Antineoplastic Agents. 554. The Manitoba Bacterium *Streptomyces* sp.[†,1]

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A *Streptomyces* sp. isolated from riverbank soil in Manitoba, Canada, was found to contain two cancer cell growth inhibitories: diazaanthraquinone **1** and 3-(hydroxyacetyl)indole (**8**). The structures were determined by interpretation of data from HRMS, UV, and high-field (400 MHz) NMR experiments. The red-colored diazaanthraquinone **1** and 3-(hydroxyacetyl)indole (**8**) were found to inhibit (0.1−3 μg/mL) growth of a minipanel of human cancer cell lines and P388 lymphocytic leukemia cells. Diazaanthraquinone **1** was also found to inhibit growth of the bacteria *Streptococcus pneumoniae* and *Neisseria gonorrheae*. However, three companion constituents, cyclo-Pro-Leu (**5**), cyclo-Pro-Phe (**6**), and cyclo-Pro-Val (**7**), did not inhibit cancer cell growth.

The actinomycete family continues to be a rich reservoir of microorganisms that produce a diverse array of biologically active metabolites effective against a broad spectrum of medical problems. Recent examples include the anticancer pladienolides,[2] gutingimycin,[3] new members of the mithramycin series,[4] and coprolactones,[5] as well as inhibitors of cell division in starfish embryos,[6] augmented by the antifungal tokanavaenes[7] and yatakemycin.[8] Earlier in our exploration of Northern microorganisms for anticancer components, we isolated and elucidated the structure of a cyclooctadepsipeptide (montanastatin) from a Montana collection of *Streptomyces anulatus*.[9] We now report the isolation of a *Streptomyces* sp. from a mud sample collected along the Ochre River in Manitoba, Canada, that contains diazaanthraquinone **1** and 3-(hydroxyacetyl)indole (**8**) as cancer cell growth inhibitors.

The total ethyl acetate extract from 372 L of fermentation broth was partitioned between hexane and 9:1 MeOH−H_2O followed by diluting the aqueous layer to 3:2 MeOH−H_2O and solvent partitioning with CH_2Cl_2. The latter fraction was found to inhibit growth of the murine P388 lymphocytic leukemia cell line (PS system where an ED_{50} of <10 μg/mL is considered significant activity), and a bioassay was employed to guide further separations by gel permeation and partition chromatographic steps on Sephadex LH-20 followed by RP HPLC to yield diazaanthraquinone **1** (2.7 mg) as a reddish-orange powder.

The molecular formula of **1**, $C_{16}H_{14}N_2O_4$, was deduced from its HRAPCI mass spectrum. The ^{1}H NMR spectrum showed three signals: one methyl connected to an olefinic carbon (δ 2.50), another methyl corresponding to an N-CH_3 group (δ 3.68), and one olefinic proton (δ 6.60). The ^{13}C NMR spectrum of **1** revealed the presence of only eight carbons, suggesting a symmetrical molecule. Detailed assignments for each carbon and proton were accomplished by analyses of 2-D NMR spectral data (Figure 1).

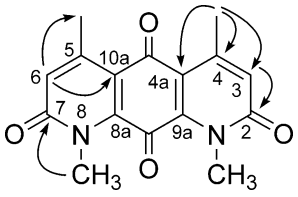
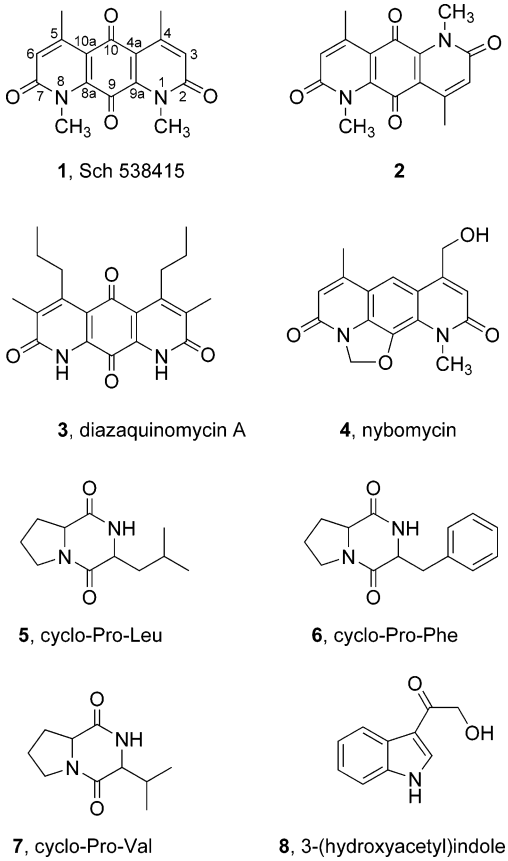


Figure 1. HMBC correlations for diazaanthraquinone **1**.

The protons of the methyl groups at δ 2.50 (4-Me, 5-Me) correlated with the quaternary carbons at δ 148.9 (C-4, C-5) and 116.8 (C-4a, C-10a) as well as the olefinic carbon at δ 126.4 (C-3, C-6). The protons of the N-CH_3 groups were found to correlate with the carbons at δ 161.3 (C-2, C-7) and 142.8 (C-8a, C-9a). Originally, the ^{13}C NMR spectrum was run under standard conditions in $CDCl_3$ and again in CD_2Cl_2 (delay is zero, PW = 1.7, and 31 424 scans), and the signal at δ 181.3 (C-10) characteristic of structure **1** (Sch 538415)[10] did not appear. That suggested an *i* (inversion) symmetry instead of the 2-fold C_2 symmetry along the *y*-axis and assignment as structure **2**. Fortunately, an expert referee suggested we reinvestigate the ^{13}C NMR spectral studies. By this time, we had available a Shigemi tube (3 mm) and then found it took 114 560 scans to reveal the ^{13}C-signal at δ 181.3. With the delay set as 2.5, PW = 1.3, surprisingly, the signal at δ 181.3 (C-10) appeared, corresponding to Sch 538415 **1**, and the new isomeric structure **2** was eliminated. The detailed interpretation of the NMR data arising from **1**[10] and prior structure determinations of the antitumor antibiotic diazaquinomycin A[10−12] (**3**) and nybomycin (**4**)[13] proved to be very helpful in confirming the structure of **1**.



† Dedicated to Professor Norman R. Farnsworth, a world-class contributor to the chemistry and pharmacology of natural products, on the occasion of his 75th birthday.

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Table 1. Murine P388 Lymphocytic Leukemia Cell Line and Human Cancer Cell Line Inhibition Values (GI_{50} in μg/mL) for Diazaanthraquinone **1** and 3-(Hydroxyacetyl)indole (**8**)[a]

	cancer cell line[b]						
	P388	BXPC-3	MCF-7	SF-268	NCI-H460	KM20L2	DU-145
1	1.4	0.54	0.49	1.3	1.2	0.32	1.0
8	2.8	0.31	0.82	1.8	0.17	1.2	0.21

[a] In DMSO. [b] Cancer type: P388 (lymphocytic leukemia); BXPC-3 (pancreas adenocarcinoma); MCF-7 (breast adenocarcinoma); SF 268 (CNS glioblastoma); NCI-H460 (lung large cell); KM 20L2 (colon adenocarcinoma); DU-145 (prostate carcinoma).

Further separation of the fraction constituents led to three cancer cell line inactive diketopiperazines, namely, cyclo-Pro-Leu (**5**),[14−16] cyclo-Pro-Phe (**6**),[14−16] and cyclo-Pro-Val (**7**),[10,14−16] which were previously isolated from other microorganisms as well as certain marine sponges and showed strong antibiotic activity against *Vibrio anguillarum*.[17] Interestingly, the sponge diketopiperazines were found to all possess D,D-chirality.[17] Since the three diketopiperazines we isolated proved uninhibitory in our cancer cell line evaluations, we did not determine their chirality. Separation was continued to yield the PS cell-line-active 3-(hydroxyacetyl)indole (**8**).[18] The diazaanthraquinone **1** and 3-(hydroxyacetyl)indole (**8**) exhibited significant growth inhibitory activity when evaluated against the murine P388 lymphocytic leukemia cell line and a minipanel of six human tumor cell lines (Table 1). This is the first report of the antineoplastic activities of **1**.

In broth microdilution susceptibility assays, **1** inhibited growth of *Streptococcus pneumoniae* ATCC 6303 (minimum inhibitory concentration [MIC] = 16−32 μg/mL) and *Neisseria gonorrheae* ATCC 49226 (MIC = 4−16 μg/mL). Diazaanthraquinone **1** did not inhibit the growth (MIC > 64 μg/mL) of *Stenotrophomonas maltophilia*, *Micrococcus luteus*, *Staphylococcus aureus*, *Escherichia coli*, *Enterobacter cloacae*, *Enterococcus faecalis*, *Candida albicans*, or *Cryptococcus neoformans* in these assays.

Experimental Section

General Experimental Procedures. Solvents used for chromatographic procedures were redistilled. The Sephadex LH-20 employed for gel permeation and partition chromatography was obtained from Pharmacia Fine Chemicals AB, Uppsala, Sweden. The silica gel GHLF Uniplates for thin-layer chromatography were supplied by Analtech, Inc. The TLC results were viewed under UV light and developed with $Ce(SO_4)_2-H_2SO_4$. Reversed-phase HPLC experiments were performed on Synergi C18 (250 × 10 mm, 4 μm) and Synergi fusion-C18 (250 × 4.6 mm, 4 μm) columns with Waters Fusion-RP 80, 00G-4424-N0 HPLC, or Hewlett-Packard 1100 Series instruments monitored with UV and/or ELSD detectors. Melting points were determined using a Fisher Scientific 12-144 micromelting point apparatus and are uncorrected. High-resolution mass spectra were obtained with a JEOL LC-Mate equipped with an APCI inlet. NMR experiments were conducted with an INOVA-400 spectrometer with tetramethylsilane as an internal reference. IR spectra were recorded with a Thermo Nicolet Avatar 360 infrared spectrometer.

***Streptomyces* sp.** In August 2001, one of us (G.R.P.) collected a mud sample from the Ochre River in Manitoba, Canada, and we isolated a microorganism from that specimen that was identified by 16S rRNA gene sequencing as a *Streptomyces* sp. The *Streptomyces* sp. was scaled up in half-strength malt extract broth (final concentration: malt extract, 3 g/L; maltose, 0.9 g/L; dextrose, 3 g/L; yeast extract, 0.6 g/L) for 13 days at room temperature with aeration.

Identification of the Producing Microbe. Bacterial colonies isolated on half-strength malt extract agar were peach/pink with a white spore mass. The bacterium was identified by 16S rRNA gene sequence similarity (Accugenix, Newark, DE). Results from the MicroSeq database based on the first 500 base pairs of the 16S gene indicated that the bacterium was in the genus *Streptomyces* (% difference = 1.2; confidence level to genus). The voucher specimen is maintained in the ASU Cancer Research Institute.

Extraction and Isolation Methods. All the separations were guided by bioassay results using the murine P388 lymphocytic leukemia and a minipanel of human cancer cell lines. *Streptomyces* sp. (372 L of fermentation broth) was extracted (3×) with EtOAc, and the extract was concentrated. After partitioning between EtOAc and H_2O, the residual extract was dissolved in 9:1 CH_3OH-H_2O (200 mL) and partitioned (3×) against hexane (200 mL). The CH_3OH-H_2O layer was adjusted to 3:2 CH_3OH-H_2O. This fraction was partitioned (3×) against CH_2Cl_2 (200 mL) to yield the active CH_2Cl_2 fraction (0.9 g) along with the inactive hexane (1.6 g) and CH_3OH-H_2O (0.33 g) fractions. The CH_2Cl_2 fraction (PS ED_{50} 0.02 μg/mL) was chromatographed on Sephadex LH-20 columns. The resulting active fraction was separated on a C18 HPLC column monitored by UV at 215 nm with CH_3CN-H_2O (5:95 to 40:60). Five constituents were isolated, namely, diazaanthraquinone (**1**, 3.5 mg), cyclo-Pro-Leu (**5**, 2.5 mg), cyclo-Pro-Phe (**6**, 5 mg), cyclo-Pro-Val (**7**, 2 mg), and 3-(hydroxyacetyl)indole (**8**, 2.4 mg).

Diazaanthraquinone 1: reddish-orange powder; mp 262−284 °C; UV (CH_3OH) λ 200, 280, 320, 350 nm; IR ν_{max} (log ϵ) 2957, 2922, 2853, 1672, 1663, 1462, 1360 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) δ 6.60 (2H, s, H-3 and H-6), 3.68 (6H, s, 1-CH_3 and 8-CH_3), 2.50 (6H, s, 4-CH_3 and 5-CH_3); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 181.3 (C-10), 178.7 (C-9), 161.3 (C-2 and C-7), 148.9 (C-4 and C-5), 142.8 (C-9a and C-8a), 126.4 (C-3 and C-6), 116.8 (C-4a and C-10a), 34.0 (N−CH_3), 22.5 (4-CH_3 and 5-CH_3); ^1H NMR (CD_2Cl_2, 500 MHz) δ 6.58 (2H, s, H-3 and H-6), 3.65 (6H, s, 1-CH_3 and 8-CH_3), 2.51 (6H, s, 4-CH_3 and 5-CH_3); ^{13}C NMR (CD_2Cl_2, 125 MHz) δ 181.3 (C-10); 178.7 (C-9), 161.3 (C-2 and C-7), 148.9 (C-4 and C-5), 142.8 (C-9a and C-8a), 126.4 (C-3 and C-6), 116.8 (C-4a and C-10a), 34.0 (N-CH_3), 22.5 (4-CH_3 and 5-CH_3); HRAPCIMS *m/z* 299.1038 $[M + H]^+$ (calcd 299.1032).

Broth Microdilution Susceptibility Testing. The antimicrobial activity of **1** was assessed by the National Committee for Clinical Laboratory Standards (NCCLS) broth microdilution assay.[19,20] Diazaanthraquinone **1** was reconstituted in a small volume of sterile DMSO and diluted in the appropriate media immediately prior to susceptibility experiments. The minimum inhibitory concentration was defined as the lowest drug concentration that inhibited all visible growth of the test organism (optically clear).

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