Pyrrolosporin A, a New Antitumor Antibiotic from *Micromonospora* sp. C39217-R109-7

I. Taxonomy of Producing Organism, Fermentation and Biological Activity

KIN SING LAM*, GRACE A. HESLER, DONALD R. GUSTAVSON, RONALD L. BERRY, KOJI TOMITA, JUDITH L. MACBETH, JOHN ROSS†, DAVID MILLER† and SALVATORE FORENZA

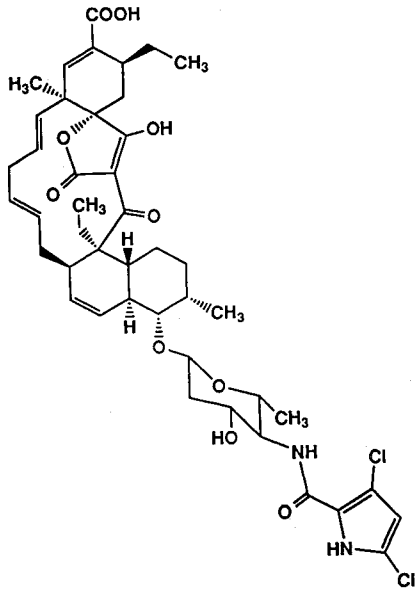
Bristol-Myers Squibb Pharmaceutical Research Institute,
5 Research Parkway, Wallingford, Connecticut 06492, U.S.A.
†Frederick Cancer Research and Development Center,
P.O. Box B, Frederick, Maryland 21702, U.S.A.

(Received for publication March 6, 1996)

Strain C39217-R109-7 (ATCC 53791) is an actinomycete strain isolated from a soil sample collected at Puerto Viejo, Peru. It produces a new antitumor antibiotic, designated pyrrolosporin A. Taxonomic studies on its morphological, cultural and physiological characteristics identified this producing strain as *Micromonospora* sp. C39217-R109-7. Pyrrolosporin A shows antimicrobial activity against Gram-positive bacteria and it is weakly active against Gram-negative bacteria. Pyrrolosporin A prolongs the life span of mice inoculated with P388 leukemia cells.

In the course of our continuing search for novel antitumor antibiotics, an actinomycete strain C39217-R109-7 (ATCC 53791) isolated from a soil sample collected at Puerto Viejo, Peru, was found to produce a new antitumor antibiotic designated pyrrolosporin A. Pyrrolosporin A (Fig. 1) is isolated as a white crystalline solid having a MW of 879 and MF of $C_{44}H_{54}Cl_2N_2O_{10}$. Pyrrolosporin A has the unique feature of a 13-membered macrolide in which a spiro-α-acyltetronic acid moiety constitutes the lactone group. Pyrrolosporin A exhibits *in vivo* antitumor activity against P388 leukemia. Pyrrolosporin A also shows activity against Gram-positive bacteria and is weakly active against Gram-negative bacteria. Taxonomic studies show that the producing strain is related to *Micromonospora narashinoensis* and *Micromonospora globosa*. Production titers of pyrrolosporin A in the shake flask and fermenter cultures are 468 μg/ml and 154 μg/ml, respectively. The present paper describes the taxonomy of the producing organism, fermentation and biological activity. The isolation and physico-chemical characterization of pyrrolosporin A will be described in the following paper[1].

Fig. 1. Structure of pyrrolosporin A.



Taxonomy of the Producing Strain

The carbon utilization pattern of strain C39217-R109-7 was determined by the method of SHIRLING and GOTTLIEB[2] excepting the inclusion of a 3-hour starvation period between the harvesting and inoculation steps. Washed vegetative cells were shaken at 250 rpm and 28°C on a rotary shaker in a liquid version of International Streptomyces Project (ISP) medium No. 9 with no carbon source. As summarized in Table 1, D-glucose, cellobiose,

Table 1. Carbon utilization of strain C39217-R109-7.

Utilization	D-Glucose, cellobiose, L-rhamnose, trehalose, soluble starch
Moderate utilization	Glycerol, D-ribose
No utilization	D-Mannitol, D-mannose, D-fructose, D-galactose, lactose, L-arabinose, D-arabinose, raffinose, xylose, sucrose, maltose, sorbose, melezitose, melibiose, salicin, dulcitol, cellulose, inositol

Table 2. Cultural characteristics of strain C39217-R109-7.

Medium	Growth of vegetative mycelium	Reverse color	Aerial mycelium	Soluble pigment
ISP No. 2	Moderate, raised	Dark reddish orange (38)	None	None
ISP No. 3	Fair	Strong yellowish pink (26)	None	None
ISP No. 4	Poor	Dark yellowish pink (30)	Scant, pink	None
ISP No. 5	Fair, raised	Deep reddish orange (36)	Scant, pink	None
ISP No. 6	Poor	Medium yellowish pink (29)	None	Bright pink (33)
ISP No. 7	Fair	Light reddish brown (42)	None	None
Glucose-asparagine agar	Fair	Medium yellowish pink (29)	None	None
CZAPEK's sucrose-nitrate agar	Scant	Grayish reddish orange (39)	None	None

Color names and numbers from the ISCC-NBS Color-Name Charts, Publication 440, Washington, D.C., 1976.

L-rhamnose, trehalose, soluble starch, glycerol and D-ribose are utilized by strain C39217-R109-7 for growth.

The cultural and physiological characteristics of strain C39217-R109-7 are given in Tables 2 and 3, respectively. Macroscopic cultural characteristics of strain C39217-R109-7 were observed after a 14 day incubation period at 28°C on various media. Slant cultures were observed further at one week intervals for a two month period. Strain C39217-R109-7 grows poorly in most descriptive media. The aerial mycelium is formed on ISP media Nos. 4 and 5. The color of the aerial mycelium is pink. Bright pink pigment is formed in ISP medium No. 7. Microscopic examination of strain C39217-R109-7 revealed a fine branched vegetative mycelium bearing monospores. Examination of liquid cultures shows fragments of hyphae bearing spores in clusters. Also observed were irregular globose structures resembling those described for *M. globosa*[3]. The temperature range for optimal growth of strain C39217-R109-7 was 30~35°C. The temperature range for growth was 22~42°C. There was no growth on yeast extract-malt extract supplemented with more than 4% NaCl. Nitrate was reduced in peptone-nitrate broth. Gelatin liquefaction and hydrolysis of starch also gave positive reactions. In Difco skimmed milk there was peptonization and coagulation.

Analysis of whole-cell hydrolyzates was performed according to the methods of SCHAAL[4]. Strain C39217-R109-7 contains *meso*-diaminopimelic acid, xylose, glucose and mannose. Phosphatidylethanolamine was the only phospholipid detected by the method of LECHEVALIER and LECHEVALIER[5]. Strain C39217-R109-7 was assigned cell wall type II_D with a P-II phospholipid pattern, consistent with members of the genus *Micromonospora*.

Table 3. Physiological characteristics of strain C39217-R109-7.

Growth temperature	22~42°C
NaCl tolerance	1~3% (+), 4% (−)
Starch hydrolysis	+
Gelatin liquefaction	+
Milk coagulation	+
Milk peptonization	+
Tyrosinase reaction	Weak
H_2S production	−

As shown in Table 4, strain C39217-R109-7 has properties resembling both *M. globosa* and *M. narashinoensis*. Strain C39217-R109-7 was more closely related to *M. narashinoensis* in the cultural characteristics on nutrient agar and tyrosine agar, and in the physiological characteristics of gelatin liquefaction and milk peptonization. However, strain C39217-R109-7 is more closely related to *M. globosa* in the colony texture, spore morphology and nitrate reduction reaction. Since the irregular swelling structures have been reported in species other than *M. globosa*[6], it is believed that strain C39217-R109-7 represents a new strain related to *M. globosa* and *M. narashinoensis*.

Production of Pyrrolosporin A in Shake Flask Culture

Working stock of strain C39217-R109-7 was prepared by growing strain C39217-R109-7 in test tubes on agar slants of yeast extract-malt extract supplemented with $CaCO_3$. This medium consisted of glucose 0.4%, yeast extract 0.4%, malt extract 1%, $CaCO_3$ 0.15% and agar 1.5%. The culture was incubated 10~14 days at 28°C until uniform production of spores was obtained. The surface growth of the slant culture was transferred into

Table 4. Comparison of strain C39217-R109-7 with published descriptions of *M. globosa* and *M. narashinoensis*.

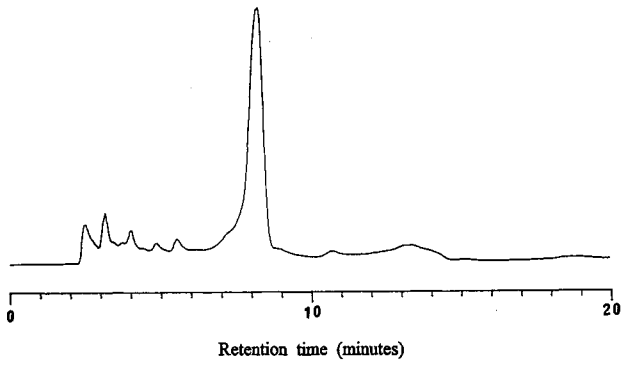
Culture or physiological characteristics	C39217-R109-7	*M. globosa*	*M. narashinoensis*
Colony color	Pink-orange to orange	Pale yellow to red-orange	Orange
Colony texture	Plicate	Leathery, becoming plicate (2 weeks)	Folded
Spore morphology	Monospores, irregular swellings and bulbs	Monospores 1~3 μm, irregular swellings and bulbs	Spherical or elongate monospores, 0.6~1.0 μm by 0.9~1.8 μm
Temperature range for growth	22~42°C	Unable to grow at 45°C	Not available
Nutrient agar	Minute, tan	Deep orange with pale orange-yellow pigment	Minute, orange to tan colonies, spore layer at periphery
Tyrosine agar	Minute, pale orange, pink/purple pigment	Pale orange-yellow, no pigment	Minute, orange colonies, purplish pigment
Starch hydrolysis	Positive	Positive (under colonies only)	Positive
Nitrate reduction	Positive	Positive (Gilkey's)	Negative
Gelatin liquefaction	Positive	Variable	Positive
Milk	Rapidly digested	Slowly peptonized	Digested

a 500-ml Erlenmeyer flask, containing 100 ml of the vegetative medium consisting of starch 2%, cerelose 0.5%, Pharmamedia 1%, yeast extract 1% and $CaCO_3$ 0.2%. The vegetative culture was incubated at 28°C for 3 days on a rotary shaker set at 250 rpm. The vegetative culture was mixed with equal volume of cryoprotective solution consisting of 10% sucrose and 20% glycerol. Five milliliter portions of this mixture were transferred to sterile cryogenic tubes (5 ml capacity) and were stored at −80°C until use.

A vegetative culture of strain C39217-R109-7 was prepared by transferring 5 ml of the cryopreserved culture to a 500-ml Erlenmeyer flask containing 100 ml of the vegetative medium. The vegetative culture was incubated at 28°C and 250 rpm on a rotary shaker. After 3 days, 5 ml aliquots were transferred to 500-ml Erlenmeyer flasks containing 100 ml of the production medium. The production medium consisted of starch 3%, Bacto-liver 1%, Pharmamedia 0.5%, NaCl 0.3%, $(NH_4)_2SO_4$ 0.1% and $CaCO_3$ 0.6%. The production cultures were incubated at 28°C and 250 rpm on a rotary shaker.

The production of pyrrolosporin A in the fermentation was monitored by HPLC using a C-18 reversed-phase column (μBondapak, 3.9 × 300 mm, Waters Associates) and UV absorption at 268 nm. The solvent system was 0.1 M ammonium acetate - CH_3OH - CH_3CN (1 : 1 : 1) with a flow rate of 2 ml/minute. The fermentation extract was processed by extracting the culture broth with an equal volume of ethyl acetate. The ethyl acetate fraction was concentrated 10-fold and 5~10 μl of the concentrated extract was used for HPLC analysis. The metabolite production profile of an extract from a

Fig. 2. HPLC of a fermentation extract of 168-hour culture of strain C39217-R109-7.

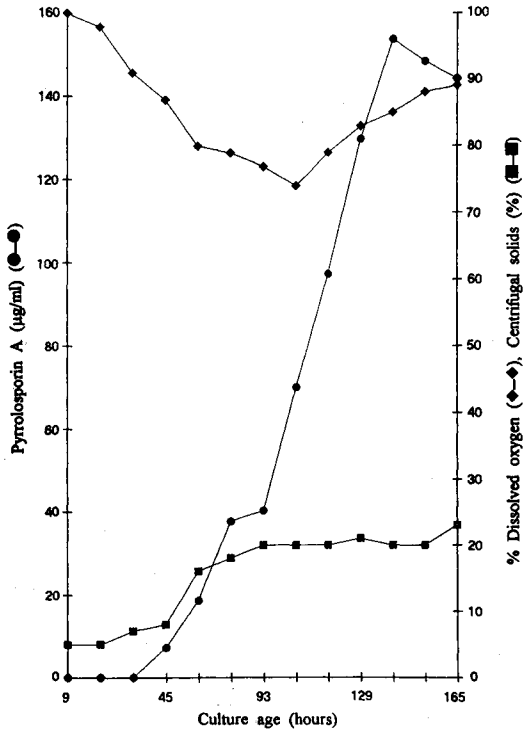


168-hour old shake flask culture analyzed by HPLC is shown in Fig. 2. The major metabolite with retention time of 8.1 minutes was pyrrolosporin A. The production of pyrrolosporin A in shake flask culture reached a maximum titer of 468 μg/ml at 168 hours.

Production of Pyrrolosporin A in Fermenter Culture

For production in a pilot plant fermentor, the first seed culture was prepared as described above. Fifty ml of this seed culture was transferred to a 4-liter Erlenmeyer flask containing 1 liter of the vegetative medium. The second seed culture was incubated at 28°C and 250 rpm on a rotary shaker for 3 days. Four flasks of the second seed culture was combined and transferred to a 100-liter fermenter containing 68 liters of the vegetative medium supplemented with 35 ml of polypropylene glycol P-2000 as antifoam. The third seed culture was incubated

Fig. 3. Time course of pyrrolosporin A fermentation by strain C39217-R109-7 in a 1000-liter fermenter containing 680 liter of production medium.



at 28°C, agitation rate of 250 rpm, aeration rate of 0.8 vvm and back pressure of 0.35 kg/cm^2, for 3 days. Thirty-six liters of the third seed culture was then inoculated into a 1000-liter fermenter containing 680 liters of production medium supplemented with 700 ml polypropylene glycol P-2000 as antifoam. The fermentation was incubated for 165 hours under the following conditions: temperature, 28°C, agitation, 125 rpm, aeration, 0.7 vvm and back pressure, 0.35 kg/cm^2.

The time course of pyrrolosporin A fermentation by strain C39217-R109-7 in the pilot plant fermenter is shown in Fig. 3. The start of the production of pyrrolosporin A was detected at around 45 hours and reaching a maximum titer of 154 μg/ml at 141 hours. Since the percent dissolved oxygen tension dropped gradually from 100% at the time of inoculation to 80% at 69 hours before started to levelling off, this time interval should correspond to the active growth phase of strain C39217-R109-7 in the fermenter culture. This is in good agreement with the values of the centrifugal solids of the fermentation which was increased from 5% at 9 hours to 16% at 69 hours before gradually levelling off indicating that the end of the active growth phase of the organism in the fermenter culture was at 69 hours. The active production phase of pyrrolosporin A was observed from 93 to 141 hours at a rate of production of >2 μg/ml. Therefore the majority of pyrrolosporin A production occurred at the stationary phase of the fermentation.

Table 5. Effect of pyrrolosporin A on P388 leukemia.

Treatment schedule	Dose, ip (mg/kg/inj)	Effect (% T/C)
QD 1 to 5 days	135	Toxic
	45	135
	15	130
	Control	100

Tumor inoculum: 10^6 ascites, ip.
Host: CDF_1 mice.
Evaluation: MST = median survival time.
Effect: % T/C = (MST treated/MST control) × 100. Control mice had an MST of 10 days.
Criteria: % T/C ≥ 125 considered significant antitumor activity.
Euthanasia was not performed on dying mice; they were allowed to die naturally. The experiment performed was approved by the Bristol-Myers Squibb Company Animal Care and Use Committee.

Table 6. Antimicrobial spectrum of pyrrolosporin A.

Test organism	MIC (μg/ml)
Enterococcus faecalis A20688	4
E. faecalis ATCC 29212	4
E. faecalis ATCC 33186	4
Staphylococcus aureus A9537	0.5
S. aureus A20698	1
S. aureus ATCC 29213	0.5
Bacillus subtilis ATCC 6633	2
Escherichia coli A15119	125
E. coli A20697	125
E. coli ATCC 33176	63
Klebsiella pneumoniae A9664	125
K. pneumoniae A20468	125
Proteus vulgaris A21559	125
Pseudomonas aeruginosa A9843	63
P. aeruginosa ATCC 27853	125

Biological Activity

The *in vivo* antitumor activity of pyrrolosporin A was evaluated against P388 leukemia in CDF_1 mice according to the previously described protocols[7,8]. There were 4 mice per treatment group and 6 mice in the control group. The results are summarized in Table 5. All doses were administered ip, once daily for five days, beginning on day 1 post-leukemia implant. The highest dosage evaluated in P388 leukemia assay, 135 mg/kg/injection, caused the death of the mice and so was considered too toxic a dose level. The effective dose ranged from 15 to 45 mg/kg with the maximum effect (35% increase of life span) achieved at a dose 45 mg/kg. The 35% increase in

lifespan was statistically significant ($P<0.01$) based on the statistical comparison using GEHAN's generalized WILCOXON test[9].

The antimicrobial spectrum of pyrrolosporin A were determined by serial broth dilution method using Nutrient broth (Difco). The results are summarized in Table 6. Pyrrolosporin A was effective against Gram-positive bacteria and only weakly against Gram-negative bacteria.

Conclusion

Culture C39217-R109-7, identified as a species of *Micromonospora* related to *M. narashinoensis* and *M. globosa* was found to produce a new antitumor antibiotic. This compound, designated pyrrolosporin A, possesses weak *in vivo* activity against P388 leukemia in CDF_1 mice, and *in vitro* activity against Gram-positive bacteria. The condition for scale-up production of pyrrolosporin A was established and titer as high as 154 μg/ml was achieved in the 680 liter tank fermentation. The isolation and physico-chemical characterization of pyrrolosporin A will be described in the following paper[1].

References

1) SCHROEDER, D. R.; K. L. COLSON, S. E. KLOHR, M. S. LEE, J. A. MATSON, L. S. BRINEN & J. CLARDY: Pyrrolosporin A, a new antitumor antibiotic from *Micromonospora* sp. C39217-R109-7. II. Isolation, physico-chemical properties, spectroscopic study and X-ray analysis. J. Antibiotics 49: 865~872, 1996
2) SHIRLING, E. B. & D. GOTTLIEB: Methods for characterization of *Streptomyces* species. Int. J. Syst. Bacteriol. 16: 313~340, 1966
3) KRISS, A. E.: The micromonospore—an actinomycete-like organism (*Micromonospora globosa* n. sp.). Mikrobiologiya 8: 178~185, 1939
4) SCHAAL, K. P.: *In* Chemical Methods in Bacterial Systematics. *Eds.*, GOODFELLOW and MINNIKIN, pp. 359~381, Academic Press, 1985
5) LECHEVALIER, M. P. & H. A. LECHEVALIER: *In* Actinomyete Taxonomy. *Eds.*, A. DIETZ and D. W. THAYER, pp. 227~291, Society for Industrial Microbiology, 1980
6) LUEDEMANN, G. M.: *Micromonospora* taxonomy. Adv. Appl. Microbiol. 11: 101~133, 1969
7) GERAN, R. I.; N. H. GREENBERG, M. M. MACDONALD, A. M. SCHUMACHER & B. J. ABBOTT: Protocols for screening chemical agents and natural products against tumors and other biological systems (3rd Ed.). Cancer Chemother. Rep. (part 3) 3: 1~103, 1972
8) BRADNER, W. T.: Transplanted animal tumors. *In* Cancer and Chemotherapy, Vol. 1, *Eds.*, S. T. CROOKE & A. W. PRESTAYKO, pp. 221~227, Academic Press, 1980
9) GEHAN, G. A.: A generalized Wilcoxon test for comparing arbitrarily singly-censored samples. Biometrika 52: 203~233, 1985