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Hyaladione, an *S*-Methyl Cyclohexadiene-dione from *Hyalangium minutum*

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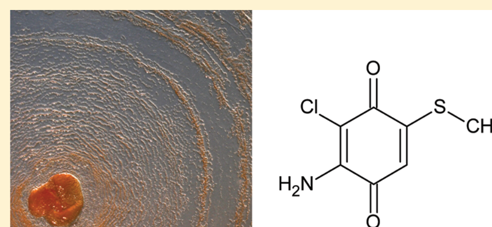
Supporting Information

ABSTRACT: A bioassay-guided fractionation of the crude methanol extract of the myxobacterium *Hyalangium minutum*, strain NOCB-2[T] (DSM 14724[T]), led to the isolation of hyaladione (**1**), a novel *S*-methyl cyclohexadiene-dione. The structure of **1** was established by HRESIMS, NMR, and IR spectroscopy as well as X-ray crystallography. Compound **1** was active against growing mammalian cell lines, with IC_{50} values ranging from 1.23 to 3.93 μM, in addition to a broad spectrum of antibacterial and antifungal activities, including inhibition of pathogenic methicillin-resistant *Staphylococcus aureus* and *Pseudomonas aeruginosa* with an MIC of 0.83 and 8.5 μg mL^{-1}, respectively.



Myxobacteria are Gram-negative soil-dwelling bacteria that move by gliding and uniquely develop fruiting bodies during starvation. Besides the actinomycetes, the genus *Bacillus*, and the pseudomonads, myxobacteria have emerged as proficient producers of novel biologically active secondary metabolites.[1,2] During the past three decades more than 100 different basic structures and over 600 structural variants have been characterized from myxobacteria, including epothilone,[1,3,4] which was developed to Ixempra, a semisynthetic derivative used as a drug against metastatic breast cancer. Within the framework of the continued screening of our myxobacterial strain collection for biologically active compounds, hyaladione (**1**) was isolated as the first biologically active secondary metabolite from *Hyalangium minutum*, strain NOCB-2[T]. The structure of **1** was elucidated by extensive HRESIMS, NMR, and X-ray crystallographic analysis, and its antibiotic, antifungal, and cytotoxic activity is reported herein.

H. minutum, strain NOCB-2[T], was isolated from a soil sample containing decaying plant material collected in the mountains of Izu and Manazuru in Japan as described.[5,6] The strain was inoculated in liquid soy-flour medium containing XAD 16 adsorber resin and fermented in a 70 L bioreactor for 7 days. The resin was recovered from the culture broth by sieving, and a raw extract was eluted from the resin with methanol. A bioassay of analytical RP-HPLC fractionations of the crude methanol extract identified **1** as antibacterial, and its purification was achieved by a series of solvent partitioning, silica gel chromatography, RP-MPLC, and finally crystallization.

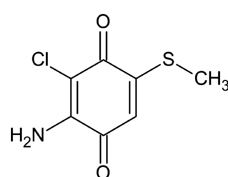


Figure 1. Hyaladione (**1**).

The molecular formula $C_7H_6ClNO_2S$ requiring five degrees of unsaturation was established by ultrahigh-resolution ESI-TOF MS in the positive-ion mode from a molecular ion peak. Supporting the empirical formula, all seven carbon atoms were present in the ^{13}C NMR spectrum of **1** in acetone-d_6. The signals of a methyl carbon and a methine carbon were found at δ_C 13.9 (C-7) and 120.3 (C-5), respectively. Five quaternary signals of two carbonyl carbons [δ_C 177.9 (C-4), 174.9 (C-1)] and three carbon signals [δ_C 106.2 (C-2), 146.4 (C-3), 159.2 (C-6)] suggested the presence of a substituted quinone-type aromatic system. This was supported by the IR spectrum, showing two strong bands of α,β-unsaturated carbonyl groups at ν_{max} 1604 and 1566 cm^{-1}. Additionally, the IR spectrum revealed two sharp bands of a primary amine at ν_{max} 3438 and 3308 cm^{-1}.

The ^{1}H NMR spectrum of **1** in acetone-d_6 presented only three singlet signals of a methyl group (δ_H 2.41, CH_3-7), a methine proton (δ_H 6.26, H-5), and a broad singlet of an amine (δ_H 6.81) (Table 1). However, all correlations from the COSY,[7] HMBC, and ROESY spectra were insufficient for a complete

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Table 1. NMR Data of Hyaladione (1) in Actone-d_6 (^{1}H 600 MHz; ^{13}C 150 MHz)

C/H	δ_C, mult	δ_H, mult	COSY[a]	ROESY[a]	HMBC[a] ($^1J_{C,H}$ in Hz)
1	174.8, qC				5
2	106.2, qC				
3	146.4, qC				5
4	177.7, qC				
5	120.3, CH	6.26, s	7	7	5 (171), 7
6	159.2, qC				7, 5
7	13.9, CH_3	2.41, s	5	5	7 (142)
NH_2		6.79, br s			

[a]Positions of correlated protons.

structure elucidation (Figure 2, Table 1). Fortunately, the compound could be crystallized from acetone to obtain pink, needle-shaped crystals for an unambiguous structure elucidation by X-ray analysis (Figure 3).

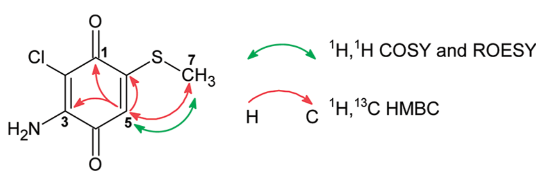

Figure 2. All ^{1}H,^{1}H COSY, ROESY, and ^{1}H,^{13}C HMBC correlations of hyaladione (1).

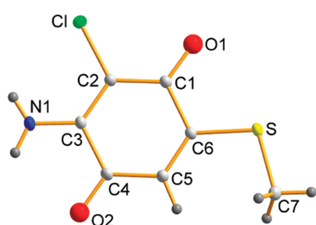

Figure 3. X-ray crystal structure of hyaladione (1).

Hyaladione (1) displayed broad antibiotic and antifungal activities, with a minimum inhibitory concentration (MIC) of 0.83 μg/mL against methicillin-resistant *Staphylococcus aureus* (Table 2). The cytotoxicity of **1** was evaluated against three growing cancer cell lines and a primary cell line using the MTT method as reported previously.[8] **1** was cytotoxic against the breast cancer cell line MCF-7 (IC_{50} 1.23 μM), the mouse subcutaneous connective tissue fibroblast cell line L929 (IC_{50} 1.47 μM), the cervix carcinoma cell line KB-3-1 (IC_{50} 3.93 μM), and the nontransformed human umbilical vein endothelial cell line HUVEC (IC_{50} 2.21 μM).

EXPERIMENTAL SECTION

General Experimental Procedures. The melting point was measured on a Büchi-510 melting point apparatus; UV data were recorded on a Shimadzu UV/vis-2450 spectrophotometer using methanol (UVASOL, Merck) as solvent; IR data were recorded on a Bruker Tensor 27 IR spectrophotometer. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker Avance DMX 600 or DPX 300 NMR spectrometer, locked to the deuterium signal of the solvent. Data acquisition, processing, and spectral analysis were performed with standard Bruker software and ACD/NMRWorkbook. Chemical shifts are given in parts per million (ppm), and coupling constants in hertz (Hz). HRESIMS data were recorded on a Maxis ESI TOF mass spectrometer (Bruker Daltonics); molecular formulas were calculated including the isotopic pattern (Smart Formula algorithm). Analytical RP HPLC was carried out with an Agilent 1260 HPLC system equipped with a UV diode-array detector and a Corona Ultra detector (Dionex). HPLC conditions: column 125 × 2 mm, Nucleodur C_{18}, 5 μm (Macherey-Nagel), solvent A: 5% acetonitrile in water, 5 mmol NH_4Ac, 0.04 mL·L^{-1} AcOH; solvent B: 95% acetonitrile, 5 mmol NH_4Ac, 0.04 mL·L^{-1} AcOH; gradient system: 10% B increasing to 100% B in 30 min, 100% B for 10 min, to 10% B post-run for 10 min; 40 °C; flow rate 0.3 mL/min. X-ray data set was gathered at 122 K on an X8-Apex Bruker-AXS diffractometer (Mo Kα radiation), collecting 18 376 reflections (independent 5432, $R_{(int)}$ = 0.031). We determined the monoclinic space group $P2_1/n$ with a unit cell of a = 3.8875(8) Å, b = 14.180(3) Å, c = 14.518(3) Å, β = 94.81(1)°. The structure was solved by direct methods.[9] Full-matrix least-squares refinement against F_o^2 with anisotropic thermal parameters and free refinement of the hydrogen positions (133 parameter) were used, resulting in R1 = 0.031 and wR2 = 0.074 with $I > 2\sigma_I$.

Table 2. In Vitro Antibacterial and Antifungal Activity of 1 and Two Control Drugs

	MIC [μg mL^{-1}]		
strain	hyaladione (**1**)	oxytetracycline hydrochloride	nystatin
Micrococcus luteus	3.3	0.104	
Staphylococcus aureus	0.83	0.104	
Escherichia coli	33.3	0.83	
Nocardia flava	3.3	1.67	
Chromobacterium violaceum	2.1	0.21	
Pseudomonas aeruginosa	8.5		
Mucor hiemalis	16.5		1.67
Schizosaccharomyces pombe	>33.3		1.67
Rhodotorula glutinis	1.7		0.42
Pichia anomala	33.3		1.67
Candida albicans	>33.3		1.67

Myxobacterial Strain. *H. minutum*, strain NOCB-2^T, was isolated in 1992 and deposited at the German Resource Centre for Biological Material (DSMZ) as a type strain with accession number DSM 14724^T.

Fermentation. Large-scale fermentation of *H. minutum* strain NOCB-2^T was performed in a medium containing 0.2% soya meal, 0.2% glucose, 0.2% yeast extract, 0.8% starch, 0.1% $CaCl_2$, 0.1% $MgSO_4 \cdot 7\ H_2O$, 8 mg/L Fe-EDTA, and 2% Amberlite XAD-16 resin in a 70 L bioreactor (B. Braun) that was inoculated with 10 L of shaken cultures grown for 7 days in the same medium. The bioreactor was kept at 30 °C, aerated at 0.05 vvm per minute, pH regulated at 7.4 with 2.5% H_2SO_4 or 2.5% KOH, and agitated with a flat-blade turbine stirrer at 100 rpm.

Extraction and Isolation. The fermentation was terminated after 7 days, and the adsorber resin (1.9 kg) was collected by sieving and extracted sequentially in a glass column (70 × 8 cm) with methanol (7 L) and finally with acetone (4 L) at a flow rate of 2 L per hour. The combined solutions were evaporated to yield 34 g of extract. Enrichment of **1** was achieved by eliminating lipophilic compounds by partitioning between methanol and *n*-heptane to give 24 g of an enriched crude methanol extract. A further solvent−solvent partitioning with EtOAc/water resulted in **1** accumulating in 8.4 g of the EtOAc layer. Two grams of this extract (8.4 g) was separated by silica gel flash chromatography (Biotage Flash+) with a gradient of 2% to 10% methanol in dichloromethane to yield 330 mg of semipure hyaladione. This was further purified by reversed-phase preparative medium-pressure liquid chromatography (RP-MPLC) on a Kronlab ODS-AQ 120/16 column (3 × 48 cm) connected to a Büchi chromatography system with a gradient of 25% B to 35% B (solvent A: MeOH/H_2O, 1:1; solvent B: MeOH) in 30 min at a flow rate of 30 mL/min and UV detection at 360 nm; **1** eluted at a retention time of

9.5 min. The fraction containing **1** was evaporated to eliminate the MeOH, and the compound was recovered from the water layer by extraction with EtOAc to yield 98 mg of **1**. A total of 23 mg of crystals was obtained from a solution in 5 mL of acetone after four days.

Hyaladione: pink needles; mp 235−236 °C; UV (methanol) λ_{max} (log ε) 353 (3.743) nm; IR (KBr) ν_{max} 3438, 3308, 3056, 1673, 1662, 1638, 1604, 1566, 1394, 1335, 1318, 1261, 1246, 1088, 1039, 952, 877 cm^{-1}; ^{1}H NMR and ^{13}C NMR data, see Table 1; HRESIMS m/z 203.9882 $[M + H]^+$ (calcd for $C_7H_6ClNO_2S$ $[M + H]^+$, 203.9880).

Biological Testing. The MIC values of **1** were determined in 96-well microtiter plates by 1:1 serial dilution in EBS medium (0.5% casein peptone, 0.5% protease peptone, 0.1% meat extract, 0.1% yeast extract, pH 7.0) for bacteria and MYC medium (1.0% glucose, 1.0% phytone peptone, 50 mM HEPES [11.9 g/L], pH 7.0) for yeasts and fungi, as previously described.[10]

Cytotoxicity assays were carried out using the MTT assay as described in the literature.[10]

■ ASSOCIATED CONTENT

Supporting Information

Copies of 1D and 2D NMR, IR, and UV spectra and X-ray crystallographic information data of hyaladione (**1**). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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