POTENT ANTITUMOR ANTIBIOTIC COMPLEX: PD 114,759, PD 115,028, PD 119,707, and PD 119,193†

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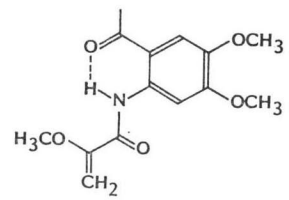
Four novel antitumor antibiotics (PD 114,759, PD 115,028, PD 119,707, and PD 119,193) are produced as a complex by a new species of *Actinomadura*. The proposed name for the culture is *Actinomadura verrucosospora* subsp. *veractimyces* ATCC 39363.

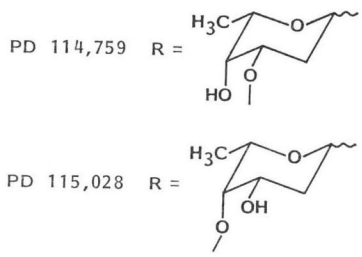
The antibiotics are extremely bioactive, with MIC values of <0.006 ng/ml against several bacteria and ID_{50} values of 0.003~0.107 ng/ml against L1210 leukemia cells *in vitro*. Antitumor activities vs. P388 leukemia *in vivo* were observed at doses of 0.313, 0.40, and 0.5 μg/kg (daily $\times$ 5) for PD 119,707, PD 115,028, and PD 114,759, respectively.

An actinomycete, culture WP 444 (ATCC 39363) was isolated from a soil collected from Knoxville, Tennessee. The fermentation broth of this culture showed activity against L1210 cells in tissue culture used in our screening program. The activity was found to be due to an antibiotic complex from which the isolation of the two major components, PD 114,759 and PD 115,028, had been previously described[1]. These compounds are isomeric, possessing the partial structures[2] as shown in Fig. 1. At least two other components have also been isolated and purified namely: PD 119,707 and PD 119,193 (J. H. WILTON, G. C. HOKANSON & J. C. FRENCH, personal communication).

Fig. 1. Partial structure showing the chromophore of PD 114,759 and PD 115,028.



The producing culture was identified as an *Actinomadura*. This paper describes the taxonomy of the producing organism, fermentation, and biological activities of the antibiotics.



Materials and Methods

Culture Characterization

The culture was isolated from a soil sample pretreated with $CaCO_3$ for 7 days before plating. The plating medium consisted of glycerol 3%, L-asparagine 0.25%, potassium chloride 0.05%, KH_2PO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05%, $FeSO_4 \cdot 7H_2O$ 0.001%, and agar 1.5%.

Culture characterization was carried out following the International Streptomyces Project procedure[3]. Morphological and color determinations of the growth of the organism were made at weekly intervals over a three-week period. Carbohydrate utilization was determined using Difco carbon utilization medium (ISP-9) containing sterilized chemically pure substrate at a final concentration of 1%[3].

† The provisional name for the antibiotic complex is veractamycin: veractamycin A (PD 114,759), veractamycin B (PD 115,028), veractamycin D (PD 119,707), and veractamycin E (PD 119,193).

Chemotaxonomy

Purified cell walls and whole-cell hydrolysates were analyzed using the methods of BECKER *et al.*[4] and LECHEVALIER[5], respectively.

Fermentation

Stock cultures of the organism were maintained in lyophilized vials and the working culture in 2-ml polypropylene cryovials (Nunc, Kamstrup, Denmark). The cryovials containing the culture were stored in a liquid nitrogen freezer (Model LR-310A, Union Carbide).

The seed medium consisted of yeast hydrolysate (Amberex 1003, Amber Labs.) 0.5%, Cerelose 0.1%, dextrin (Amidex $B_4$11, Corn Products) 2.4%, hydrolyzed peptone (N-Z case, Humko-Sheffield) 0.5%, spray dried meat solubles (Daylin Labs.) 0.3%, and $CaCO_3$ 0.2%. The production of the antitumor complex was carried out in shake-flasks, stirred-jars or pilot plant fermentors. The production medium consisted of dextrin (Maltrin, Grain Processing) 2.5%, cotton seed meal (Pharmamedia, Traders Protein) 0.65%, fish meal (Zapata-Haynie) 0.5%, sodium succinate 0.25%, dried whey (Toruway 30, Pure-Culture Products) 0.25%, castor oil (Welch, Holme and Clark) 0.5%, $CoCl_2 \cdot 6H_2O$ 0.0001%, and $FeSO_4 \cdot 7H_2O$ 0.005%. The fermentation conditions were as follows: Shake-flask, 50-ml/250-ml Erlenmeyer flask, 200 rpm shaker (Model G-53, New Brunswick); 30-liter stirred-jars, 16 liters/jar, 1 v/v/minute, 300 rpm; and 760-liter fermentor, 600 liters/tank, 0.75 v/v/minute (570 liters/minute), 155 rpm. The fermentation was carried out for 6~7 days at 33°C.

Assay and Antitumor Activity

The antibiotic complex in the fermentation broth was assayed both by high pressure liquid chromatography (HPLC)[1] and *in vitro* bioactivity vs. L1210 murine leukemia cells (Mason Res. Inst. Worcester, Mass) in tissue culture.

The *in vivo* antitumor activity was evaluated vs. P388 murine lymphocytic leukemia tumor cell lines in CDF_1 mice[6]. The tumor cells were injected intraperitoneally (ip) on day 0, and the antibiotics were administered ip on days 1 through 5.

Antimicrobial Activity

The antimicrobial activities were evaluated by the broth dilution method[7]. Because of the low water solubility, the antibiotics were dissolved in DMSO or ethanol then diluted with distilled water to bring the final solvent concentration to 10%. Subsequent dilutions were made in the media contained in the microdilution trays.

Results

Culture Characterization

Cell wall analysis showed the presence of *meso*-diaminopimelic acid, and whole cell analysis revealed madurose (Table 1). Preliminary cultural and morphological characterization of ATCC 39363 indicated some similarity of *Actinomadura verrucosospora*. For this reason a side-by-side comparison was carried out between ATCC 39363 and the type strain, *A. verrucosospora* ATCC 27299.

Several significant differences were noted between ATCC 39363 and the type strain ATCC 27299: Lack of aerial mycelia for ATCC 39363 on ISP-4 medium (Table 1); poor growth for ATCC 39363 on mannose (Table 2); differences in the substrate mycelial color, particularly on glycerol lactose, and maltose (Table 2); and elliptical spores for ATCC 39363 as compared to the globose to oval spores with the ATCC 27299 (Fig. 2).

The spores of both cultures had warty surfaces (Fig. 2). The spore chains consisted of hooks and spirals with 5~6 spores/chain.

Fermentation

The fermentation yield of the antibiotic complex was very low. A typical shake-flask yield in a

Table 1. Cultural and physiological characteristics of ATCC 39363 as compared to *Actinomadura verrucosospora* ATCC 27299.

Basis of characterization		Isolate WP-444	*Actinomadura verrucosospora* ATCC 27299
Aerial mycelium	Yeast extract-malt extract agar (ISP-2)	Oyster white (b)	Oyster white (b)
	Oatmeal agar (ISP-3)	Oyster white (b)	White (a)
	Inorganic salts starch agar (ISP-4)	None	White (a)
	Glycerol-asparagine agar (ISP-5)	None	None
Reverse (substratal)	ISP-2	Wheat (2fb)	Pastel yellow (1db)
	ISP-3	White (a)	Pastel yellow (1db)
	ISP-4	Clove brown (3ni)	Yellow maple (3le)
	ISP-5	Rose beige (4gc)	Rose beige (4gc)
Physiological	Melanine pigmentation: ISP-6	Negative	Negative
	ISP-7	Negative	Negative
	Gelatin liquefaction	Positive	Positive
	Nitrate reduction	Positive	Positive
	Milk coagulation	Negative	Negative
Soluble pigments	ISP-2	None	None
	ISP-3	None	None
	ISP-4	None	None
	ISP-5	None	None
	ISP-7 (tyrosine agar)	None	None
Morphology	Spore chain	Hooks and spiral	Hooks and spiral
	Spore ornamentation	5～6 spores/chain, elliptical warty surface	5～6 spores/chain, globose to oval, warty surface
Whole cell analysis	Cell wall	*meso*-DAP[a]	*meso*-DAP
	Whole cell sugar	Madurose[b]	Madurose

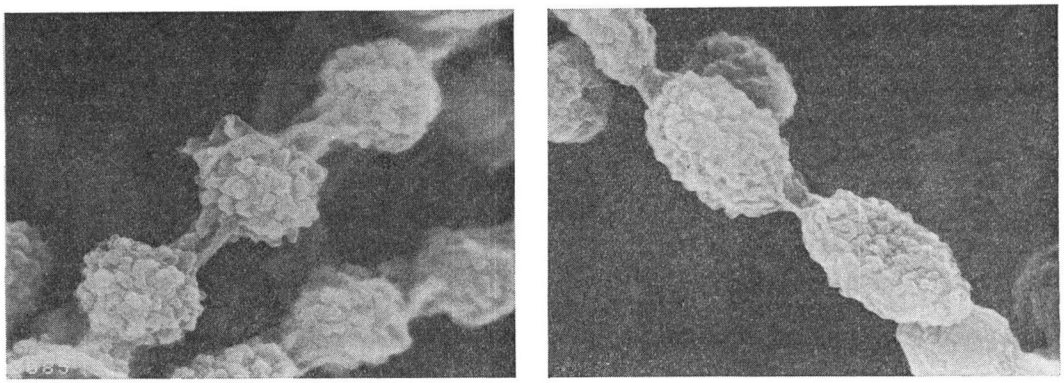
[a] *Meso*-diaminopimelic acid.
[b] Madurose: 3-*O*-methyl-D-galactose.

Table 2. Comparison of carbon utilization pattern and substrate mycelial color between ATCC 39363 and *Actinomadura verrucosospora* ATCC 27299.

Carbohydrate substrate	Growth rating[a]		Substrate mycelial color[b]	
	ATCC 39363	ATCC 27299	ATCC 39363	ATCC 27299
L-Arabinose	4+	3+	Nv	Nv
D-Galactose	3+	4+	Cherry red (7na)	Cherry red (7na)
D-Glucose	2+	2+	Nv	Nv
Glycerol	2+	4+	Coral red (6nc)	Nv
i-Inositol	2+	4+	Brick red (6 1/2 ng)	Brick red (6 1/2 ng)
Inulin	−	±	Nv	Nv
Lactose	2+	2+	Catchup (6 1/2 ng)	Coral red (6nc)
D-Fructose	4+	4+	Nv	Nv
Maltose	3+	4+	Nv	Coral red (6nc)
D-Mannitol	3+	4+	Nv	Nv
Mannose	±	3+	Nv	Nv
Raffinose	−	±	Nv	Nv
L-Rhamnose	3+	2+	Nv	Nv
Salicin	−	−	Nv	Nv
Sucrose	4+	4+	Colonial rose (7ic)	Colonial rose (7ic)
Xylose	2+	4+	Nv	Nv

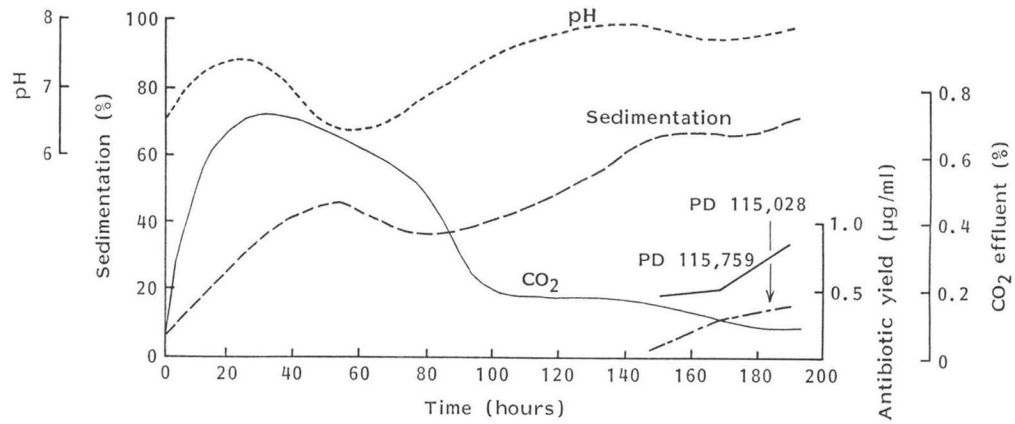
[a] Scale of 1～4, 4 as best; − no growth, ± marginal growth.
[b] Color designation from Color Harmony Manual 4th Ed. Container Corporation of America, 1958. Nv, no vivid color.

Fig. 2. Electron micrographs of the spores of ATCC 39363 and *Actinomadura verrucosospora* ATCC 27299 (14-day old cultures on water agar).



Actinomadura verrucosospora ATCC 27299 (30,600×) ATCC 39363 (30,600×)

Fig. 3. Fermentation pattern of *Actinomadura veractimyces* ATCC 39363 in a 760-liter fermentor.



144-hour fermentation was 400 ng/ml of PD 114,759 and 300 ng/ml of PD 115,028. Significantly smaller quantities of PD 119,707 and PD 119,193 were produced. Typical shake-flask yields in a 144-hour fermentation were 400 ng/ml, 300, 20, 10 for PD 114,759, PD 115,028, PD 119,707, and PD 119,193, respectively.

A fermentation profile of ATCC 39363 in a 760-liter fermentor is shown in Fig. 3. There was an initial growth phase with peak growth achieved about the 48-hour period followed by a brief decline. A second growth phase resumed at about the 80-hour period.

The antibiotic complex was first detectable in the fermentation beer at about the 96-hour period by antimicrobial assay using *Micrococus luteus*. Detectable levels were assayed by HPLC at the 120-hour period, and with peak yield at about the 186-hour period. The antibiotic yields obtained in the 760-liter fermentor were 880 and 400 ng/ml for PD 114,759 and PD 115,028, respectively. PD 119,707 and PD 119,193 components were detected, along with other minor components, but were produced in lesser amounts.

Table 3. Antimicrobial activities of the different antibiotics in the complex.

Microorganisms	Antibiotic components, MIC (ng/ml)			
	PD 114,759	PD 115,028	PD 119,707	PD 119,193
Escherichia coli 04863	1,000	1,000	111	111
Salmonella typhimurium TA 1535	37	333	37	37
Branhamella catarrhalis 03596	<0.46	12.3	<0.46	<0.46
Pseudomonas aeruginosa 05111	1,000	1,000	111	111
Micrococcus luteus 05064	<0.46	<0.46	<0.46	<0.46
Staphylococcus aureus 02482	<0.46	<0.46	<0.46	<0.46
Streptococcus pyogenes C203	<0.46	12.3	<0.46	<0.46
S. pneumoniae SV1	<0.46	<0.46	<0.46	<0.46
Enterococcus faecalis 05045	12.3	37	<0.46	<0.46
Bacillus cereus 04810	<0.46	<0.46	<0.46	<0.46
B. megaterium 066	<0.46	12.3	<0.46	<0.46
Saccharomyces cerevisiae S 288C-α	333	1,000	111	37
Schizosaccharomyces pombe M 1388	1.4	333	<0.46	0.46
Rhodotorula aurantiaca M 1508	1,000	1,000	<0.46	1.4
Torulopsis albida M 1390	1,000	333	111	111
Mucor parasiticus M 2652	1,000	1,000	333	333
Rhizopus japonicus M 1577	1,000	<1,000	333	1,000

Table 4. Side-by-side comparison of antimicrobial activities of antibiotic PD 114,759 and CC-1065.

Microorganisms	MIC (ng/ml)	
	CC-1065	PD 114,759
Escherichia coli 04863	333	1,000
Salmonella typhimurium TA 1535	333	111
Branhamella catarrhalis 03596	12.3	<0.006
Pseudomonas aeruginosa 05111	>1,000	1,000
Micrococcus luteus 05064	4.1	<0.006
Staphylococcus aureus 02482	12.3	<0.006
Streptococcus pyogenes C203	37	<0.006
S. pneumoniae SV1	12.3	<0.006
Enterococcus faecalis 05045	111	<0.006
Bacillus cereus 04810	333	<0.006
B. megaterium 066	333	<0.017
Saccharomyces cerevisiae S 288C-α	1,000	1,000
Schizosaccharomyces pombe M 1388	333	1.4
Rhodotorula aurantiaca M 1508	1,000	1,000
Torulopsis albida M 1390	1,000	1,000
Mucor parasiticus M 2652	1,000	1,000
Rhizopus japonicus M 1577	1,000	1,000

Table 5. *In vitro* activity of the different antibiotics in the complex vs. L1210 murine lymphoid leukemia in tissue culture.

Antibiotic	ID_{50} (ng/ml)
PD 114,759	0.003
PD 115,028	0.011
PD 119,707	0.022
PD 119,193	0.107

Antimicrobial Activity

The antibiotics showed a broad antimicrobial spectrum vs. bacteria, yeast and filamentous fungi. The antibiotics were extremely potent with MIC values of <0.006 ng/ml vs. certain bacteria (Table 4). The filamentous fungi and yeasts showed higher MIC values than the bacteria.

Results of a side-by-side study, comparing the antimicrobial activities of PD 114,759 and Upjohn's CC-1065, demonstrated the marked potency of PD 114,759 (Table 4). The lowest MIC obtained from CC-1065 was 4.1 ng/ml vs. *M. luteus* compared to <0.006 ng/ml for PD 114,759 vs. several Gram-positive and Gram-negative bacteria.

Antitumor Activity

The activities of the different antibiotics vs. L1210 murine lymphoid leukemia in tissue culture

Table 6. *In vivo* antitumor activity of the different antibiotics in the complex vs. P388 lymphocytic leukemia[a].

Dosage[c]	T/C Value[b]			
	PD 114,759	PD 115,028	PD 119,707	PD 119,193
6.4	—	141	—	—
5.0	—	—	—	213
4.0	205	—	—	—
3.2	—	164	—	—
2.5	—	—	204	150
2.0	155	—	—	—
1.6	—	169	—	—
1.25	—	—	188	158
1.00	139	—	—	—
0.80	—	161	—	—
0.625	—	—	141	—
0.50	145	—	—	—
0.40	—	171	—	—
0.313	—	—	145	—

[a] Data provided by NCI (Drug Evaluation Branch, Division of Cancer Treatment).
[b] T/C is the quotient (expressed in %) of the survival time of treated animals (T) and the survival time of control animals (C). T/C values of ≥ 130 is considered active.
[c] μg/kg/injection. Daily$\times 5$.

are shown in Table 5. Of the four antibiotics, PD 114,759 showed the greatest activity, with an ID_{50} value of 0.003 ng/ml.

Table 6 shows the activity of the four antibiotics vs. P388 murine lymphoid leukemia *in vivo*. T/C values of 205, 171, 204, and 213 were obtained with PD 114,759, PD 115,028, PD 119,707, and PD 119,193, respectively. The data reported here were the optimum doses for the different antibiotics. Toxicity was generally observed at >16 μg/kg in the described treatment schedule.

A detailed description of the biochemical and antitumor activities of these antibiotics in other tumor systems will be reported in a separate communication[8].

Discussion

The culture ATCC 39363 has a chemotype III wall and contains the sugar madurose (3-*O*-methyl-D-galactose). Moreover, this isolate has a branching substrate mycelium and exhibits a chain of spores in the form of loops and spirals. The above characteristics of ATCC 39363 classify the organism as an *Actinomadura*[9]. The cultural and physiological characteristics of isolate ATCC 39363 generally resembles that of *Actinomadura verrucosospora**. However, the growth characteristics of ATCC 39363 on the different carbon substrates, and the substrate mycelial color are significantly different from *A. verrucosospora* ATCC 27299. For this reason, we designate ATCC 39363 as a subspecies of *Actinomadura*; the proposed name is *A. verrucosospora* subsp. *veractimyces*. This isolate was deposited with the American Type Culture Collection (Rockville, MD).

The antibiotic complex demonstrated a broad antimicrobial spectrum in the subnanogram level. The antibiotics in the complex appear to be the most potent antitumor antibiotic described to date, exceeding CC-1065[10]. A comparative evaluation of PD 114,759 and known antibacterial and antifungal antibiotics vs. several microbial strains clearly demonstrate the overall superior potency of PD

* A strain of *Actinomadura verrucosospora* has been reported also as a producer of BBM-1675, an antitumor antibiotic complex (German Pat. DE 3,418,023, May 16, 1983; U.S. Pat. 495,231, Japan Kokai 84-232,094, Dec. 26, 1984).

Table 7. Antimicrobial activity of PD 114,759 as compared to known antibacterial (streptomycin, gentamicin) and antifungal (candicidin, griseofulvin) antibiotics.

Microorganisms	MIC (ng/ml)				
	PD 114,759	Streptomycin	Gentamicin	Candicidin	Griseofulvin
Salmonella typhimurium TA 1535	37	<500	<500	—	—
Branhamella catarrhalis 03596	<0.46	<500	<500	—	—
Pseudomonas aeruginosa 05111	1,000	111,000	<500	—	—
Enterococcus faecalis 05045	12.3	36,000	12,000	—	—
Bacillus cereus 04810	<0.46	4,000	500	—	—
B. megaterium 066	<0.46	4,000	500	—	—
Saccharomyces cerevisiae S 288C-α	333	—	—	<150	500
Schizosaccharomyces pombe M 1388	1.4	—	—	150	4,000
Rhodotorula aurantiaca M 1508	1,000	—	—	150	36,000

114,759 (Table 7).

The fermentation pattern of the organism shows two distinct growth phases indicative of a diauxic phenomenon. The production of the antibiotic complex, which seems associated with the second growth phase, started about 96 hours into the fermentation cycle. Such onset of antibiotic production is relativly long, particularly for an actinomycete fermentation.

The fermentation yield of the antibiotic complex was very low, in the submicrogram level. Preliminary studies indicate that the antibiotic complex inhibited the growth of the producing organism when the culture is streaked on to an agar plate containing the antibiotic. It is not known whether the inhibitory effect contributes to the low yield or a feedback inhibition is involved during the fermentation.

Acknowledgments

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