The Clecarmycins, New Antitumor Antibiotics Produced by *Streptomyces*: Fermentation, Isolation and Biological Properties

NOBORU FUJII*, TOSHIYUKI KATSUYAMA, EIJI KOBAYASHI, MITSUNOBU HARA† and HIROFUMI NAKANO†

Pharmaceutical Research Laboratories, Kyowa Hakko Kogyo Co., Ltd., 1188 Shimotogari, Sunto, Shizuoka 411, Japan
†Tokyo Research Laboratories, Kyowa Hakko Kogyo Co., Ltd., 3-6-6 Asahimachi, Machida, Tokyo 194, Japan

(Received for publication January 25, 1995)

In the course of screening for microbial products with antitumor activity, new antitumor agents, clecarmycins, were isolated from culture broth of *Streptomyces* sp. DO-114. The antibiotics were produced in a fermentation medium supplemented with a highly porous polymer resin which adsorbs antibiotics and results in a increase of titer. Active materials were separated from the polymer resin by solvent extraction procedure and two components named clecarmycin A1 and C were isolated by silica gel column chromatography. These were active against bacteria, and showed antiproliferative activities against human HeLa S3 cells. Clecarmycins exhibited antitumor activity against leukemia P388 and sarcoma 180 in mice.

Most clinically useful antitumor agents including mitomycin C, adriamycin, bleomycin and cis-platin function by initiating DNA damage[1]. With this knowledge, we focused our efforts on the discovery of novel DNA-active antitumor antibiotics, and have screened cultures of actinomycetes and fungi using a microbial prescreen (DC screening) with both wild type of *Bacillus subtilis* and strains which are supersensitive to DNA-active drugs[2].

In the course of our continued DC screening program, a culture designated DO-114 was isolated from a soil of Japan and was found to produce antitumor compounds (Fig. 1). Two active components named clecarmycin A1 and C were isolated. The clecarmycins inhibited the growth of rec^- and $polA^-$ strains of *Bacillus subtilis* more selectively than that of wild type, suggesting that DNA is the primary target of the clecarmycins. Clecarmycins showed antimicrobial and anticellular activity *in vitro*, and antitumor activity *in vivo*.

We report here the taxonomy of the producing strain, fermentation, isolation and biological activity of clecarmycins. Physico-chemical properties and structure determination of these compounds are reported in the following paper[3].

Materials and Methods

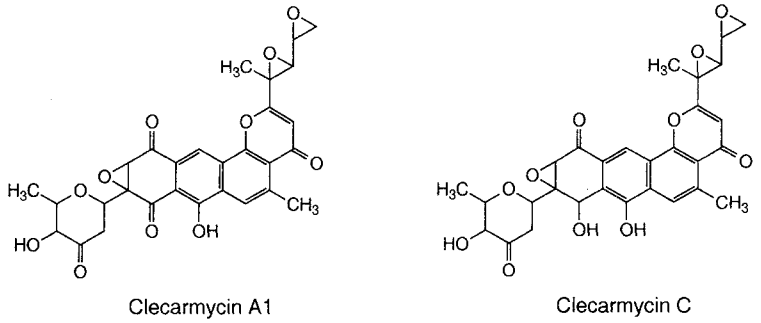
Microorganism

The producing microorganism was isolated from a soil sample collected at Ami-cho, Ibaragi, Japan.

Taxonomic Studies

Growth characteristics and carbohydrate utilization were determined by the methods of the International Streptomyces Project (ISP)[4]. Color codes were assigned to the substrate and aerial mass pigments according to the Color Harmony Manual, 4th Ed., 1958 (Container Corporation of America, Chicago). The spores and mycelia were observed with a scanning electron

Fig. 1. Structures of clecarmycin A1 and C.



Clecarmycin A1 Clecarmycin C

microscope (Model S-570, Hitachi Co., Ltd.).

Diaminopimelic acid of the cell wall was analyzed on the hydrolysate of cultures grown in a medium (glucose 10 g, starch 10 g, beef extract 3 g, yeast extract 5 g, $CaCO_3$ 2 g per liter of tap water, pH 7.0) for 48 hours at 28°C by the method of BECKER *et al.*[5].

Fermentation

Seed medium contains glucose 10 g, soluble starch 10 g, Bacto-tryptone 5 g, yeast extract 5 g, beef extract 3 g and $CaCO_3$ 2 g per liter (pH 7.2 prior to sterilization). Fermentation medium contains glycerol 25 g, glucose 25 g, dry yeast 15 g, KH_2PO_4 0.5 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, $CaCO_3$ 5 g and antifoam agents LG 109, 0.3 ml (Asahi Denka Kogyo, Japan) and KM70, 0.3 ml (Shinetsu Kagaku, Japan) per liter (pH 7.0 prior to sterilization).

Growth of the organism was evaluated as packed cell volume (PCV) by centrifuging the fermentation broth in a 10-ml graduated conical tube at 1200 × *g* for 10 minutes. The PCV was recorded as % of total broth volume.

To monitor the production of antibiotics during the fermentation, whole broth was combined with a half volume of *iso*-PrOH and the antibiotics thus extracted from Diaion HP-20 were determined by high performance liquid chromatography (HPLC). HPLC was performed using a column of YMC-Pack ODS-AM-312 (150 × 6 mm, 5 μm particle size, YMC Co.) at 25°C with an eluate of water-MeOH (3:7) containing 5 mM ammonium acetate-acetic acid buffer (pH 4.0). The flow rate was 1 ml per minute, and UV absorption of the eluate was monitored at 245 nm. Clecarmycin C, the major component of clecarmycins was eluted at a retention time of 10.2 minutes.

Antimicrobial Activity

The *in vitro* antimicrobial activities of the clecarmycins were determined on the nutrient agar (0.1% glucose, 0.3% Bacto Tryptone, 0.3% meat extract, 1.6% agar) by a twofold serial dilution method[6]. The lowest concentration that inhibited growth of a bacterial strain after 18 hours of incubation at 37°C was recorded as the MIC.

Anticellular and Antitumor Activity

Human uterine cervix carcinoma HeLa S3 cells were obtained from American Type Culture Collection through Dainippon Pharmaceutical Co. (Osaka, Japan). Cytotoxicity was determined as described previously[7].

Antitumor activity *in vivo* was determined as follows[8,9]. P388 cells (1×10^6 cells/mouse) were transplanted ip into $CD2F_1$ mice on day 0, and drugs were administered ip on day 1. Antitumor efficacy was expressed as a percentage of increase in life span (ILS). Sarcoma 180 (5×10^6 cells/mouse) was inoculated sc at the axillary region in ddY mice on day 0. Drugs were administrated ip or iv on day 1 and the tumor volume was measured on day 7. These experiments were conducted with 5 mice in a group.

Results and Discussion

Taxonomy of the Producing Strain

The appearance of strain DO-114 on nine solid media is presented in Table 1. The vegetative mycelia grew on both synthetic and complex media. The aerial mycelium was gray to white colored and branched. It bore chains of 10 to 30 or more spores which were in the form of curves or loops. Scanning electron micrographs indicated that the spore was oval, in size 0.5 × 0.7 μm, and smooth-surfaced (Fig. 2). The substrate mycelium was branched but not fragmented.

Physiological properties of strain DO-114 are summarized in Table 2. Temperature range for growth was from 16~37°C with the optimum in a range of 28~32°C. Starch hydrolysis, milk peptonization and cellulose decomposition were positive, while gelatin liquefaction and milk coagulation could not be detected. D-glucose, D-xylose, D-mannitol, L-arabinose, L-rhamnose, raffinose, lactose, sucrose and D-galactose were utilized as a sole carbon source but not inositol and salicin. Analysis of the whole-cell hydrolysate by TLC revealed the presence of the L,L-isomer of diaminopimelic acid, indicating that the cell wall belongs to type I.

From the cultural, morphological and physiological characteristics observed, strain DO-114 was identified as *Streptomyces*[10~14]. Compared with the published descriptions of *Streptomyces* species[10~14], strain DO-114 closely resembles to *Streptomyces galilaeus*. The strain has been deposited in the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Japan with the name of *Streptomyces* sp. DO-114. The accession number of strain DO-114 is FERM BP-2641.

Fermentation

The culture of strain DO-114 on agar slant (ISP MEDIUM 4, Difco Laboratories, Detroit, U.S.A.) was inoculated into a 300-ml Erlenmyer flask containing 50 ml of the seed medium. After 3 days incubation on a rotary shaker (200 rpm) at 28°C, 6 ml of the resulting seed culture was inoculated into a 2-liter Erlenmyer flask containing 300 ml of the same seed medium and culture as described above. After 24 hours, 1.8 liters of the second culture was inoculated into a 200-liter fermenter containing 100 liters of the same seed medium and cultured at 28°C for 22 hours under aeration at 60 liters per minute with agitation at 150 rpm. 100 liters of the third seed culture was transferred into a 2000-liter tank fermenter containing 1000 liters of fermentation medium and cultured at 27°C under aeration at 400 liters per

Table 1. Cultural characteristics of strain DO-114.

Sucrose-nitrate agar	G:	good
	AM:	abundant, white
	SM:	camel (3ie)
	P:	found, light yellow
Glucose-asparagine agar	G:	normal
	AM:	abundant, cobalt gray (2fe)
	SM:	cobalt gray (2fe) to bisque (3ec)
	P:	not found
Glycerol-asparagine agar	G:	good
	AM:	abundant, cobalt gray (2fe)
	SM:	toast tan (4lg) to natural (2dc)
	P:	not found
Inorganic salts-starch agar	G:	good
	AM:	abundant, cobalt gray (2fe)
	SM:	bamboo (2gc)
	P:	not found
Tyrosine agar	G:	good
	AM:	abundant, cobalt gray (2fe)
	SM:	dark brown (2pn)
	P:	found, light yellow
Nutrient agar	G:	good
	AM:	abundant, cobalt gray (2fe)
	SM:	camel (3ie)
	P:	not found
Yeast extract-malt extract agar	G:	good
	AM:	abundant, cobalt gray (2fe)
	SM:	golden brown (3pi) to yellow maple (3ng)
	P:	found, yellow
Oatmeal agar	G:	good
	AM:	abundant, dark cobalt gray (2ih)
	SM:	light brown (3lg)
	P:	found, brown
Peptone-yeast extract-iron agar	G:	good
	AM:	not found
	SM:	camel (3ie)
	P:	found, brown

Abbreviations: G, growth degree; AM, formation and color tone of aerial mycelium; SM, color tone of substrate mycelium; P, color tone of soluble pigment.

Fig. 2. Scanning electron micrographs of strain DO-114.

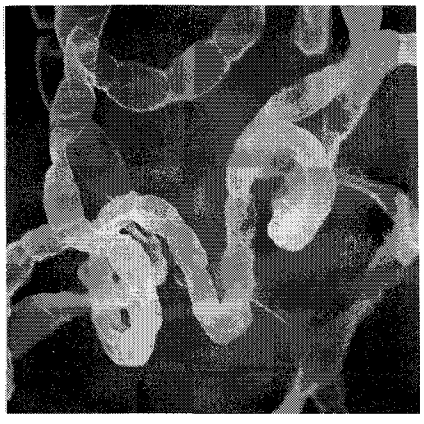


Table 2. Physiological properties of strain DO-114.

Temperature range for growth	16~37℃ (Optimum, 28~32℃)
Liquefaction of gelatin	-
Hydrolysis of starch	+
Coagulation of milk	-
Peptonization of milk	+
Decomposition of cellulose	+
Utilization of carbon source	
D-Glucose	+
D-Xylose	+
D-Mannitol	+
L-Arabinose	+
L-Rhamnose	+
Raffinose	+
Lactose	+
Sucrose	+
D-Galactose	+
Inositol	-
Salicin	-
Type of diaminopimelic acid	LL

+: Positive, −: Negative.

minute with agitation at 120 rpm. High porous polymer resin Diaion HP-20 (50 liters) was added at 18 hours of incubation, because examination in shake flasks indicated that the addition of Diaion HP-20 resin (5 volume %) to the fermentation medium resulted in a significant increase in accumulation of clecarmycins (data not shown). Fig. 3 shows a typical time course for production of clecarmycins in a 2000-liter tank fermentation, The clecarmycin C production increased concomitantly with gradual increase of mycelium volume and reached a maximum at 46 hours.

Isolation

The clecarmycins were isolated from the culture broth

described above by the following procedure. The whole culture broth was passed through a vibrating screen (Kawasaki heavy industries, LTD. Japan) in order to isolate resin from mycelia. The recovered Diaion HP-20 resin was suspended in deionized water and then packed into a column. The column was washed with 150 liters of deionized water - MeOH (1 : 1) and eluted with 150 liters of MeOH. The active eluate was concentrated under reduced pressure to give an aqueous solution (5 liters). The aqueous solution was diluted with an equal volume of water and extracted twice with 10 liters of EtOAc. The active EtOAc extract was concentrated after dehydration over anhydrous Na_2SO_4 to give a crude powder. The clecarmycins crude powder was applied to a silica gel column chromatography (10 liters, BW-300, Fuji Davison, Japan). The column was washed with 30 liters of toluene - acetone (8 : 1) and the first active fractions containing clecarmycin A1 were eluted with 30 liters of toluene - acetone (4 : 1) and the second active fractions containing clecarmycin C were eluted with 30 liters of toluene - acetone (2 : 1). The first active fractions containing clecarmycin A1 were pooled and further purified by silica gel chromatography (1 liter, LiChroprep Si60, Merk) with 3 liters of toluene - acetone (4 : 1). Each active fraction was concentrated to give precipitates. The resulting precipitates were collected and dried *in vacuo* to afford clecarmycin A1 (0.67 g) and clecarmycin C (3.6 g).

Fig. 3. Time course of clecarmycins fermentation in a 2000-liter tank fermentor.

pH (○), PCV (□), clecarmycin C production (●).

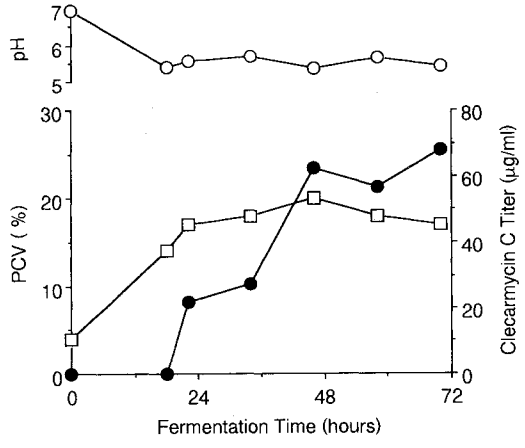


Table 3. Antimicrobial spectrum of the clecarmycins.

Organism	MIC (μg/ml) A1	C
Staphylococcus aureus ATCC 6538P	0.04	0.16
Enterococcus faecium ATCC 10541	0.16	0.16
Bacillus subtilis No.10107	0.33	0.33
Klebsiella pneumoniae ATCC 10031	5.2	5.2
Escherichia coli ATCC 26	5.2	21
Pseudomonas aeruginosa Bin H No. 1	5.2	42
Salmonella typhi ATCC 9992	5.2	21
Proteus vulgaris ATCC 6897	2.6	5.2
Shigella sonnei ATCC 9290	5.2	21
Candida albicans ATCC 10231	5.2	> 83

Biological Activity

Antimicrobial Activity

The antimicrobial activity of the clecarmycins is shown in Table 3. The clecarmycins exhibited antimicrobial activity, particularly against Gram-positive bacteria. Clecarmycin A1 was more active than clecarmycin C against Gram-negative bacteria and *C. albicans* with MIC values in the μg/ml range.

Anticellular Activity

Clecarmycins were potent cytotoxic agents in the ng/ml range against HeLa S3 cells *in vitro* (Table 4). The IC_{50} value of clecarmycin A1 and C against HeLa S3 cells were 6.58 ng/ml and 11.6 ng/ml respectively which were 10 ~ 20-fold more active than adriamycin.

Antitumor Activity on Ascitic Tumor (P388) in Mice

The antitumor activity of the clecarmycins against

Table 4. Cytotoxicity of the clecarmycins.

Compounds	IC_{50} (ng/ml)
Clecarmycin A1	6.58
Clecarmycin C	11.5
Adriamycin	121

Table 5. Antitumor activity of clecarmycins against P388 leukemia.

Compounds	Dosage (mg/kg)	Mean survival (days ± SD)	ILS[a] (%)
Clecarmycin A1	1.5	12.8 ± 2.2	36
	3.0	14.4 ± 1.7	53
	6.0	13.0 ± 2.0	38
	12.0	10.4 ± 3.6	11
Clecarmycin C	0.75	13.8 ± 2.0	47
	1.5	16.6 ± 2.9	77
	3.0	16.2 ± 2.3	72
	6.0	15.0 ± 2.6	60
Adriamycin	3.3	16.2 ± 3.4	72
	6.3	20.2 ± 5.5	115
	13.0	> 26.0 ± 5.5 (3)[b]	> 177
	25.0	6.6 ± 0.5	-30
Control	0.0	9.4 ± 0.5	0

[a] ILS (%) = [(T/C) − 1] X 100
T : survival days of treated mice, C : survival days of control mice
[b] Survivors more than 30 days.

Table 6. Antitumor activity of clecarmycins against sarcoma 180.

Compounds	Route	Dosage (mg/kg)	T/C[a] (%)	B.W.[b] (g)
Clecarmycin A1	i.p.	0.75	65	+7.6
		1.5	77	+5.4
		3.0	38	+2.2
		6.0	12(4)[c]	0.0
Clecarmycin C		1.5	88	+9.4
		3.0	59	+6.2
		6.0	39	+5.0
		12	Toxic	-
Clecarmycin A1	i.v.	0.38	82	+8.6
		0.75	78	+8.2
		1.5	56	+8.0
		3.0	Toxic	-
Clecarmycin C		1.5	70	+8.8
		3.0	41	+8.2
		6.0	20	+5.6
		12	Toxic	-
Adriamycin		6.8	67	+6.8
		14	31	+1.0
Control	-	0	100[d]	+7.0

[a] Treated *versus* control value.
[b] Body weight change between day 1 and day 7.
[c] Mortality (5 mice in a group).
[d] Tumor volume : 3445 mm^3.

P388 leukemia were examined and compared with that of adriamycin by single ip administration (Table 5). As shown in Table 5, clecarmycin A1 and C prolonged the life span of mice bearing P388 leukemia with the maximum ILS of 53% and 75% respectively which were less active than that (>177%) observed for adriamycin.

Antitumor Activity on Murine Solid Tumor (Sarcoma 180) in Mice

The antitumor activity of the clecarmycins against Sarcoma 180 were examined by single ip or iv administration (Table 6). When the drugs were given by ip administration, clecarmycin A1 and C showed antitumor activity against sarcoma 180 with T/C values less than 40%. While by iv administration, clecarmycin C showed the strongest activity with the minimum T/C of 20%, which was superior to that (T/C 31%) of adriamycin, whereas clecarmycin A1 showed the marginal regression (T/C 58%) in this system. Further studies on the antitumor activities of clecarmycins are in progress.

Acknowledgements

We thank N. MORISHIMA and M. KUSUNOKI for skillful technical assistance.

References

1) FISHER, J. F. & P. A. ARISTOFF: The chemistry of DNA modification by antitumor antibiotics. Prog. Drug. Res. 32: 411~498, 1988
2) NAKANO, H.; T. TAMAOKI & F. TOMITA: Mechanism based screens for national product leads as sources for antitumor drugs. ACS Conference Proceeding Series: 72~75, 1992
3) UOSAKI, Y.; N. FUJII, M. HARA, H. NAKANO & Y. SAITOH: Clecarmycins, new antitumor antibiotic produced by *Streptomyces*. Structure determination. J. Antibiotics, in preparation
4) SHIRLING, E. B. & D. GOTTLIEB: Methods for characterization of *Streptomyces* species. Int. J. Syst. Bacteriol. 16: 313~340, 1966
5) BECKER, B.; M. P. LECHEVALIER, R. E. GORDON & H. A. LECHEVALIER: Rapid differentiation between *Nocardia* and *Streptomyces* by paper chromatography of whole-cell hydrolysates. Appl. Microbiol. 12: 421~423, 1964
6) FUJII, N.; Y. YAMASHITA, Y. ARIMA, M. NAGASHIMA & H. NAKANO: Induction of topoisomerase II-mediated DNA cleavage by the plant naphthoquinones plumbagin and shikonin. Antimicrob. Agents Chemother. 36: 2589~2594, 1992
7) KOBAYASHI, E.; A. OKAMOTO, A. MASAO, M. OKABE, S. NAGAMURA, A. ASAI, H. SAITO, K. GOMI & T. HIRATA: Characteristics of antitumor activity of KW-2189, a novel water-soluble derivatives of duocarmycin, against murine and humor tumors. Cancer Res. 54: 2404~2410, 1994
8) FUJIMOTO, K. & M. MORIMOTO: Antitumor activity of trioxacarcin C. J. Antibiotics 36: 1216~1221, 1983
9) GERAN, R. I.; N. H. GREENBERG, M. M. MACDONALD, A. M. SCHUMACHER & B. J. ABBOTT: Protocols for screening chemical agents and natural products against animal tumors and other biological system. Cancer Chemother. Rep. Part 3 3: 1~103, 1972
10) LECHEVALIER, M. P. & H. A. LECHEVALIER: Chemical composition as a criterion in the classification of aerobic actinomycetes. Int. J. Syst. Bacteriol. 20: 435~443, 1970
11) SHIRLING, E. B. & D. GOTTLIEB: Cooperative description of type cultures of *Streptomyces*. II. Species description from the second, third and fourth studies. Int. J. Syst. Bacteriol. 18: 69~189, 1968
12) SHIRLING, E. B. & D. GOTTLIEB: Cooperative description of type cultures of *Streptomyces*. IV. Species description from the second, third and fourth studies. Int. J. Syst. Bacteriol. 19: 391~512, 1969
13) NONOMURA, H.: Key for classification and identification of 458 species of the streptomyces included in ISP. J. Ferment. Technol. 52: 78~92, 1974
14) WILLIAMS, S. T.; M. GOODFELLOW, G. ALDERSON, E. M. H. WELLINGTON, P. H. A. SNEATH & M. J. SACKIN: Numerical classification of *Streptomyces* and related genera. J. Gen. Microbiol. 129: 1743~1813, 1983