Antineoplastic Agents. 488. Isolation and Structure of Yukonin from a Yukon Territory Fungus

George R. Pettit,* Rui Tan, Delbert L. Herald, John Hamblin,† and Robin K. Pettit

Cancer Research Institute and Department of Chemistry and Biochemistry, Arizona State University, P.O. Box 872404, Tempe, Arizona 85287-2404

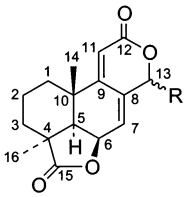
Received April 3, 2002

Cancer cell line bioassay-guided separation of an extract from a Yukon Territory fungus resulted in the isolation of a new C_{16}-terpene dilactone designated yukonin (**1**) accompanied by two previously known, structurally related constituents (**2** and **3**). The structure of each was determined by X-ray crystallographic analysis. Dilactone **2** was found to correspond to LL-Z1271α isolated from fungi in the genera *Oidiodendron*, *Acrostalagmus*, and *Holwaya*, while dilactone **3** had earlier been prepared by reduction of an α-lactol derivative. Each of the dilactones was found to inhibit growth of human cancer cell lines (pancreas, breast, CNS, lung, colon, and prostate) and some pathogenic fungi.

During an exploration in 1995 in the Yukon Territory, Canada, for new sources of microorganism anticancer constituents, a soil specimen near the Mayo River was collected and a fungus subsequently isolated that yielded extracts containing terpene lactones with activity against human cancer cell lines and pathogenic fungi. Previously, terpene lactones have been isolated from the fungal genera *Oidiodendron*,[1,2] *Acrostalagmus*,[3] and *Holwaya*.[4] The reported biological activities of such derived terpene lactones include herbicidal[5] and fungicidal.[2,3] On the basis of macroscopic and microscopic evaluation, the Yukon territory fungus was placed in the genus *Phialophora*, but it did not group well within any genus found in the MicroSeq database (Table 1). The isolate was 4% different than the closest genus, *Ovadendron*, and there was a 17% difference from *Phialophora americana*. The GenBank database, which contains many *Phialophora* species, was searched but provided no match. The Yukon fungus is therefore either a new species or a species whose LSU rRNA gene sequence is not present in either database.

The isolated soil fungus was cultured and fermented on a large scale at room temperature with shaking. The CH_2Cl_2-soluble extract of the fermentation broth was then partitioned between hexane and CH_3OH-H_2O (9:1). After dilution with water to CH_3OH-H_2O (3:2), the aqueous phase was extracted with CH_2Cl_2. Bioassay results showed that the CH_2Cl_2 fraction displayed the highest activity with a GI_{50} level of 10^{-1} μg/mL against human cancer cell lines. The active CH_2Cl_2 fraction proved to be only partially soluble in CH_3OH, but the readily soluble portion showed increased activity against the human cancer cell lines. Reversed-phase HPLC separation of this active fraction employing elution with a $CH_3CN-CH_3OH-H_2O$ system followed by further separation by HPLC yielded a new dilactone named yukonin (**1**, 8 mg), the previously known LL-Z1271α[6] (**2**, 23 mg), and a derivative[1,3] (**3**, 9 mg).

The EIMS and NMR spectra of dilactones **1**−**3** suggested a very close structural relationship that was accurately elucidated by X-ray crystal structure determination. After successful crystallization from CH_3OH solutions, the X-ray analyses led to structures for yukonin (**1**, Figure 1), LL-Z1271α (**2**), and **3** as C_{16} terpenoid dilactones. Although yukonin (**1**, 13β-OH) may have occurred in an intermediate mixture during the semisynthesis of LL-Z1271γ (13α-OH),[4,6,7] to our knowledge there is no report of its isolation from a natural source.



1 R = β-OH (Yukonin)
2 R = α-OCH_3 (LL-Z1271α)
3 R = H

Evaluation of dilactones **1**−**3** against the murine P388 lymphocytic leukemia cell line and a minipanel of human cancer cell lines gave interesting preliminary SAR results (Table 2). The P388 cell line was less sensitive than the human lines to lactones **1** and **2**. Also the lactol methyl ether (**2**) and dilactone **3** were about 10-fold more inhibitory to the human cancer cell lines than yukonin (**1**). To our knowledge, this is the first report of cancer cell line inhibitory activity for these compounds.

In broth microdilution assays, all three compounds had activity against leading agents of human fungal infection. Compound **2** was the most potent, with minimum inhibitory concentrations (MICs) of 2 μg/mL for *Cryptococcus neoformans* and 8 μg/mL for *Candida albicans*. Compound **3** had MICs of 16 μg/mL for *C. neoformans* and 64 μg/mL for *C. albicans*. Compound **1** had marginal antifungal activity with a MIC of 64 μg/mL for *C. neoformans* and a MIC of >64 μg/mL for *C. albicans*.

Experimental Section

General Experimental Procedures. Solvents used in the extraction and isolation processes were freshly redistilled or filtered for HPLC prior to use. Thin-layer chromatography employed Analtech silica gel GHLF Uniplates, which were visualized under long- and short-wave UV and developed using an acidic ceric sulfate solution (with heating). Melting points were measured using an uncorrected Kofler melting point apparatus. Optical rotation values were recorded using a Perkin-Elmer 241 polarimeter. NMR spectra were recorded

* To whom correspondence should be addressed. Tel: (480) 965-3351. Fax: (480) 965-8558. E-mail: bpettit@asu.edu.
† 13 Terners Dr., Apt. 33, Sausalito, CA 94965.

10.1021/np020144m CCC: $25.00
Published on Web 12/06/2002

Table 1. LSU rRNA Gene Sequence Alignment with MicroSeq Database

percent difference	
4.04	*Ovadendron sulphureoochraceum*
5.59	*Geomyces asperulatus*
7.14	*Scytalidium lignicola*
9.94	*Oosporidium margaritiferum*
12.89	*Chaetomium atrobrunneum*
13.17	*Myceliophthora thermophila*
13.79	*Chaetomium aureum*
14.11	*Achaetomium strumarium*
14.42	*Ophiostoma piceae*
17.39	*Phialophora americana*

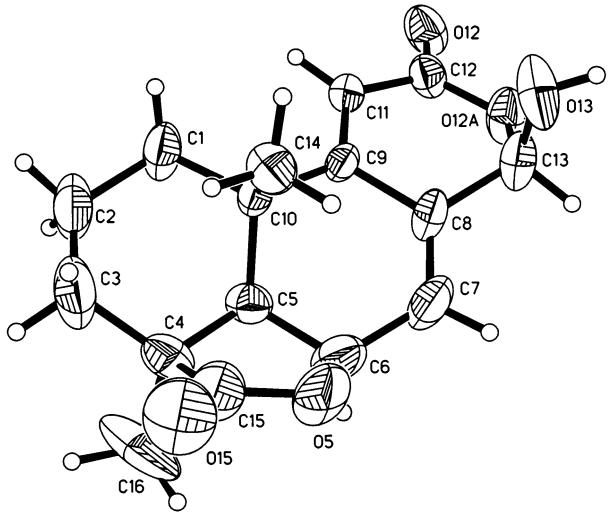


Figure 1. X-ray molecular structure and numbering scheme of yukonin **1**. Thermal ellipsoids are drawn at 50% probability.

Table 2. Inhibition of Cancer Cell Line Growth (GI_{50} μg/mL) by Dilactones **1**−**3**

cancer cell line	yukonin (**1**)	LL-Z1271α (**2**)	dilactone **3**
murine P388 lymphocytic leukemia	>10	4.1	0.31
pancreas-a BXPC-3	5.6	0.36	1.8
breast MCF-7	4.0	0.33	0.69
CNS gliobl., SF268	2.9	0.24	0.57
lung NSC NCI-H460	4.1	0.24	0.48
colon KM20L	3.6	0.21	0.45
prostate DU-145	2.9	0.14	0.34

with a Varian Unity Inova 500 instrument. EIMS were determined on a MAT-312 mass spectrometer.

Collection and Fermentation. Soil samples were collected (September 1995) in clean plastic bags near Mayo on the banks of the Mayo River in the Yukon Territory, Canada, and shipped to our laboratory. Soils were aseptically diluted and spread on half-strength potato dextrose agar (Difco). Isolated colonies were subcultured and fermented in potato dextrose broth, and the extracts were screened against the murine P388 lymphocytic leukemia cell line and a mini-panel of human cancer cell lines.[8] Prior to large-scale fermentation, the human cancer cell line activity of the fungus was determined to be optimum in half-strength potato dextrose broth for 1 week. All activity peak experiments and large-scale fermentations were performed at room temperature with shaking.

Fungal Identification. On potato dextrose agar, the fungal isolate was wooly and dark olive with a dark brown reverse. Microscopically, septate hyphae that bore vase-shaped phialides were observed. Phialides that typically displayed collarettes were usually found solitary at approximately 90° to the hyphae. Conidia were solitary or were attached to the phialades in sticky circular masses of ≥8 conidia. However, solitary conidia may have been an artifact of sample preparation. As colonies aged, an arrangement of several phialades emanating from one location on the hyphae was also observed. All conidia were unicellular, and no other reproductive structures were present.

The isolate was sent to the Fungal Testing Laboratory (UTHSC, San Antonio), where the placement in *Phialophora* was confirmed microscopically. More recently, an attempt was made to identify the fungus by large subunit (LSU) rRNA gene sequencing (Accugenix, Newark, DE). The top 10 alignment matches are presented in descending order of similarity percentage (Table 1). A low percentage indicates a close match. A rRNA sequence homology of greater than 99% is indicative of a species level match. For sequence identification, genomic DNA was extracted, and approximately 300 bp of the large subunit (LSU) rRNA gene starting at position 3334 was PCR amplified and sequenced. Specific sequences were identified with PE Applied Biosystems MicroSeq analysis software and database. Voucher specimens (ASU-B849440) are available from RKP.

Extraction and Isolation. The culture broth was extracted with CH_2Cl_2. The CH_2Cl_2 solution was concentrated to dryness and partitioned using a modification of the procedure first developed by Bligh and Dyer[9] as follows. The residue was partitioned between CH_3OH-H_2O (9:1) and hexane. After separation the CH_2OH-H_2O was diluted with H_2O to 3:2. The resulting aqueous solution was extracted with CH_2Cl_2. The cancer cell line active CH_2Cl_2 fraction (2.24 g) was triturated with CH_3OH to yield soluble and sparingly soluble phases. Repeated reversed-phase HPLC separation of the CH_3OH-soluble fraction using a C_{18} column with acetonitrile−methanol−water (3:3:4) as mobile phase afforded yukonin (**1**, 8 mg, t_R 3.6 min), dilactone **3** (9 mg, t_R 4.5 min), and LL-Z1271α (**2**, 23 mg, 5.4 min).

Yukonin (1): colorless needles (CH_3OH); mp 224−227 °C; $[\alpha]^{23}_D$ −146.4° (*c* 0.53, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 500 MHz) δ 6.50 (1H, s, br, H-7), 6.13 (1H, s, H-13), 5.81 (1H, s, H-11), 5.05 (1H, t, J = 4.5 Hz, H-6), 2.28 (1H, m, H-3a), 1.95 (1H, d, J = 5 Hz, H-5), 1.87 (1H, m, H-2a), 1.73 (1H, m, H-1a), 1.66 (1H, m, H-2b), 1.63 (1H, m, H-1b), 1.55 (1H, m, H-3b), 1.35 (3H, s, H-16), 1.20 (3H, s, H-14); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 180.8 (CO-15), 163.2 (CO-12), 157.1 (C-9), 134.0 (C-8), 124.7 (C-7), 111.7 (C-11), 95.9 (C-13), 71.2 (C-6), 48.0 (C-5), 42.7 (C-4), 35.0 (C-10), 29.7 (C-1), 27.7 (C-3), 24.3 (C-14), 24.2 (C-16), 17.4 (C-2); EIMS *m*/*z* 290 [M^+] (35), 272 (10), 262 (30), 244 (100), 185 (50), 145 (60), 115 (54).

X-ray Crystal Structure Determinations. All X-ray data collections were performed at ambient temperature (298 K) on a Bruker AXS SMART 6000 diffractometer with graphite-monochromated Cu Kα radiation (λ = 1.54178 Å) from a normal focus sealed tube. The crystal−detector distance was 4.52 cm. Frames of data, 512 pixels in width, were collected in 0.40° steps in either ω or ϕ, using the MULTIRUN data collection procedure of the SMART[10] software. The data were processed using the SAINT+[11] software employing the narrow frame algorithm, and the integrated data were corrected for absorption using SADABS.[12] All structures were solved and refined using SHELXTL NT[13] software.

Yukonin (1; 7-Hydroxy-3a,10b-dimethyl-1,2,3,3a,5a,7,-10b,10c-octahydro-5,8-dioxa-acephenanthrylene-4,9-dione). An irregular, block-shaped X-ray sample (∼0.16 × 0.16 × 0.16 mm), obtained by cleavage from a crystalline cluster grown from a methanol solution, was mounted on the tip of a glass fiber. An initial set of cell constants was calculated from reflections harvested from three sets of 60 frames. The cell parameters indicated an orthorhombic space group. Subsequent data collection, using 30 s scans/frame, resulted in a total of 10 433 reflections being harvested from the total data collection. Final cell constants were calculated from a set of 1120 strong, unique reflections from these data. Subsequent statistical analysis of the complete reflection data set using the XPREP[13] program indicated the space group was $P2_12_12_1$. Crystal Data: $C_{16}H_{18}O_5$, orthorhombic, $P2_12_12_1$, a = 7.8066(8) Å, b = 9.6686(10) Å, c = 18.8138(16) Å, V = 1420.0(2) Å^3, λ = (Cu Kα) = 1.54178 Å, μ(Cu Kα) = 0.837 mm^{-1}, ρ_c = 1.358 g cm^{-3} for Z = 4 and M_r = 290.30, F(000) = 616.

After data reduction, merging of equivalent reflections, and rejection of systematic absences, 2556 unique, independent reflections remained (R_{int} = 0.21), which were used in the subsequent structure solution and refinement. An absorption correction was applied to the data with SADABS;[12] the ratio of minimum to maximum apparent transmission = 0.602. All non-hydrogen atoms for dilactone **1** were located using the default settings of that program. Hydrogen atom coordinates were calculated at optimum positions and forced to ride the atom to which they were attached. Full-matrix least-squares anisotropic refinement of the model shown in Figure 1 resulted in a final standard residual value of 0.0670 for observed data (1354 reflections) and 0.1183 for all data (2556 reflections). The difference Fourier map showed insignificant residual electron density, the largest difference peak and hole being +0.235 and −0.216 e/Å^3, respectively. Final bond distances and angles were all within acceptable limits.

Dilactone 2 (LL-Z1271α; 7-Methoxy-3a,10b-dimethyl-1,2,3,3a,5a,7,10b,10c-octahydro-5,8-dioxa-acephenanthrylene-4,9-dione). Cell constants were calculated from reflections harvested from three sets of 90 frames at 298(2) K on a sample (~0.66 × 0.40 × 0.10 mm). Subsequent data collection was performed with 5 s scans/frame. Crystal Data: $C_{17}H_{20}O_5$·2CH_3OH, monoclinic, space group *C*2, a = 18.2230(12) Å, b = 7.7207(5) Å, c = 12.0442(7) Å, β = 101.967(3)°, V = 1657.72(18) Å^3, λ = (Cu Kα) = 1.54178 Å, μ(Cu Kα) = 0.787 mm^{-1}, ρ_c = 1.284 g cm^{-3} for Z = 4 and M_r = 320.35, F(000) = 684, reflections collected 6420, independent reflections 2496 (R_{int} = 0.09) Å. SADABS[12] absorption correction was made with T_{min}/T_{max} = 0.564. A standard decay, 18.9%, was also performed. Refinement method full-matrix least squares on F^2, data/restraints/parameters 2496/1/208, H atom treatment applied in a manner analogous to compound **1** above, goodness-of-fit on F^2 0.991, final R indices ($I > 2\sigma(I)$ (1753 reflections), R_1 = 0.0878, wR_2 = 0.2174, R indices (all data) (2496 reflections) R_1 = 0.1080, wR_2 = 0.2341. An extinction correction was applied as final diffuse solvent parameters (SWAT). Final residual electron density as largest peak and hole, +0.619 and −0.416. The model used for the dilactone **2** is shown in Figure 2 and found in the Supporting Information. The compound was found to be identical to the previously reported dilactone LL-Z1271α.[6] In addition to a molecule of the parent compound, a half molecule of disordered methanol solvent was found to be present in each asymmetric unit, the carbon atom of which was positioned at a special site of 0.5, 0.712, 0.5. The oxygen atom of methanol was disordered over two general sites related by a crystallographic 2-fold axis of symmetry down the *y* axis. Final bond distances and angles were all within acceptable limits.

Dilactone 3 (3a,10b-Dimethyl-1,2,3,3a,5a,7,10b,10c-octahydro-5,8-dioxa-acephenanthrylene-4,9-dione). Data were collected on an X-ray sample (~0.50 × 0.28 × 0.08 mm), obtained by cleavage from a much larger crystal which had been grown from methanol solution. An initial set of cell constants was calculated from reflections harvested from three sets of 90 frames at 298(2) K. The cell parameters indicated a monoclinic space group. Subsequent data collection was performed using 5 s scans/frame. Crystal Data: $C_{16}H_{18}O_4$, monoclinic, *C*2, a = 18.3413(13) Å, b = 7.7494(6) Å, c = 12.7538(8) Å, β = 130.277(3)°, V = 1379.62(13) Å^3, λ = (Cu Kα) = 1.54178 Å, μ(Cu Kα) = 0.771 mm^{-1}, ρ_c = 1.317 g cm^{-3} for Z = 4 and M_r = 274.30, F(000) = 584, reflections collected 5315, independent reflections 2263 (R_{int} = 0.0962), SADABS[12] absorption correction ratio of maximum and minimum effective transmission: 1.000 and 0.6404; refinement method full-matrix least squares on F^2, data/restraints/parameters 2263/1/182, H atom treatment applied in a manner analogous to compound **1** above, goodness-of-fit on F^2 0.908, final R indices ($I > 2\sigma(I)$ (1451 reflections), R_1 = 0.0596, wR_2 = 0.1346, R indices (all data) = 2263 reflections, R_1 = 0.0830, wR_2 = 0.1458, extinction coefficient 0.0016(4), largest peak and hole +0.231 and −0.227 e/Å^3. The model used for dilactone **3** is shown in Figure 3 (included in the Supporting Information). This compound was found identical to a previously reported dilactone prepared synthetically.[1,3]

Additional physical data for all three dilactones, including both ^{1}H and ^{13}C spectra, atomic coordinates, bond lengths and angles, and thermal displacement parameters, may be found in the Supporting Information.

Antimicrobial Susceptibility Testing. Compounds were screened against the bacteria *Stenotrophomonas maltophilia* ATCC 13637, *Micrococcus luteus* Presque Isle 456, *Staphylococcus aureus* ATCC 29213, *Escherichia coli* ATCC 25922, *Enterobacter cloacae* ATCC 13047, *Enterococcus faecalis* ATCC 29212, *Streptococcus pneumoniae* ATCC 6303, and *Neisseria gonorrhoeae* ATCC 49226 and the fungi *Candida albicans* ATCC 90028 and *Cryptococcus neoformans* ATCC 90112, according to established broth microdilution susceptibility assays.[14,15] The minimum inhibitory concentration was defined as the lowest concentration of compound that inhibited all visible growth of the test organism (optically clear). Assays were repeated on separate days.

Acknowledgment. We are pleased to acknowledge the very important financial support provided by Outstanding Investigator Grant CA 44344-05-12 and Grant R01 CA 90441-01 with the Division of Cancer Treatment and Diagnosis, NCI, DHHS; the Arizona Disease Control Research Commission; the Fannie E. Rippel Foundation; the Robert B. Dalton Endowment Fund; Gary L. and Diane R. Tooker; Dr. John C. Budzinski, Polly Trautman, and Sally Schloegel. We also thank the Government of the Yukon Territory (especially, Mr. Jeff Hunston, Director, Heritage Branch); Professors Cherry L. Herald, Fiona Hogan, and Jean M. Schmidt; and Dr. J-C. Chapuis, Eric Herbig, and Linda Richert for other helpful assistance.

Supporting Information Available: Crystallographic data, containing fractional coordinates, isotropic and anisotropic displacement parameters, and bond lengths and angles are available for compounds **1**−**3**. This material may be obtained free of charge via the Internet at htpp://pubs.acs.org.

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NP020144M