metabolites of *Dictyostelium discoideum*.[13,14] Furthermore, there is a biosynthetical analogy to diverse dihydroisocoumarins, such as 5-chloro-6-hydroxymellein isolated from *Plectophomella* sp.[11] and ochratoxin A from *Aspergillus ochraceus* (Figure S1).[15] They all have a chlorinated and multiply oxygenated aromatic nucleus and result from polyketide biosynthesis. Interestingly, compound **1** was previously described as a synthetic precursor involved in the synthesis of differanisole A, but to date it is unknown as a natural product.[16]

Compound **2** represents a dihydroisocoumarin with a rarely occurring 7,8-dihydroxy moiety, to date found only in melleins from the plant *Azadirachta indica* and from the fungi *Septoria nodorum*, *Cytospora eucalypticola*, and *Cephalosporium* sp.[8,9,17,18] The unusual ortho hydroxyl substitution probably arises through an additional oxidation at C-7 after the formation of the polyketide skeleton. Accordingly, the fungus *Nodulisporium* sp. is one of a few known organisms capable of producing melleins with this unusual structural feature.

Experimental Section

General Experimental Procedures. Optical rotation was 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. All NMR spectra were recorded on Bruker Avance 500 DRX or 300 DPX spectrometers in methanol-d_4, acetone-d_6, or chloroform-d_1. Spectra were referenced to residual solvent signals. HREIMS were recorded on a Kratos MS 50 spectrometer. ESIMS measurements were recorded employing an API 2000, Applied Biosystems/MDS Sciex. HPLC was carried out using a Waters system consisting of a 600 pump, a 996 photodiode array detector, a 717 plus autosampler, and a fraction collector or a Waters 515 HPLC-pump with a Knauer differential-refractometer A0298.

Origin of the Algal Sample and Isolation and Taxonomy of the Fungus. An algal sample belonging to the genus *Plocamium* was collected near Helgoland, Germany. A second algal species (taxonomy not determined) originated from Corfu, Greece. Algal samples were processed immediately after collection. The isolation of fungi was carried out using an indirect isolation method. Algal samples were rinsed three times with sterile H_2O. After surface sterilization with 70% EtOH for 15 s the alga was rinsed in sterile artificial seawater (ASW). Subsequently, the alga was aseptically cut into small pieces and placed on agar plates containing isolation medium: 15 g/L agar, ASW 800 mL/L, glucose 1 g/L, peptone from soymeal 0.5 g/L, yeast extract 0.1 g/L, benzyl penicillin 250 mg/L, and streptomycin sulfate 250 mg/L. Fungi growing out of the algal tissue were separated on biomalt medium (biomalt 20 g/L, agar 10 g/L, ASW 800 mL/L) until the culture was pure. The fungal strain 273/H3 09 isolated from the Helgoland alga was identified as *Acremonium* sp. (related to *A. alabamense*, G. MORGAN-JONES). The strain 707/GrKo3 was assigned as *Nodulisporium* sp. and came from the Greek alga. Identification of fungi was done by Dr. R. A. Samson, Centralbureau voor Schimmel Cultures, Utrecht, The Netherlands.

Cultivation. The fungal strain *Acremonium* sp. was cultivated on 600 mL (20 Petri dishes) solid biomalt medium containing 50 g/L biomalt (Villa Natura Gesundheitsprodukte GmbH, Germany), 0.1 g/L yeast extract, and 15 g/L agar at room temperature for 9 weeks. The *Nodulisporium* sp. was cultivated for 10 weeks on 10 L of solid Czapek-Dox medium (Becton Dickinson, France) with 15 g/L agar at room temperature in Fernbach flasks.

Extraction and Isolation. Fungal biomass and media were homogenized using an Ultra-Turrax apparatus and extracted with EtOAc. Extraction of *Acremonium* sp. gave 220 mg of crude extract. An isocratic fractionation (2 mL/min, MeOH/H_2O, 80/20) with a reversed-phase C_{18} column (Macherey-Nagel Nucleodur 100, 250 mm × 10 mm, 5 μm) was performed yielding seven fractions. Fraction 4 gave 11.0 mg of pure compound **1**.

The EtOAc extract (16.35 g) of the *Nodulisporium* fermentation was separated by liquid–liquid extraction (*n*-hexane/MeOH). The MeOH extract was fractionated using NP-vacuum liquid chromatography (Merck silica gel 60, 63–200 μm, 7 × 23 cm) with a CH_2Cl_2/acetone/MeOH gradient, yielding 12 fractions. Fraction 4 (1.53 g) was further fractionated by RP18-VLC (Macherey-Nagel Polygoprep 60–50 C_{18}, 4 × 23 cm) with a MeOH/H_2O gradient. Subfraction 4 was then purified via RP18 HPLC (Knauer C_{18} Eurosphere 100 250 × 8 mm, 5 μm) with a mobile phase (2 mL/min) consisting of 55/45 MeOH/H_2O, resulting in 18 fractions. Fraction 4 afforded the pure compound **2** (55.1 mg).

Acremonisol A, 3-chloro-4,6-dimethoxy-2-propylbenzoic acid (1): colorless oil (11.0 mg, 18 mg/L); UV (MeOH) λ_{max} (log ϵ) 208 (4.62), 288 (3.59) nm; IR (ATR) ν_{max} 2960, 1697, 1589, 1331, 1207, 1080 cm^{-1}; ^1H NMR (500 MHz, CD_3OD) δ 6.68 (1H, s, H-7), 3.97 (3H, s, H_3-12), 3.90 (3H, s, H_3-11), 2.74 (2H, t, J = 7.3 Hz, H_2-4), 1.64 (2H, sext, J = 7.3 Hz, H_2-3), 1.01 (3H, t, J = 7.3 Hz, H_3-9); ^{13}C NMR (75.5 MHz, MeOH-d_4) δ 171.4 (C-1), 158.0 (C-8), 157.0 (C-6), 140.0 (C-4a), 119.0 (C-5), 115.1 (C-8a), 95.9 (CH-7), 56.8 (CH_3-12), 56.7 (CH_3-11), 34.6 (CH_2-4), 24.0 (CH_2-3), 14.6 (CH_3-9); ESIMS *m/z* 259 $[M + H]^+$, 257 $[M - H]^-$; EIMS *m/z* 258 (100), 260 (33), 241 (88), 243 (28), 225 (50); HREIMS *m/z* 258.0663 (calcd for $C_{12}H_{15}{}^{35}ClO_4$, 258.0659).

(3*R*)-7-Hydroxy-5-methylmellein (2): brownish solid (55.1 mg, 5.5 mg/L); $[\alpha]^{20}_D$ −57.4 (*c* 0.19, $CHCl_3$); UV ($CHCl_3$) λ_{max} (log ϵ) 257 (3.66), 338 (3.32) nm; IR (ATR) ν_{max} 2920, 1665, 1305, 1171, 1135, 1023 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 11.0 (s, OH-8), 6.98 (1H, s, H-6), 4.69 (1H, dqd, J = 3.7, 6.6, 11.7 Hz, H-3), 2.98 (1H, dd, J = 3.7, 16.5 Hz, H-4β), 2.68 (1H, dd, J = 11.7, 16.5 Hz, H-4α), 2.17 (3H, s, H_3-10), 1.54 (3H, d, J = 6.6 Hz, H_3-9); ^{13}C NMR (75.5 MHz, acetone-d_6) δ 171.5 (C-1), 149.1 (C-7), 144.8 (C-8), 128.3 (C-4a), 125.8 (C-5), 124.0 (CH-6), 109.0 (C-8a), 77.1 (CH-3), 31.6 (CH_2-4), 20.9 (CH_3-9), 17.9 (CH_3-10); ESIMS *m/z* 209 $[M + H]^+$, 207 $[M - H]^-$; EIMS *m/z* 208 (100), 190 (13), 179 (41), 164 (15), 162 (44); HREIMS *m/z* 208.0741 (calcd for $C_{11}H_{12}O_4$, 208.0736).

Biological Assays. Compounds **1** and **2** were tested in agar diffusion assays[4] against the bacteria *Bacillus megaterium* and *Escherichia coli*, the fungi *Microbotryum violaceum*, *Eurotium rubrum*, and *Mycotypha microspora*, and the green microalga *Chlorella fusca*.

Cytotoxicity of the compounds was investigated using 36 cancer cell lines.[19,20] The pure compounds showed no activity in any of these assays at concentrations of 1 and 10 μg/mL, respectively.

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Supporting Information Available: ^{1}H and ^{13}C NMR spectra of compounds **1** and **2** and a figure with related compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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