PD 116,152, A NEW PHENAZINE ANTITUMOR ANTIBIOTIC

STRUCTURE AND ANTITUMOR ACTIVITY

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A new, highly substituted phenazine with antitumor activity was isolated from the culture broth of a *Streptomyces* sp. This compound, whose structure was determined by spectroscopic methods and verified by X-ray diffraction analysis, was found to be methyl 6-formyl-4,7,9-trihydroxy-8-methyl-1-phenazinecarboxylate.

During the course of our screening for novel antitumor agents, a culture broth of a *Streptomyces* sp. (NRRL 15783) was found to be active against an *Escherichia coli* mutant that is especially sensitive to agents that cause DNA damage. The compound, PD 116,152[1], responsible for this activity could be readily isolated by extraction of the fermentation beer with EtOAc at pH 3 followed by concentration and crystallization. The structure of PD 116,152 was shown to be methyl 6-formyl-4,7,9-trihydroxy-8-methyl-1-phenazinecarboxylate (**1**) on the basis of spectral and X-ray diffraction analysis. In addition to its antimicrobial activity, PD 116,152 possesses significant antitumor activity in a variety of *in vitro* and *in vivo* test systems.

Experimental

General

^1H and ^{13}C NMR spectra were recorded on a Varian XL-200 spectrometer at 200 MHz and 50.3 MHz, respectively. Chemical shifts are reported as ppm downfield from internal 2,2,3,3-tetradeuterio-3-(trimethylsilyl)propionate (TSP). High resolution mass spectra were recorded on a VG Analytical Model 7070E/HF instrument. UV spectra were run on an IBM Model 9420 spectrophotometer and IR spectra were recorded using a Nicolet SX-60 FTIR spectrophotometer.

Isolation of PD 116,152 (1)

Unfiltered fermentation beer (4,800 liters) was adjusted to pH 3.0 and stirred for 1 hour with 3,000 liters of EtOAc. Celite (660 kg) was added and the mixture was filtered. The organic layer was separated, diluted with an EtOAc wash (380 liters) of the filter cake, and then concentrated *in vacuo* to 337 liters. The concentrate was stored at −20°C for 65 hours. The precipitate that formed was filtered off, washed with 3 liters of EtOAc, 4 liters of MeOH, and finally with 23 liters of $CHCl_3$. The partially crystalline product (155 g) was recrystallized from 18 liters of $CHCl_3$-MeOH (9: 1) to afford 63 g of crystalline PD 116,152: MP 255°C (dec); EI-MS *m/z* 328.0704 (M^+; $C_{16}H_{12}N_2O_6$ 328.0695), 296.0436, 268.0504, 240.0529, 212.0562; IR ν_{max}^{KBr} cm^{-1} 3380, 1705, 1630, 1550, 1240, 1180; UV λ_{max}^{MeOH} nm (ε) 226 (15,940), 271 (26,540), 385 (6,070); UV $\lambda_{max}^{MeOH+KOH}$ nm (ε) 262 (21,030), 294 (23,810), 351 (19,840), 415 (5,080), 440 (4,690); ^1H NMR (D_2O+NaOD) δ 2.04 (3H, s), 3.86 (3H, s), 6.61 (1H, d, J=8.0 Hz, 3-H), 8.03 (1H, d, J=8.0, 2-H), 9.83 (1H, s); ^{13}C NMR (DMSO-d_6) δ 8.14, 52.10, 105.22, 110.35,

113.05, 119.96, 130.90, 133.62, 133.95, 137.94, 139.90, 156.25, 157.33, 166.20, 170.85, 194.24.

Anal Calcd for $C_{16}H_{12}N_2O_6 \cdot \frac{1}{2}$MeOH: C 57.55, H 4.07, N 8.13.
Found: C 58.06, H 3.91, N 8.36.

Concentration and cooling of the mother liquor afforded an additional 23 g of crystalline PD 116,152. HPLC analysis of this product was performed using a 4.1×250 mm PRP-1 column (Hamilton Co., Reno, Nevada) with UV detection at 254 nm. The mobile phase consisted of a linear gradient from 0.025 M borate buffer (pH 9.5) - MeCN - MeOH (90: 5: 5) at time zero to 0.025 M borate buffer (pH 9.5) - MeCN - MeOH (70: 25: 5) over a course of 7 minutes. At a flow rate of 2.0 ml/minute, the retention time of PD 116,152 is 3.5 minutes.

X-Ray Diffraction Analysis

Crystals of PD 116,152 suitable for a single crystal X-ray diffraction analysis could be grown from DMSO - MeOH solutions. A crystal of approximate dimensions 0.2×0.5×0.9 mm was selected for the analysis. Preliminary X-ray photographs displayed monoclinic symmetry, and accurate lattice constants of $a=13.328(6)$, $b=6.5034(2)$, $c=33.657(8)$ Å, and $\beta=79.62(3)°$ were determined from a least-squares fit of 15 diffractometer measured 2θ-values. Systematic extinctions and crystal density were uniquely accommodated by space group C2/c with one molecule of composition $C_{16}H_{12}N_2O_6$ forming the asymmetric unit. All diffraction maxima with $2\theta \leq 114°$ were collected on a computer controlled four-circle diffractometer using graphite monochromated Cu $K\bar{\alpha}$ radiation (1.54178 Å) and variable speed, 1° ω-scans. A total of 1919 reflections was collected in this manner and after correction for Lorentz, polarization, and background effects, 1582 (82%) were judged observed ($|F_o| \geq 3\sigma(F_o)$) and used in subsequent calculations*. A phasing model was found uneventfully using a multisolution sign determining approach, and all nonhydrogen atoms were clearly visible in the first E-synthesis (Fig. 1). Hydrogen atoms were located in a ΔF-synthesis following partial refinement of the nonhydrogen atoms. Block diagonal least-squares refinements with anisotropic nonhydrogen atoms and isotropic hydrogens converged to a conventional crystallographic residual of 0.082 for the observed reflections. Crystallographic parameters have been deposited with the Cambridge Crystallographic Data File, University Chemical Laboratory, Lensfield Road, Cambridge, CB2 1EW, England and are available from them.

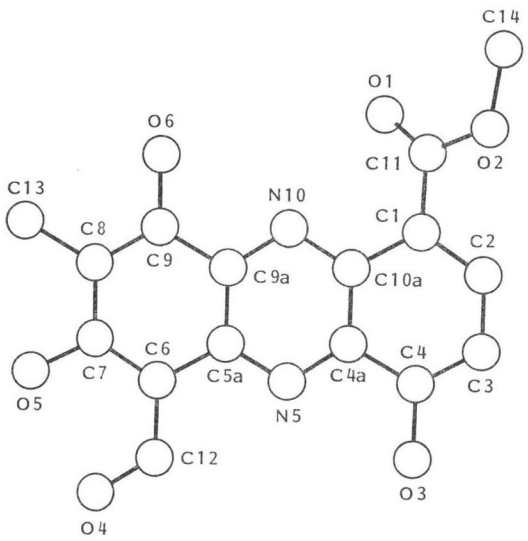
Structure Determination

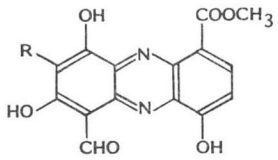
The molecular formula of PD 116,152 was established as $C_{16}H_{12}N_2O_6$ on the basis of elemental analysis and high resolution mass spectral data. UV absorption maxima were observed at 226, 271 and 385 nm in MeOH shifting to 262, 294, 351, 415 and 440 nm in alkali. These data, coupled with the presence of two nitrogen atoms, suggested that PD 116,152 is a phenazine bearing at least one phenolic group.

The ^{1}H NMR spectrum of PD 116,152 in D_2O+NaOD exhibits signals for an aromatic methyl group (s, 2.04 ppm), a methoxyl group (s, 3.86 ppm), two vicinal aromatic protons (doublets at 6.61 and 8.03 ppm $J=8$ Hz) and an aldehydic proton (s, 9.83 ppm). Corresponding signals in the ^{13}C NMR at 8.14, 52.10, 110.35 (d), 133.95 (d) and 194.24 ppm, respectively, support the presence of these functionalities. The presence of an aromatic formyl group was confirmed by the ready conversion of PD 116,152 to imine derivatives in the presence of various amines. In addition, the methoxyl group was determined

* All crystallographic calculations were done on a PRIME 9950 computer operated by the Cornell Chemistry Computing Facility. Principal programs employed were: REDUCE and UNIQUE, data reduction programs by M. E. LEONOWICZ, Cornell University, 1978; MULTAN 78, MULTAN 80, and RANTAN 80, systems of computer programs for the automatic solution of crystal structures from X-ray diffraction data (locally modified to perform all Fourier calculations including Patterson syntheses) written by P. MAIN, S. E. HULL, L. LESSINGER, G. GERMAIN, J. P. DECLERCQ and M. M. WOOLFSON, University of York, England, 1978 and 1980; BLS78A, an anisotropic block diagonal least squares refinement written by K. HIROTSU and E. ARNOLD, Cornell University, 1980; PLUTO78, a crystallographic illustration program by W. D. S. MOTHERWELL, Cambridge Crystallographic Data Centre, 1978; and BOND, a program to calculate molecular parameters and prepare tables written by K. HIROTSU and G. VAN DUYNE, Cornell University, 1985.

Fig. 1. Structure of PD 116,152.





PD 116,152 (**1**) R=CH_3
Lomofungin (**2**) R=H

Table 1. *In vivo* activity of PD 116,152 against P388 lymphocytic leukemia[a].

Dosage[b] (mg/kg/injection)	T/C (%)
25	Toxic
12.5	149
6.25	130
3.12	119

[a] Tumor inoculated ip on day 0.
[b] Administered ip on days 1~9.

to be part of a carbomethoxy group by treatment of PD 116,152 with aqueous NaOH to afford an acidic product lacking a methoxyl signal in its ^{1}H NMR spectrum. Additional signals in the ^{13}C NMR spectrum of PD 116,152 include those for quarternary carbons at 156.25, 157.33, 166.20 and 170.85 ppm. One of these signals must be assigned to the carbonyl carbon of the carbomethoxy group while the other three signals are assigned to aromatic carbon atoms bearing phenolic hydroxyl groups. Signals for the remaining seven carbon atoms required by the molecular formula are observed as singlets between 105.22 ~139.90 ppm. The above data account for the complete molecular formula of PD 116,152 and indicate that it is a hexasubstituted phenazine bearing CH_3, CHO, $COOCH_3$, and three OH groups.

A search of the literature showed that lomofungin (**2**), an antibiotic isolated at the Upjohn Laboratories in 1967[2], is a phenazine very similar to PD 116,152. Indeed, all of the substituents found in PD 116,152, with the exception of the aromatic methyl group, are also present in lomofungin. A comparison of the ^{1}H NMR spectra of PD 116,152 and an authentic sample of lomofungin showed that the former compound differed only by the presence of a methyl substituent at C-8, suggesting that structure **1** could be assigned to PD 116,152. This structure was confirmed by X-ray crystallographic analysis.

A computer generated drawing for the final X-ray model of PD 116,152 is shown in Fig. 1. Hydrogen atoms have been omitted for clarity. The entire molecule, except for the carbomethoxy group is planar within experimental error. The carbomethoxy group is rotated approximately 27° out of this plane. The most important fact gained from the X-ray analysis was the placement of the various substituents about the phenazine nucleus which confirms that PD 116,152 is methyl 6-formyl-4,7,9-trihydroxy-8-methyl-1-phenazinecarboxylate (**1**).

Discussion

The discovery of phenazines as microbial metabolites has a long history, and recently new phenazines such as senacarcin (DC-59A)[3], saphenamycin (A-32256)[4,5], and DC-86M[6] have been isolated and found to have antitumor activity. Most of these products, however, are di- or at most trisubstituted

phenazines. A prominent exception is lomofungin as noted above. Although PD 116,152 is structurally very similar to lomofungin, a careful examination of PD 116,152-containing beers using HPLC failed to detect any lomofungin. Analogously, fermentation of the lomofungin producer, *Streptomyces lomondensis* (NRRL 3252), afforded good yields of lomofungin but no detectable quantity of PD 116,152. PD 116,152 is very insoluble in most solvents and has weak broad spectrum antimicrobial activity. However, it possesses cytotoxic activity against L1210 lymphocytic leukemia and human colon adenocarcinoma (HCT-8) cells with IC_{50} values of 5.2×10^{-7} M and 7.1×10^{-7} M, respectively. Although PD 116,152 displays only moderate *in vivo* antitumor activity against P388 lymphocytic leukemia (Table 1), some of its derivatives are very active antitumor agents. The results of these *in vivo* tests will be reported separately.

Acknowledgments

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References

1) TUNAC, J. B.; S. W. MAMBER, B. D. GRAHAM & W. E. DOBSON: PD 116,152, a novel phenazine antitumor antibiotic. Discovery, fermentation, culture characterization and biological activity. J. Antibiotics 39: 192~197, 1986
2) TIPTON, C. D. & K. L. RINEHART, Jr.: Lomofungin. I. Degradative studies of a new phenazine antibiotic. J. Am. Chem. Soc. 92: 1425~1426, 1970
3) NAKANO, H.; M. YOSHIDA, K. SHIRAHATA, S. ISHII, Y. ARAI, M. MORIMOTO & F. TOMITA: Senacarcin A, a new antitumor antibiotic produced by *Streptomyces endus* subsp. *aureus*. J. Antibiotics 35: 760~762, 1982, Japan Kokai (Kyowa Hakko) 82-4,975, Jan. 11, 1982
4) KITAHARA, M.; H. NAKAMURA, Y. MATSUDA, M. HAMADA, H. NAGANAWA, K. MAEDA, H. UMEZAWA & Y. IITAKA: Saphenamycin, a novel antibiotic from a strain of *Streptomyces*. J. Antibiotics 35: 1412~1414, 1982
5) K. H. MICHEL & M. M. HOEHN (Eli Lilly): A-32256 phenazine antibiotic. U.S. 4,400,510, Feb. 23, 1982
6) TOMITA, F.; K. TAKAHASHI, I. KAWAMOTO, K. ASANO, M. MORIMOTO, T. ASHIZAWA & K. FUJIMOTO (Kyowa Hakko): Antitumor 4-hydroxyethyl-phenazine-9-carboxylic acid compounds obtained by fermentation of a *Streptomyces* strain. Japan Patent 012,383, Jan. 28, 1983