Mayamycin, a Cytotoxic Polykide from a Streptomyces Strain Isolated from the Marine Sponge Halichondria panicea

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A new benz[a]anthracene derivative called mayamycin (1) was identified in cultures of Streptomyces sp. strain HB202, which was isolated from the marine sponge Halichondria panicea and selected because of its profound antibiotic activity. The ability to produce aromatic polyketides was indicated by genetic analyses, demonstrating the presence of a type II polyketide synthase. The production of mayamycin (1) was induced by variation of the culture conditions. The structure of 1 was elucidated by HPLC-UV/MS and NMR spectroscopy. Mayamycin (1) exhibited potent cytotoxic activity against eight human cancer cell lines and showed activity against several bacteria including antibiotic-resistant strains.

Marine actinomycetes are among the most promising natural producers of new antibiotics with clinical relevance. Therefore, we have performed a systematic analysis of Actinobacteria isolated from marine habitats with regard to their potential to produce biologically active natural products. This strategy included the selection of promising producers based on bioassay-guided analyses and the detection of genes encoding for the biosynthesis of secondary metabolites. Within such an approach, the marine Streptomyces sp. strain HB202, which was isolated from the sponge Halichondria panicea, showed a wide antibiotic activity spectrum while also containing one type II polyketide synthase gene fragment with a similarity of 92% to the jadomycin polyketide ketosynthase gene from Streptomyces venezuelae ISP5230 (AAB36562). Even though many different cultivation experiments were conducted with this strain, stimulation with subinhibitory concentrations of antibiotics was demonstrated, and no aromatic polyketide was observed. The aim of our study was to demonstrate that by further varying the cultivation conditions, strain HB202 has the ability to biosynthesize a type II PKS product.

Previous investigations had demonstrated the production of streptophenazines A–H by Streptomyces sp. strain HB202. In this former study, the biosynthesis was induced by subinhibitory concentrations of the antibiotics bacitracin and tetracycline, but former study, the biosynthesis was induced by subinhibitory production of the new benz[a]anthracene derivative mayamycin.

Cultivation of the bacterium in penicillin flasks containing GYM medium without using shaking conditions led to the production of the new benz[a]anthracene derivative mayamycin.

(1R,3R,4S,5R)-mayamycin (1)

1. No further compounds were observed under these conditions. The culture broth and cells were homogenized and extracted with EtOAc. The extract was separated by preparative reversed-phase (C18) HPLC to yield 1.

Mayamycin (1) was an amorphous, brown solid with a molecular formula of C26H25NO7, as determined by HRESIMS (m/z 464.1707 [M + H]+) and verified by 13C NMR and 1H–13C HMBC spectra. According to its UV–vis spectrum, metabolite 1 was likely to belong to the anthraquinone group. This was supported by a set of downfield resonances occurring in the 13C NMR spectrum between δ 100 and 170 ppm, which belonged to aromatic carbon atoms, and by two carbonyl resonances that were observed at δ 194.2 and 188.0 ppm, consistent with a quinone skeleton. According to their chemical shifts, three of the aromatic carbon atoms mentioned above (δ 154.4, 165.6, and 162.9 ppm) carried hydroxy groups. In addition, one methyl residue (2.45, s) attached to C-3 to the aromatic carbon skeleton was observed. Comprehensive analyses of 2D NMR (1H–1H COSY, HSQC, 1H–13C HMBC, and NOESY) data showed that compound 1 was an angucycline-type polykide with a C-glycosidically bound amino sugar. The aromatic protons H-9, H-10, and H-11 were located at the D-ring of the angucycline skeleton and showed HMBC couplings to the remaining carbons (C-7a, C-8, and C11a) of ring D as well as to the carbonyl carbon C-12 of the C-ring (Figure 1). Additionally, 1H–1H COSY correlations were observed from H-9 to H-10 and from H-10 to H-11. The remaining two aromatic protons together with the methyl group were found to be located at ring A. Long-range H,C-couplings revealed their exact position at this ring (C-2, C-4, and C-3, respectively) (Figure 1). This angucycline skeleton of 1 is identical to the known dehydrorabelomycin.5

Pivotal correlations that proved the connection site of the amino sugar at C-5 were the long-range H,C-couplings of H-4 to C-5 and C-12b as well as the couplings of H-1′ to C-4a, C-5, and C-6. A
have the same angucyclinone core. Although the absolute con-
group, is present in benzanthrins A and B, related compounds that

correlations to H-5 (74.5 ppm) in combination with the molecular formula of the

of the structure of the sugar moiety was revealed by long-range

couplings between the N-CH₃ and C-3 methylamino residue was evident from the long-range H,C-

of H-4

Further evaluation of the NMR data showed the amino sugar to

the benz[α]anthracene skeleton of I by a C-glycosidic bond.

Further evaluation of the NMR data showed the amino sugar to be a 2,3,6-trideoxy-3-methylamino pyran. The position of the methylamino residue was evident from the long-range H-C-couplings between the N-CH₃ and C-3' as well as NOESY correlations between the N-CH₃ and both protons at C-2'. The rest of the structure of the sugar moiety was revealed by long-range H-C-couplings of both protons at H-2' with C-1', C-3', and C-4', of H-4' with C-3', C-5', and 5'-CH₃, and of H-5' with C-3', C-4', and 5'-CH₃. The methyl group at C-5' showed HMBC couplings with C-4' and C-5'. Additionally, a series of 1H→3H COSY as well as NOESY correlations were observed for the sugar moiety from H-1' to H-2'a and H-2'b, from H-2' to H-3', from H-3' to H-4', and from H-4' to H-5'. The methyl group at C-5' showed NOESY correlations to H-5' as well as H-4'. The chemical shift of C-4' (74.5 ppm) in combination with the molecular formula of the compound indicated a free hydroxy group at this position. The 1J-coupling constants of 9.0 Hz for H-5'/H-4' as well as for H-4'/H-3' showed axial orientations for these protons. The likewise axial orientation of H-1' was inferred from the NOE interaction H-3'/H-1'. These results indicate the sugar is N-demethylangolosamine, and a relative configuration of (1'R*,3'R*,4'S*,5'R*) is assigned to mayamycin (I). Angolosamine, which has a dimethylamino group, is present in benzanthrins A and B, related compounds that have the same angucycline core. Although the absolute configuration has not been determined, the glycoside has been depicted in the d-configuration because in other cases where the configuration of angolosamine has been established d-angolosamine has been present.

Most of the reported angucyclines are O-deoxoglycosides, and a much smaller group are C-glycosides. In the case of mayamycin (I) the sugar binding site is C-5. At this position so far, no C-glycoside bound sugar is known, although some examples exist with S- or O-glycoside bound sugars. Deoxynamninosugars are quite frequently found in antibiotics. Examples with D-angolosamine are angolamycin and medermycin. In our case the aminosugar is the mono N-demethylated form of angolosamine. Other examples of angucyclines with an aminosugar are the antibiotic P371A and the marmycins. With regard to the combination of the angucyclinone core structure with this particular aminosugar and the site of glycosylation as well as the character of the glycosylation (C-glycosidic bond) mayamycin (I) is unusual compared to previously reported angucycline amino glycoside metabolites.

Members of the angucycline class are polyketide-derived tetracyclic benz[a]anthraquinones that exhibit a wide range of biological activities including antibacterial, enzyme-inhibitory, and cytostatic effects. In a previous bioassay-guided approach it was shown that the extract of strain HB202 inhibited the growth of various Gram-positive and Gram-negative bacteria (data not shown). Therefore, the antimicrobial activity of mayamycin (I) was analyzed using a wide range of Gram-positive and Gram-negative bacteria and yeast strains. The results revealed strong inhibitory activity against several test strains, including clinically relevant bacteria, such as Pseudomonas aeruginosa and methicillin-resistant Staphylococcus aureus (MRSA), the causative agents of acne vulgaris, Propionibacterium acnes, the human skin flora forming bacteria Dermabacter hominis and Brevisbacterium epidermidis, and the phytopathogenic bacterium Xanthomonas campestris, as summarized in Table 2. On the other hand, I was found to be inactive against the yeast Candida glabrata and the fungus Septoria tritici as well as the Gram-negative bacteria Escherichia coli, Pseudomonas syringae, and Ralstonia solanacearum. In addition, I showed an inhibitory activity against the phytopathogenic oomycete Phytophthora infestans, with an IC₅₀ value of 15.2 µM (positive control: cycloheximid, IC₅₀ 0.36 µM) in a growth-inhibitory assay.

Furthermore, mayamycin (I) showed potent in vitro cytotoxicity against eight human cancer cell lines (Table 3). The determined IC₅₀ values were within a concentration range of 0.13 to 0.33 µM. Mayamycin (I) was also cytotoxic toward the mouse fibroblast cell line NIH-3T3. The IC₅₀ value of I was 0.22 µM. Tamoxifen was used as positive control and showed an IC₅₀ value of 23.7 µM.

### Experimental Section

**General Experimental Procedures.** The optical rotation was measured on a Perkin-Elmer model 241. The UV spectra were obtained on a NanoVue (GE Healthcare). NMR spectra were recorded on a
Isolation and identification of strain HB202 was described by Mitova et al. 

System coupled to an ESI-ion trap detector (Esquire 4000, Bruker).

HCOOH added to both solvents (gradient: 0 min 5% B, 4 min 60% B, 100 min 100% B respectively), using the signals of the residual solvent protons and the δH 3.31 and 3.00 mm applying an H2O (A)/MeCN (B) gradient with 0.1% solvent the extract was redissolved in 200 µl of MeOH. An aliquot of 15 µl was analyzed by HPLC/UV-MS.

Additional cultivation approaches were performed, but no production of I was observed under any of the following conditions: Erlenmeyer flasks in GYM, MB (37.4 g of Marine Broth, Difco), and TSB10 medium (3 g of BD tryptic soy broth (TSB), 10 g of NaCl, 1 L of water, pH 7.2) in volumes of 1000, 300, and 25 mL, respectively, under shaking conditions (120 rpm).

**Fermentation.** For chemical analyses strain Streptomyces sp. HB202 was grown in GYM medium for 7 days at 28 °C in multiple 4 L penicillin flasks. Each flask was inoculated with a 3 x 5 cm piece from a GYM agar plate with a 4-week-old culture.

**Purification of Mayamycin (I).** The culture broth (5 L) was homogenized by Ultra Turrax T25 basic (IKA-Werke GmbH and Co., Staufen, Germany) at 16 000 rpm for 30 s and extracted with 3.5 L of EtOAc. Evaporation of the solvent yielded 400 mg of extract. An aliquot was analyzed by HPLC-MS. The extract was subjected to preparative HPLC (Phenomenex Gemini-NX C18 110A, 110 Å, 30 × 2.00 mm) applying an H2O (A), MeCN (B); gradient 0 min 10% B, 17 min 60% B, 22 min 100% B; flow 100 mL/min; UV detection at 236 nm. Mayamycin (166.5 mg) eluted at 8.4—9.0 min.

**Antimicrobial Activities.** Antimicrobial assays were performed using Bacillus subtilis (DSM 347), Brevisbacterium epidermidis (DSM 20660), Dermbacter hominis (DSM 7083), Escherichia coli K12 (DSM 498), Pseudomonas aeruginosa (DSM 1987), Pseudomonas syringae pv aptata (DSM 50252), Ralstonia solanacearum (DSM 9544), Staphylococcus epidermidis (DSM 20044), Staphylococcus lentus (DSM 6672), Xanthomonas campestris (DSM 2405), and the yeast Candida glabrata (DSM 6425) and Septoria tritici. The assays were prepared by transferring 10 µL of a 2 mM methanolic solution of the sample compounds into one well of a 96-well microtiter plate and evaporating the solvent in a vacuum centrifuge. Overnight cultures of the test organisms in tryptic soy broth were diluted to an OD600 of 0.02—0.05, and 200 µL of the resulting suspension was added to the wells. After incubating the microtiter plates for 14—16 h at 28 °C, in the case of the Dermbacter hominis for 24 h at 37 °C, 10 µL of a resazurin solution (0.2 mg mL⁻¹ PBS) was added to each well and the plate incubated at 28 °C for 1—2 h. For evaluation of the cell viability the reduction of resazurin to resorufin was assessed by measuring the intensity of fluorescence at 560Ex/590Em nm. The resulting values were compared to a positive (50 mg of chloramphenicol or tetracycline for bacteria; 50 mg of cycloheximide for Candida glabrata, Septoria tritici, and Phytophthora infestans) and a negative control (no compound) on the same plate. Propionibacterium acnes was grown anaerobically (Anaerocult A mini, Merck) in PYG medium (modified DSMZ medium 104) at 37 °C for 48 h. The bacterial culture was diluted to an OD600 of 0.03, 200 µL of the inoculum was added to each well, and the microtiter plate was incubated anaerobically at 37 °C for 48 h. The assays against Phytophthora infestans were prepared by transferring 190 µL of a spore suspension with a concentration of 1 × 10⁶ spores/mL into one well of a 96-well microtiter plate, respectively. From a 10 mM DMSO stock solution of mayamycin, a dilution series with PBS buffer was established down to 100 µM. Then 10 µL of this solution was added into the assay. After incubating the microtiter plate for 48 h at 20 °C in the dark, the absorption was measured at 600 nm. As positive control, cycloheximide was used.

The antibacterial assays against the clinically relevant strains Staphylococcus epidermidis (clinical isolate, Dr. S. Schubert), Staphylococcus aureus (ATCC 12600), Staphylococcus aureus (ATCC 33593), Klebsiella pneumoniae (ATCC 700603), and Pseudomonas aeruginosa (ATCC 10145) were performed as described by Sahly et al., 2003.
Cytotoxic Activities. The sensitivity of the cell lines NIH-3T3, HepG2, and HT-29 to the isolated compound (1) was evaluated by monitoring of the metabolic activity using the CellTiter-BlueCell viability assay (Promega). The mouse fibroblast cell line was kindly provided by G. Rimbach (University of Kiel, Germany). The human hepatocellular carcinoma cell line HepG2 and the human colon adenocarcinoma cell line HT-29 were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). HepG2 and NIH-3T3 cells were maintained in RPMI 1640 medium, and HT-29 cells were cultured in Mccoy’s 5A medium. Media were supplemented with 10% fetal bovine serum (Promocell), 100 U/mL penicillin, and 100 µg/mL streptomycin (Invitrogen). The cultures were maintained at 37 °C under a humidified atmosphere and 5% CO₂. The cell lines were transferred twice per week. For experimental procedures, cells were seeded in 96-well plates at concentrations of 7500 cells (NIH-3T3) and 10 000 cells (HepG2 and HT-29) per well. After 24 h incubation the medium was removed, and 100 µL of the test sample adjusted to final concentrations ranging from 10 µM to 40 nM by dilution in growth medium was added to the cells. Each concentration was tested in triplicate. Tamoxifen, as a standard reference compound, was added to the cells. The sensitivity of the cell lines NIH-3T3, HepG2, and HT-29 to the isolated compound (1) was determined by a modified propidium iodide charge via the Internet at http://pubs.acs.org.

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Supporting Information Available: Spectroscopic data and additional information such as NMR spectra (1H, 13C, NOESY, and HMBC) of (2′R*,4′R*,5′S*,6′R*)-mayamycin (1) are available free of charge via the Internet at http://pubs.acs.org.

References and Notes


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