Isolation and Structure of Ruprechstyril from *Ruprechtia tangarana*[†,1]

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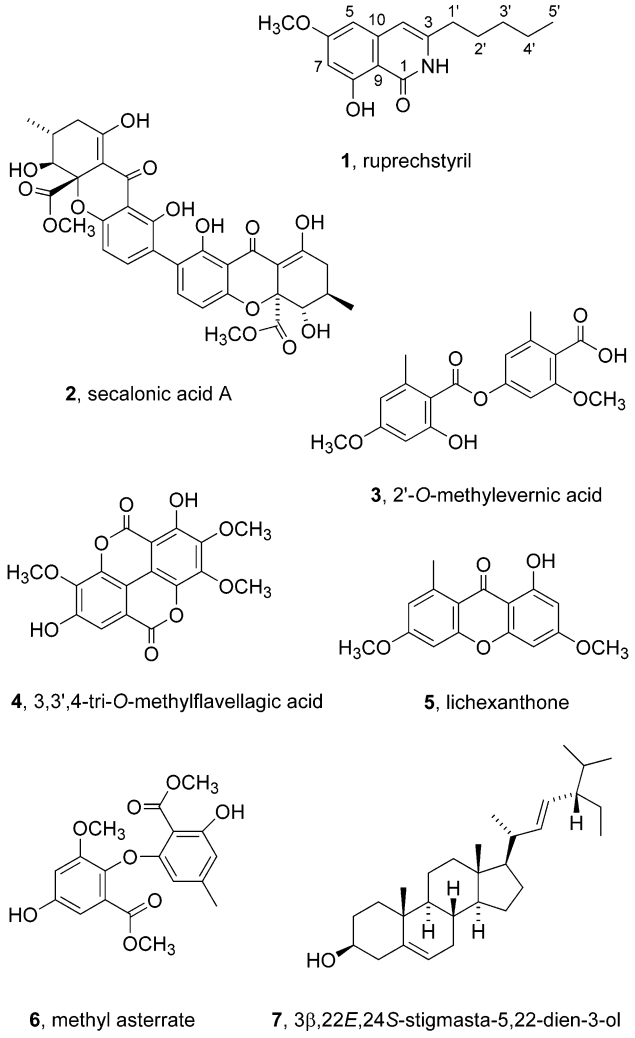
Bioassay (P388 lymphocytic leukemia cell line and human cancer cell lines)-guided separation of an extract prepared from the stem bark and twigs of the previously uninvestigated *Ruprechtia tangarana* led to the isolation of a new isocarbostyril designated ruprechstyril (**1**), secalonic acid A (**2**), 2′-*O*-methylevernic acid (**3**), 3,3′,4-tri-*O*-methylflavellagic acid (**4**), lichexanthone (**5**), methyl asterrate (**6**), and 3*β*,22*E*,24*S*-stigmasta-5,22-dien-3-ol (**7**). Only secalonic acid A exhibited cancer cell and microbial growth inhibition. The structure of ruprechstyril (**1**) was determined by HRMS and 1D and 2D NMR spectra and confirmed by single-crystal X-ray analysis. The structures and absolute stereochemistry of five of the other compounds were also established by X-ray crystal structure determination.

The plant family Polygonaceae comprises some 800 species (primarily herbs, a small number of shrubs, and a few trees) divided among 40 genera. A few of these rhubarb family genera such as *Rheum*,[2a,b] *Rumex*,[3a−c] and *Polygonum*[4a−f] have received the majority of investigations directed at detecting, and in some cases isolating, biologically active constituents. Primary attention has been devoted to antioxidant,[2a,3a,4e] antifungal,[4c] analgesic/antipyretic,[3c] estrogenic,[4d] protein kinase C inhibitory,[4d] and cancer cell growth inhibitory properties.[3b,4a] These anticancer studies did not lead to the isolation of active substances, but a stilbene glycoside reported in 1971[2b] may have cancer cell growth inhibition properties.[5]

Ruprechtia is one of the smallest and least explored Polygonaceae genera and encompasses some 17 species distributed from Mexico to northern Argentina and Uruguay. Only *R. salicifolia* from Uruguay has led to any prior report of medicinally active constituents and is believed to contain an alkaloid (perhaps accounting for its traditional use as a "stomachal bitter").[6] In 1978−79, extracts of *Ruprechtia tangarana* Standl., collected in Peru as part of the U.S. National Cancer Institute's worldwide evaluation of terrestrial plants as sources of new anticancer agents, gave evidence of some activity against the KB cell line (ED_{50} 2.3−9.2 μg/mL) and P388 lymphocytic leukemia (in vivo, T/C 120−126). October 1979 and May 1980 recollection (153 and 72 kg, respectively) of *R. tangarana* stem bark and twigs gave extracts that led to inconsistent results with our P388 cell line bioassay system. Hence, further research was deferred until recently when our panel of human cancer cell lines gave consistent results during bioassay-guided separation of *R. tangarana* extracts.

Results and Discussion

A MeOH−CH_2Cl_2 (1:1) extract of the stem bark and twigs of *R. tangarana* was partitioned between 1:2 MeOH−H_2O and CH_2Cl_2. The CH_2Cl_2 layer showed a marginal response to the human tumor cell lines (BXPC-3 and DU-145). Human cancer cell line bioassay-guided separation of the CH_2Cl_2 fraction followed by a series of gel permeation and partition separations on Sephadex LH-20 columns and final purification using recrystallization afforded a new 1(2*H*)-isoquinolinone named ruprechstyril (**1**, 3.3 × 10^{-6} %). Also isolated and characterized were secalonic acid A (**2**, 1.0 × 10^{-6} %, P388 ED_{50} 3.5 μg/mL)[7] and five phenols: 2′-*O*-methylevernic acid (**3**, 2.0 × 10^{-6} %),[8,9] 3,3′,4-tri-*O*-methylflavellagic acid (**4**, 4.8 × 10^{-6} %),[10,11] lichexanthone (**5**, 2.7 × 10^{-5} %),[12,13] methyl asterrate (**6**, 4.1 × 10^{-5} %),[14,15] and 3*β*, 22*E*,24*S*-stigmasta-5,22-dien-3-ol (**7**, 2.3 × 10^{-6} %).[16] Compounds **2**−**7** were identical in all respects to those



[†] Dedicated to the memory of Dr. Harry B. Wood (1919−2002), a U.S. National Cancer Institute pioneer in anticancer drug development.

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Table 1. ^{1}H and ^{13}C NMR Assignments for Ruprechstyril (**1**) in $CHCl_3$[a]

position	δ ^{1}H	^{1}H−^{1}H COSY	δ ^{13}C	HMBC[b]
1			166.94	H-5, H-7
2	12.5s (1H)			
3			141.73	H-4, H-1′, H-2′
4	6.25s (1H)		105.43	H-5, H-1′
5	6.37d (1H, 3.0)		98.98	H-4, H-7
6			164.81	OCH_3, H-5, H-7
7	6.42d (1H, 3.0)		99.50	H-5
8			162.81	H-7, H-4(w), H-5(w)
9			105.17	H-4, H-5, H-7, OH
10			141.06	H-4
1′	2.55t (2H, 9.5)	H-2′	33.34	H-4, H-2′
2′	1.72m (2H)	H-1′, H-3′	28.07	H-1′
3′	1.35m (2H)	H-2′, H-4′	31.26	H-2′, H-4′, H-5′
4′	1.38m (2H)	H-3′, H-5′	22.44	H-3′, H-4′, H-5′
5′	0.91t (3H, 8)	H-4′	14.03	H-6′
OH	10.86s (1H)			H-2′
OCH_3	3.86s (3H)		55.55	H-2′, H-6′

[a] Measured at 500 MHz. [b] w = weak.

reported in the literature. All that were evaluated except ester **2** were inactive against our P388 and human cancer cell lines.

Ruprechstyril (**1**) was isolated as colorless needles. The molecular formula was assigned $C_{15}H_{19}NO_3$ on the basis of high-resolution APCI$^+$ mass spectroscopy ($[M + 1]^+$ at *m*/*z* 262) and ^{1}H and ^{13}C NMR spectra (Table 1). Analysis of ^{1}H and ^{13}C NMR (DEPT) and the HMQC (Table 1) spectra provided evidence that isocarbostyril **1** possessed 15 carbon atoms, which included one methyl, four aliphatic methylenes, one methoxy, three olefinic methines, five olefinic quaternary carbons, and one carbonyl carbon. The ^{1}H NMR spectrum showed two coupled aromatic doublets at δ 6.37 (1H, d, 3 Hz) and 6.42 (1H, d, 3 Hz), indicating the presence of a 1,2,3,5-tetrasubstituted benzene ring. Strong absorption shown at 1619 cm^{-1} in the IR spectrum, together with the upfield-shifted carbonyl signal at δ_C 166.9 and the downfield-shifted proton signal at δ_H 12.5 (NH), indicated the presence of an N-substituted benzamide. The ^{1}H−^{1}H COSY spectrum (Table 1) indicated the presence of an *n*-pentyl group, and both HMQC and HMBC (Table 1) spectra confirmed the pentyl group was connected to the quaternary carbon on a trisubstituted vinyl group −CH═C<. It was established that the quaternary carbon was also connected to the NH group in the benzamide fragment by the singlet NH signal (δ_H 12.5). A second ring was deduced from the value of 7 for the degrees of unsaturation. Thus, it was necessary for the methine in the vinyl group >C═CH− to be connected to the adjacent carbon of the amide group on the benzene ring to form a six-membered lactam. Therefore, ruprechstyril (**1**) was considered to be a 1(2*H*)-isoquinolinone. The HMQC and HMBC spectra confirmed that the δ-lactam ring was fused with the benzene ring at C-9 and C-10 through the strong correlations of H-5 and H-7 to C-9, and H-4 to C-10 and C-9. HMQC and HMBC spectra also confirmed that the methoxy group was linked to C-6 by showing the correlations to C-6, as well as H-5 and H-7 to C-6 and C-9. The ^{1}H NMR spectrum showed the presence of one hydroxyl group at δ_H 10.86, which was confirmed as joined to C-8 by the correlations of the OH to C-9 and H-7 to C-8 observed in the HMBC spectrum. Thus, the structure was determined to be 3-*n*-pentyl-6-methoxy-8-hydroxy-1(2*H*)-isoquinolinone, designated ruprechstyril. The structure was subsequently and unequivocally confirmed by X-ray crystal structure determination.

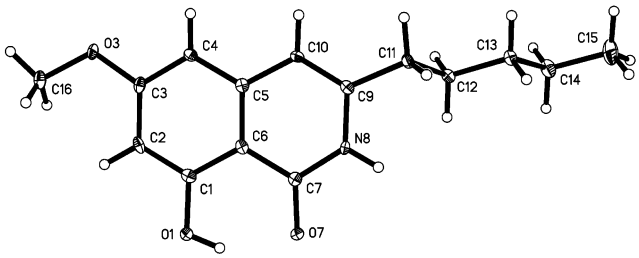

Figure 1. X-ray crystal structure of ruprechstyril (**1**) with 50% thermal probability ellipsoids.

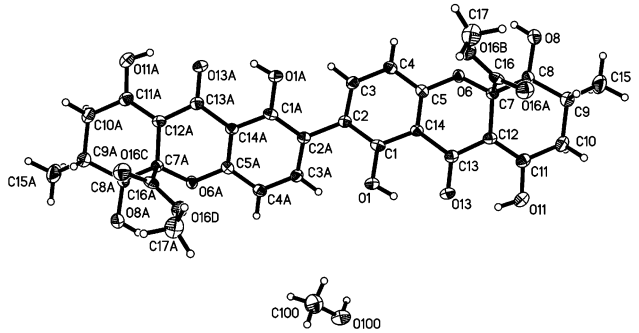

Figure 2. X-ray crystal structure of secalonic acid A (**2**) with 50% thermal probability ellipsoids.

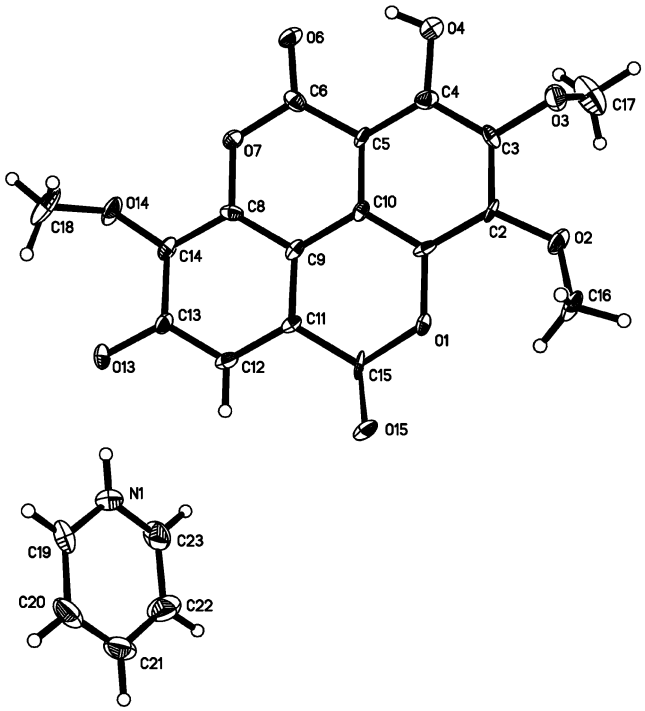

Figure 3. X-ray crystal structure of 3,3′,4-tri-*O*-methyflavellagic acid pyridinium salt (**4**) with 50% thermal probability ellipsoids.

Crystals of compound **1** were readily obtained via crystallization from acetone as colorless plates. Figure 1 shows the stereochemical drawing of **1** obtained from the X-ray analysis. In the crystalline state, there are two molecules of ruprechstyril (**1**) in the unit cell, with intramolecular hydrogen bonding between the hydroxyl hydrogen on O1 and the O7 carbonyl and intermolecular hydrogen bonding between the amide N8 hydrogen and the O7 carbonyl of an adjacent molecule.

The structures of secalonic acid A (**2**) (unstable and easily tautomerized in polar solvents), 3,3′,4-tri-*O*-methylflavellagic acid (**4**), lichexanthone (**5**), methyl asterrate (**6**), and 3-β,22*E*,24*S*-stigmasta-5,22-dien-3-ol (**7**) were also deter-

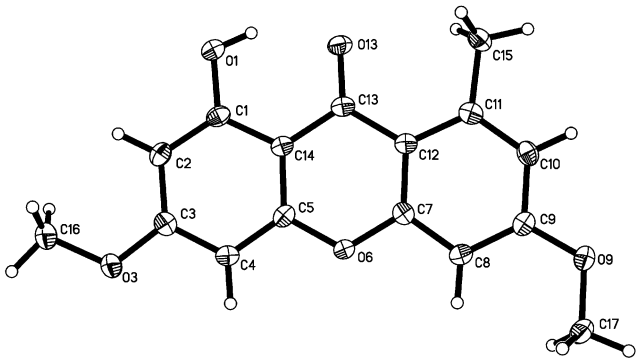

Figure 4. X-ray crystal structure of lichexanthone (**5**).

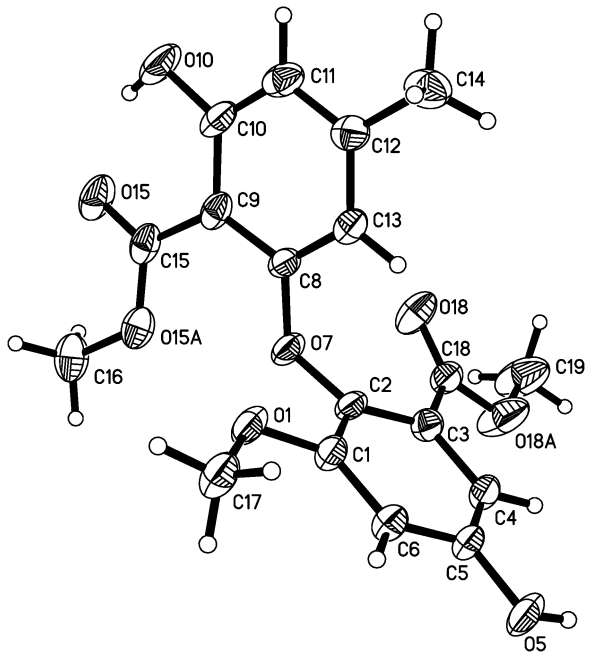

Figure 5. X-ray crystal structure of methyl asterrate (**6**), showing the contents of an asymmetric unit at 50% thermal probability ellipsoids.

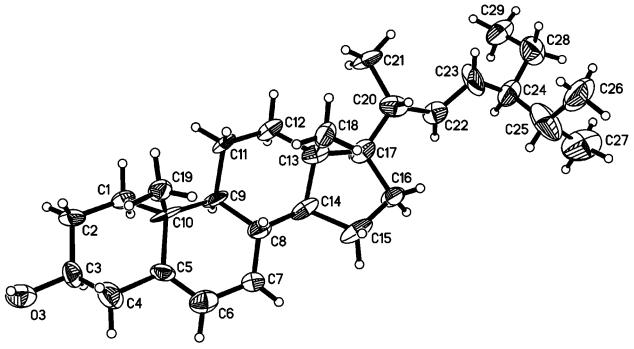

Figure 6. X-ray structure of 3β,22*E*,24*S*-stigmasta-5, 22-dien-3-ol (**7**).

mined by single-crystal X-ray analysis using the same experimental conditions employed for the X-ray determination of ruprechstyril (**1**). The structural results of these experiments are shown in the corresponding Figures 2−6, respectively. Finally, the structure of 2′-*O*-methylevernic acid was established by HRMS, IR, and 1D and 2D ^{1}H and ^{13}C NMR spectral analysis and by comparing its NMR data with those published.[17]

All seven compounds (**1**−**7**) isolated from *R. tangarana* were examined using the murine P388 lymphocytic leukemia cell line and a selection of human cancer cell lines. Only secalonic acid A (**2**) exhibited P388 cancer cell inhibitory activity (ED_{50} 3.5 μg/mL), and the supply was not sufficient for human cancer cell line evaluation. Secalonic acid A, isolated mainly from fungi,[18,19] was previously reported to inhibit growth of the murine L1210 lymphocytic leukemia cell line.[20] The other six compounds were inactive against both P388 and human cancer cell lines.

Compounds **1**−**6** were available in sufficient quantity for antimicrobial evaluation. In broth microdilution susceptibility assays,[21,22] secalonic acid A (**2**) inhibited growth of the opportunistic bacteria *Micrococcus luteus* (minimum inhibitory concentration 4−8 μg/mL) and *Enterococcus faecalis* (minimum inhibitory concentration 32 μg/mL). In these assays, ruprechstyril (**1**) and **3**−**6** were not active against *Staphylococcus aureus, Streptococcus pneumoniae, Enterococcus faecalis, Micrococcus luteus, Enterobacter cloacae, Escherichia coli, Stenotrophomonas maltophilia, Neisseria gonorrhoeae, Cryptococcus neoformans,* or *Candida albicans* (up to 64 μg/mL).

Experimental Section

General Experimental Methods. All chromatographic solvents were redistilled. Sephadex LH-20 used for partition column chromatography was obtained from Pharmacia Fine Chemicals AB. Melting points were measured on an Olympus electrothermal melting point apparatus and are uncorrected. UV spectra were collected with a Perkin-Elmer Lambda 3B UV/vis spectrometer. IR spectra were recorded with a Thermo Nicolet Avatar 360 infrared spectrometer. NMR spectra were obtained with a Varian XL-300 or a Varian UNITY INOVA-500 spectrometer with tetramethylsilane (TMS) as an internal reference. High-resolution mass spectra were obtained using a JEOL LCMate magnetic sector instrument either in the FAB mode, with a glycerol matrix, or by APCI with a poly(ethylene glycol) reference.

Plant Collection. The stems and twigs of *R. tangarana* Standl. employed in the following experiments were collected in Peru in 1979 and 1980 under the direction of Drs. James A. Duke (USDA), John D. Douros (U.S. NCI), and Matthew Suffness (U.S. NCI). Voucher specimens of *R. tangarana* have been retained by the United States Department of Agriculture, Beltsville, MD 20705, under Accession Numbers PR-54212 and PR-52705.

Extraction and Initial Separation. A 135.7 kg portion of the 1980 collection of stem bark and twigs of *R. tangarana* Standl. was extracted (2×) with (1:1) CH_2Cl_2−CH_3OH. The first extraction was for 10 days, and the second for 5 days. After each extraction, water was added to make the partition between 1:2 CH_3OH−H_2O and CH_2Cl_2, the CH_2Cl_2 layer separated, and solvent was evaporated in vacuo. The first (133.5 g, P388 inactive) and second CH_2Cl_2 extracts (220.4 g, P388 inactive) were combined, and 1.6 L of CH_3OH was added. The sample and CH_3OH mixture was stirred and agitated thoroughly to effect solution. The insoluble fraction (A, 212 g, P388 inactive) was separated from the methanol-soluble fraction (B, 108 g, P388 ED_{50} 27 μg/mL). The CH_3OH-insoluble fraction A (212 g) was partitioned between CH_3OH−H_2O (9:1 → 1:1) and hexane followed by CH_2Cl_2 to afford 114 g of a CH_2Cl_2 fraction (C) with P388 ED_{50}, 24 μg/mL. The solvent partitioning sequence was a modification of the original procedure of Bligh and Dyer.[23]

Isolation. The CH_3OH-soluble fraction B (108 g) was passed through a Sephadex LH-20 column, using CH_3OH as eluent. Four human cancer cell line inhibitory fractions were obtained. Colorless needles separated from one of the fractions and recrystallized (2×) from CH_3OH to yield 3.1 mg of steroid **7**. The other three bioactive fractions were combined and again passed through a Sephadex LH-20 column in CH_3OH with the same solvent as eluent; two bioactive fractions were obtained. These two fractions were combined and rechromatographed on a Sephadex LH-20 column, using toluene−acetone−*n*-hexane−CH_3OH (4:1:1:0.5) as eluent. Four bioactive fractions

(*a*, *b*, *c*, and *d*) were obtained. Fraction *a* was recrystallized from acetone to provide a pale brown crystalline fraction, which was recrystallized (2×) from acetone to yield ruprechstyril (**1**, 4.5 mg) as colorless needles. Fraction *b*, upon recrystallization from acetone, led to a pale brown solid. Recrystallization (3×) from toluene−*n*-hexane−CH_3OH (3:1:1) afforded 55.1 mg of methyl asterrate (**6**) as colorless needles. Fraction *c*, when recrystallized from CH_3OH, gave a yellow powder. Recrystallization from CH_3OH−acetone followed by recrystallization from pyridine yielded 6.5 mg of phenol **4**. Fraction *d*, recrystallized from toluene−*n*-hexane−CH_3OH (3:1:1), gave a pale green solid, which was recrystallized (2×) from CH_3OH to yield 2.8 mg of phenol **3**. The mother liquid from recrystallizations of compounds **1**, **3**, **4**, and **6** were combined and rechromatographed on a Sephadex LH-20 column, using toluene−*n*-hexane−CH_3OH (4:1:1) as solvent and eluent. One active fraction was obtained and rechromatographed by the same procedure. Again, one active fraction was obtained and recrystallized (2×) from CH_3OH−CH_2Cl_2 to provide yellow needles (**2**, 1.4 mg). The CH_2Cl_2 fraction C (114 g) in CH_3OH was passed through a Sephadex LH-20 column. Elution was conducted using CH_3OH, and one fraction recrystallized (2×) from CH_3OH yielded phenol **5** as colorless needles (36.4 mg).

Ruprechstyril (**1**): colorless needles from acetone; mp 139−141 °C; UV λ_{max} (CH_3OH) 244 (log ϵ 4.7) nm; HRAPCI (positive ion mode) *m*/*z* 262.1443 $[M + H]^+$ (calcd for $C_{15}H_{19}NO_3$, 262.4232); ^1H and ^{13}C NMR (see Table 1).

Crystal Structure of Ruprechstyril (1). A large, colorless block-shaped crystal, obtained via slow evaporation of an acetone solution, with approximate dimensions of 0.80 × 0.54 × 0.48 mm, 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 at 123(1) K on a Bruker 6000 diffractometer. Cell parameters indicated a triclinic space group. Subsequent data collection, using 1 s scans/frame and 0.396° steps in ω, was conducted in such a manner as to completely survey a complete sphere of reflections. This resulted in >92.6% coverage of the total reflections possible to a resolution of 0.83 Å. A total of 4862 reflections were harvested from the total data collection, and final cell constants were calculated from a set of 3031 reflections from these data. Subsequent statistical analysis of the complete reflection data set using the XPREP[24] program indicated the space group was $P\bar{1}$. Crystal data: $C_{15}H_{19}NO_3$, $a = 6.96680(10)$ Å, $b = 8.6605(2)$ Å, $c = 11.4897(2)$ Å, $V = 662.09(2)$ Å^3, λ (Cu Kα) = 1.54178 Å, μ(Cu Kα) = 0.739 mm^{-1}, $\rho_c = 1.311$ g cm^{-3} for $Z = 2$ and $M_r = 261.31$, $F(000) = 280$. After data reduction, merging of equivalent reflections, and rejection of systematic absences, 2315 unique reflections remained ($R_{int} = 0.0307$), of which 1950 were considered observed ($I_o > 2\sigma(I_o)$) and were used in the subsequent structure solution and refinement. An absorption correction was applied to the data with SADABS.[25] Direct methods structure determination and refinement were accomplished with the SHELXTL NT ver. V6.12[24] suite of programs. All non-hydrogen atoms for ruprechstyril (**1**) were located using the default settings of that program. The remaining hydrogen atom coordinates were calculated at optimum positions using the program SHELXL.[24] These latter atoms were assigned thermal parameters equal to either 1.2 or 1.5 (depending upon chemical type) of the U_{iso} value of the atom to which they were attached, then both coordinates and thermal values were forced to ride that atom during the final cycles of refinement. All non-hydrogen atoms were refined anisotropically in a full-matrix least-squares refinement process. The unit cell contains two independent molecules of the parent compound. The final standard residual R_1 value for the model shown in Figure 1 was 0.0847 (for observed data) and 0.0896 (for all data). The corresponding Sheldrick *R* values were wR_2 of 0.1999 and 0.2081, respectively. The difference Fourier map showed some residual electron density, the largest difference peak and hole being +1.292 and −0.624 e/Å^3, respectively. However, these peaks and holes were within 1 Å of the carbonyl O7 atom and were consequently attributed to this atom. The final bond distances and angles for the structural model, as shown in Figure 1, were all within acceptable limits.

Cancer Cell Line Procedure. The National Cancer Institute's standard sulforhodamine B assay was used to assess inhibition of human cancer cell growth as previously described.[26] The murine P388 lymphocytic leukemia cell line results were obtained as described previously.[27]

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Supporting Information Available: Crystallographic data, containing fractional coordinates, isotropic and anisotropic displacement parameters, and bond lengths and angles, are available for compound **1**. This material may be obtained free of charge via the Internet at htpp://pubs.acs.org.[28]

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(28) Crystallographic data for structures **1**−**7** reported in this paper have been deposited with the Cambridge Crystallographic Data Centre. Copies of the data can be obtained free of charge on application to the Director, CCDC, 12 Union Rd., Cambridge CB2 1EZ, UK (fax: +44-(0)1223-336033 or e-mail: deposit@ccdc.cam.ac.uk).

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