PD 114,759 AND PD 115,028, NOVEL ANTITUMOR ANTIBIOTICS WITH PHENOMENAL POTENCY

I. ISOLATION AND CHARACTERIZATION

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The isolation of two new antibiotics, PD 114,759 and PD 115,028, exhibiting *in vivo* antitumor activity at extremely low doses is described. The physico-chemical properties of these sulfur-containing compounds show that they represent a novel class of antitumor agents.

During the course of our anticancer drug discovery program, a fermentation broth produced by a novel *Actinomadura* sp. (ATCC 39363) was found that exhibited excellent activity against P388 leukemia in mice. Fractionation of these fermentation beers showed that the antitumor activity was due to a complex of related antibiotics from which two major components, PD 114,759 and PD 115,028, were isolated as pure compounds.

As summarized in Table 1, these components are active against P388 lymphocytic leukemia in mice over a wide range of extremely low dosages. *In vivo* activity against P388 leukemia and various solid tumors in mice is reproducibly demonstrated at doses less than 1 μg/kg. In tissue culture, the IC_{50} values vs. L1210 cells for PD 114,759 and PD 115,028 are 7.4×10^{-13} M (1.0 pg/ml) and 2.2×10^{-12} M (3.0 pg/ml), respectively. Thus, on a molar basis, these compounds are approximately three times more potent *in vivo* and six times more potent *in vitro* than CC-1065, heretofore, the most potent antitumor antibiotic described in the literature[1~3].

The present report describes the isolation and characterization of PD 114,759 and PD 115,028 which were found to be unique, sulfur-containing antibiotics representing a novel class of antitumor agents. Future reports will describe the taxonomy of the producing organism, fermentation parameters, biological and structural aspects of these compounds, and the characterization of other closely related components coproduced with the title compounds.

Table 1. Antitumor activity of PD 114,759 and PD 115,028 vs. P388 lymphocytic leukemia[a].

% T/C (Dose[b])			
PD 114,759		PD 115,028	
Test-1	Test-2	Test-1	Test-2
205 (4)	171 (4)	165 (6.4)	141 (6.4)
149 (2)	155 (2)	132 (3.2)	164 (3.2)
130 (1)	162 (1)	118 (1.6)	169 (1.6)
		128 (0.8)	161 (0.8)
			171 (0.4)

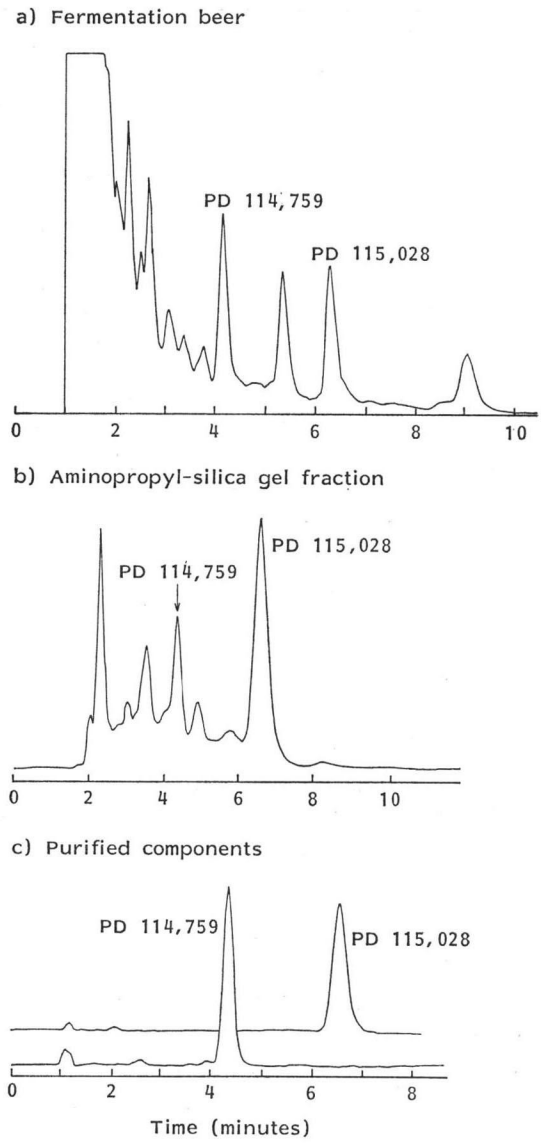
[a] Tumor inoculated intraperitoneally on day 0.
[b] μg/kg/injection, intraperitoneally; single doses given on days 1~5.

Analytical

The complex of antitumor antibiotics, which includes PD 114,759 and PD 115,028, can be detected in fermentation beers by testing for antimicrobial activity vs. *Micrococcus luteus* or *Bacillus subtilis*, or by cytotoxicity assays using L1210 cells. The results of these assays, however,

do not always correlate directly with the specific concentrations of PD 114,759 and PD 115,028. High pressure liquid chromatography (HPLC) accurately assays the concentration of these compounds in chromatographic fractions and semi-purified concentrates and defines approximate titers of PD 114,759 and PD 115,028 in fermentation beers. Good resolution of these and other components is obtained using a 5 μm C-18 Novapak column (0.39×15 cm; Waters Associates, Milford, MA) and 0.05 M NH_4OAc (pH 4.4) buffer - MeOH - MeCN - AcOH (28: 42: 30: 0.50) as the mobile phase (Fig. 1). Retention times, detected by UV absorbance at 254 nm at a flow rate of 1.0 ml/minute, of PD 114,759 and PD 115,028 are approximately 4.2 and 6.5 minutes, respectively. Column eluates were assayed by direct injections. Fermentation broths (10 ml of whole beer) were thoroughly mixed with EtOAc (30 ml). After centrifugation the organic layer was concentrated to dryness *in vacuo* and the residue was reconstituted in a final volume of 0.2 ml MeOH. An injection of 5~15 μl of this solution was sufficient for HPLC analysis (Fig. 1a). Using this procedure and pure samples of each component as standards, the titers of both PD 114,759 and PD 115,028 in fermentation beers varied between 0.1~2 μg/ml.

Fig. 1. HPLC of PD 114,759 and PD 115,028 using the procedure described in the Analytical section.



Initial Fractionation

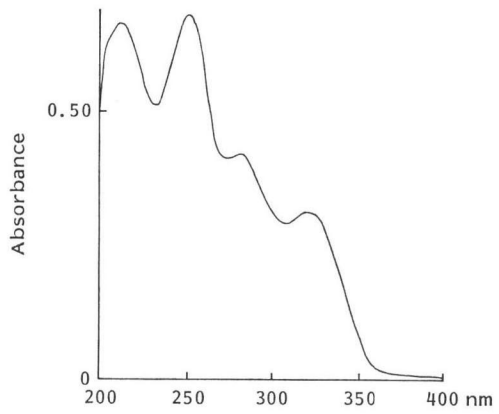
Unfiltered fermentation beer (4,500 liters) was stirred for two hours with 3,200 liters EtOAc. The mixture was treated with 115 kg Celite 545 and then filtered through a plate and frame filter press. The filter cake was washed twice with 280 liter portions of EtOAc and the washes were added to the filtrate. The lower aqueous layer was separated and the organic extract was concentrated *in vacuo* to 29.5 liters. This concentrate was diluted with 91 liters petroleum ether and the mixture was extracted with 22 liters of MeOH - H_2O (1: 1). The lower aqueous MeOH layer (25 liters) was extracted with 7 liters petroleum ether and then concentrated to 3 liters. All of the organic soluble material that remained was carefully transferred to 9 liters EtOAc. This solution was dried (Na_2SO_4) and concentrated to 2 liters. The concentrate was diluted with 4 liters CH_2Cl_2 and stored overnight at −20°C. Insoluble material was removed by filtration using 200 g Celite 545. After filtration the Celite pad was washed with 1 liter CH_2Cl_2 -

Table 2. Physico-chemical properties of PD 114,759 and PD 115,028.

	PD 114,759	PD 115,028
Appearance	Off-white solid	Off-white solid
Solubility Soluble	MeOH, $CHCl_3$	MeOH, $CHCl_3$
Insoluble	Hexane, H_2O	Hexane, H_2O
UV λ_{max}^{MeOH} nm (log ε)	215 (4.57), 252 (4.58), 282 (4.36), 318 (4.20)	215 (4.56), 252 (4.56), 282 (4.34), 325 (4.21)
IR (KBr) ν_{max}	2970, 2930, 1690, 1630, 1610, 1600, 1530, 1450, 1410, 1310, 1255, 1215, 1160, 1080, 1020, 990, 880, 850, 800 cm^{-1}	2970, 2930, 1690, 1630, 1610, 1600, 1530, 1450, 1410, 1310, 1255, 1215, 1160, 1080, 1020, 940, 880, 780 cm^{-1}
Elemental analysis	C 53.26, H 6.04, N 3.82, S 8.98	C 54.81, H 6.33, N 3.86, S 8.96
FAB-MS (m/z)	1,357 (M+H)	1,357 (M+H)
MP °C (dec)	185~195	170~195
$[\alpha]_D^{24}$ (% in $CHCl_3$)	−198° (1.25)	−189° (0.63)
TLC[a] Rf	0.50	0.43

[a] Stationary phase, C_{18}-silica gel (Whatman $KC_{18}F$); mobile phase, MeOH - 0.1 M NH_4OAc buffer (pH 6.8) (9: 1).

Fig. 2. UV spectrum of PD 114,759 in MeOH.

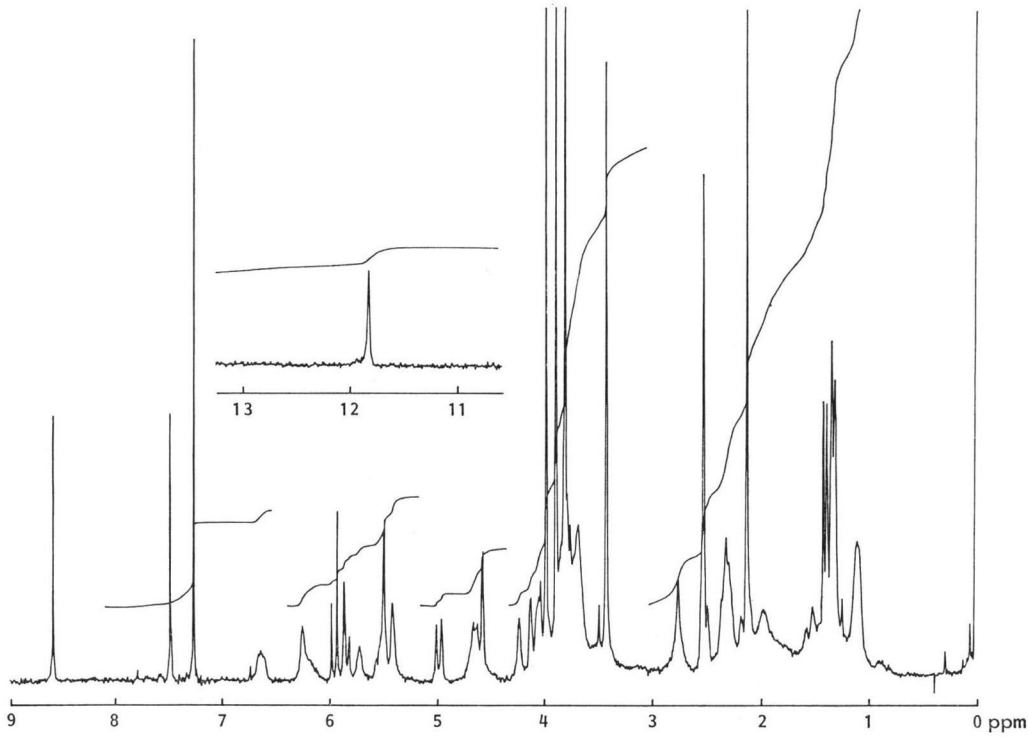


EtOAc (2 : 1). The washes and filtrate were combined (7 liters) and diluted with 1.5 liters CH_2Cl_2. This solution was added to a 10 cm (inside diameter) column containing 8.2 kg of 40 μm aminopropyl-silica gel (Analytichem International, Inc., Harbor City, CA) packed in CH_2Cl_2. The resin was then eluted sequentially with 44 liters CH_2Cl_2; 80 liters of $CHCl_3$ containing 35~20% CH_2Cl_2; and finally with 30 liters of $CHCl_3$ - EtOH (95: 5). A head pressure of 0.70 kg/cm^2 was used to maintain a flow rate of approximately 400 ml/minute. A total of 24 fractions were collected in volumes of 2~16 liters. HPLC analysis of each fraction showed that the majority of the PD 114,759 was eluted with $CHCl_3$ - CH_2Cl_2 (80: 20) (Eluate A) and most of the PD 115,028 was eluted with $CHCl_3$ - EtOH (95: 5) (Eluate B). An HPLC chromatogram of a major portion of Eluate B is shown in Fig. 1b. Eluate A (61 liters) was concentrated *in vacuo* to 150 ml. The concentrate was treated with 1.2 liters petroleum ether to precipitate 3.04 g of a solid (Product A). HPLC showed that Product A contained 1.29 g of PD 114,759 and 0.396 g of PD 115,028. Eluate B (19.5 liters) was similarily processed to yield 4.15 g of a solid (Product B) that contained 0.47 g of PD 114, 759 and 1.75 g of PD 115,028.

Isolation of PD 114,759

A 7 cm (i.d.) × 88 cm stainless steel column was dry-packed with 1.9 kg of 40 μm C_{18}-silica gel (Analytichem). After the adsorbent was washed with 10 liters of MeOH - 0.05 M NH_4OAc (pH 6.8)

Fig. 3. 200 MHz ^1H NMR spectrum of PD 114,759 in $CDCl_3$.



buffer (75: 25), a solution of Product A (3.04 g) in 20 ml of MeOH - H_2O (9: 1) was applied to the top of the column. For chromatography, MeOH - 0.05 M NH_4OAc (pH 4.8) buffer (70: 30) was used as the eluent at a head pressure of approximately 10.6 kg/cm^2. Each of the 20 fractions collected (total volume 39 liters) was analyzed by HPLC. The majority of the PD 114,759 eluted between 7~18 liters; the smaller amount of PD 115,028 present in Product A was subsequently eluted using 15.5 liters of MeOH - 0.1 M NaOAc (pH 4.8) buffer (75: 25). The fractions (11 liters) containing most of the PD 114,759 were combined and concentrated *in vacuo* to remove MeOH. The aqueous mixture was extracted with $CHCl_3$. The organic extract was washed with H_2O, dried over Na_2SO_4 and concentrated to about 150 ml. Ten volumes of cyclohexane was added to precipitate 892 mg of a solid that contained about 500 mg of PD 114,759 and <50 mg of PD 115,028. This product was further purified by reverse phase chromatography on C_{18}-silica gel using a Prep LC/System 500 instrument (Waters Associates, Milford, MA) fitted with a PrepPAK-500/C_{18} column. The charge (0.88 g) was triturated with 9 ml MeOH. Insoluble material was removed by centrifugation. The supernatant solution was diluted with 1 ml H_2O and added to the top of the column. Chromatography was effected using 17 liters of MeCN - MeOH - 0.1 M NaOAc (pH 4.4) buffer (38: 8: 54), adjusted to pH 4.8 with AcOH prior to use. The fractions eluting between 9~15 liters contained PD 114,759 as the only UV-absorbing component. These were combined (7 liters) and concentrated at <30°C to remove organic solvents. The concentrate was extracted three times with $CHCl_3$. The extracts were combined, extracted twice with one-third volumes of H_2O, and dried over Na_2SO_4. The $CHCl_3$ solution was concentrated to 25 ml (<25°C) and then treated with 500 ml *n*-pentane to precipitate 273 mg of PD 114,759 (>95% pure by HPLC).

The properties of PD 114,759 are listed in Table 2. The UV and NMR spectra are shown in Figs. 2 and 3.

Isolation of PD 115,028

A solution of Product B (4.0 g), described above, in 30 ml MeOH - H_2O (9: 1) was chromatographed over 1.9 kg of 40 μm C_{18}-silica gel contained in a 7 cm (i. d.) column. The eluent was 51 liters of MeOH - 0.05 M NH_4OAc (pH 6.8) buffer (75: 25). Each of the 25 fractions collected was analyzed by HPLC. PD 114,759 was eluted between 13.5~24 liters and PD 115,028 was eluted between 31~50 liters. The latter eluate was concentrated *in vacuo* to 1 liter. The turbid concentrate was extracted three times with $CHCl_3$. The organic extracts were combined, washed twice with H_2O, and dried over anhydrous Na_2SO_4. The $CHCl_3$ solution was concentrated to 50 ml and then treated with 500 ml of cyclohexane to precipitate 930 mg of a pale yellow solid. HPLC showed that this product contained 760 mg of PD 115,028. This material was further purified, in three separate runs, using a Prep LC/System 500 instrument fitted with a PrepPAK-500/C_{18} column. Each portion (310 mg) of the starting charge was dissolved in 5.4 ml MeOH. Insoluble material was removed by centrifugation. The supernatant solution was treated with 0.6 ml H_2O and applied to the C_{18}-silica gel column. The mobile phase was prepared by adjusting a 67: 33 mixture of MeOH - 0.1 M NaOAc (pH 4.4) buffer to pH 4.8 with AcOH. Approximately 9 liters of eluent was required for each of the three column runs which were completed and worked up within 12 hours. The course of each of the three chromatographic separations was monitored by HPLC. The majority of PD 115,028 eluted between 5~8.5 liters. The fractions that contained PD 115,028 as the only UV absorbing component were combined and quickly concentrated *in vacuo* to remove MeOH. The aqueous mixture was extracted twice with $CHCl_3$. The $CHCl_3$ extracts were combined, dried (Na_2SO_4), and concentrated to 45 ml. Cyclohexane (2 liters) was added to precipitate 503 mg of PD 115,028 which was >98% pure by HPLC analysis. The properties of this material are listed in Table 2.

Discussion

A molecular weight of 1,356 has been determined for both PD 114,759 and PD 115,028 by FAB mass spectrometry. This information, in conjunction with elemental analytical data, indicates that these compounds contain four atoms each of nitrogen and sulfur although exact molecular formulas could not be established. These data as well as the distinctive UV absorption spectra clearly distinguish PD 114,759 and PD 115,028 from previously described antibiotics. Structure elucidation studies of PD 114,759, PD 115,028, and related antibiotics are in progress in our laboratories. Structural aspects of these unique, highly potent antitumor compounds will be the subject of a future report.

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