PYRROINDOMYCINS, NOVEL ANTIBIOTICS PRODUCED BY *Streptomyces rugosporus* LL-42D005

II. BIOLOGICAL ACTIVITIES

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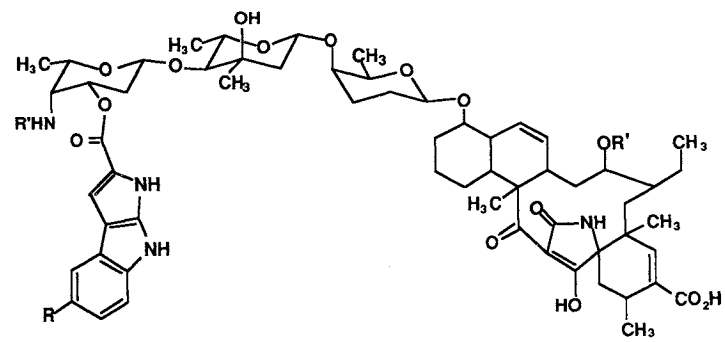
The pyrroindomycins, a complex of novel antibiotics identified in fermentation broths of "*Streptomyces rugosporus*" LL-42D005, demonstrated excellent *in vitro* activity against Gram-positive bacteria. The semisynthetic diacetyl derivative of pyrroindomycin B (pyrroindomycin B-Ac_2) was bactericidal for exponential-phase cells, but not for stationary-phase cells. This compound also exhibited marginal protection against a lethal *Staphylococcus aureus* challenge in mice. The poor *in vivo* activity of this antibiotic complex may be related to binding to blood components, as suggested by elevated MICs observed in blood-containing media. Incorporation of radiolabeled precursors into DNA, RNA, and protein was inhibited in an exponential-phase culture of *Bacillus subtilis* within ten minutes of exposure to pyrroindomycin B-Ac_2. Microscopic examinations of drug-treated cells revealed lysis within the same ten minute period. These data are consistent with an effect of pyrroindomycin B-Ac_2 on the integrity of the bacterial membrane.

The evolution and spread of antibiotic-resistant pathogens remains a major clinical problem[1]. Although the discovery of new antimicrobial agents has become increasingly difficult, the search for unique metabolites from microorganisms remains an attractive venture[2,3]. During the course of our screening program for novel antibacterial agents, fermentation samples of culture LL-42D005 exhibited activity against methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococcus faecium*[4]. This culture was subsequently identified as a new *Streptomyces* species, *S. rugosporus*[5] which produced a novel class of antibiotics, the pyrroindomycins[6]. The pyrroindomycins (Fig. 1) contain a tetramic acid nucleus and are structurally related to other antibiotics containing a tetronic acid moiety and a macrocyclic ring. The latter include tetrocarcin[7], kijanimicin[8], antlermicin[9], and the recently isolated antibiotics PA-46101 A

Fig. 1. Pyrroindomycins (A, R and R′=H; B, R=Cl and R′=H; B-diacetyl, R=Cl and R′=Ac).



and B[10]. In the present paper, we report the *in vitro* and *in vivo* antibacterial activities and mechanistic studies of the pyrroindomycins.

Materials and Methods

Bacterial Strains

Clinical isolates were collected between 1987~1993 from various medical centers in the United States, and quality control strains were obtained from the American Type Culture Collection, Rockville, MD. *B. subtilis* (*trpC*2) BGSC1A1 and *Escherichia coli* (*imp*) BAS849 were obtained from the Bacillus Genetic Stock Center and Dr. S. A. BENSON[11], respectively. Identification of each culture was done by conventional methods: Gram-negative rods by API 20E (Analytab Products, Plainview, NY) and NF systems (Remel, Lenexa, Kansas), and staphylococci by Staph Trac (Analytab Products). All clinical isolates were stored frozen in skim milk at −70°C, and other strains were stored frozen at −80°C in 20% DMSO.

Media

All media were prepared in distilled deionized (DI) water. Mueller-Hinton (MH) medium was purchased from Becton Dickinson Microbiology Systems, Cockeysville, MD. Modified minimal medium contained per liter: dextrose 4 g, NH_4Cl 1 g, KH_2PO_4 3 g, Na_2HPO_4 6 g, $MgSO_4 \cdot 7H_2O$ 0.25 g, $FeSO_4 \cdot 7H_2O$ 0.5 mg, vitamin-free Casamino Acids 2 g, and L-tryptophan 0.05 g. All ingredients used in the minimal medium were purchased from Sigma Chemical Co., St. Louis, MO., with the exception of Casamino Acids from Difco Laboratories, Detroit, MI.

Chemicals

^{3}H-thymidine (^{3}H-Tdr, TRK.686, 90 Ci/mmol), ^{3}H-uridine (^{3}H-Udr, TRK.410, 49 Ci/mmol), and ^{3}H-amino acids (^{3}H-AA, TRK. 550; mixture of leucine, lysine, phenylalanine, proline, and tyrosine with specific activities of 135, 83, 123, 103, and 118 Ci/mmol, respectively) were purchased from Amersham Corporation, Arlington Heights, IL. All antimicrobial agents were purchased from Sigma Chemical Co., St. Louis, MO, with the exception of pyrroindomycins, which were provided by Dr. W. DING, American Cyanamid Company, Pearl River, NY.

In vitro Susceptibility Testing

The *in vitro* antibacterial activities were determined by the agar or microbroth dilution method as recommended in the National Committee for Clinical Laboratory Standards[12]. Mueller-Hinton II agar was used for nonfastidious aerobic bacteria, and this medium was supplemented with 5% sheep blood for *Streptococcus* spp. or for the determination of the effect of blood on antibacterial activity. Inocula were adjusted to a density of 10^7 CFU/ml and 3 μl were then applied to the surface of agar plates with a Steers' replicator. The test plates were incubated at 35°C for 18 hours. The agar minimum inhibitory concentration (MIC) was defined as the lowest concentration of antimicrobial agent that completely inhibited visible growth of the organism. Broth MICs were determined by adding 5 μl of an exponential-phase bacterial culture ($1 \sim 5 \times 10^7$ CFU/ml) to 0.1 ml of minimal medium or MHB containing the drug at 0.002 to 128 μg/ml. The MIC was defined as the lowest concentration of antibiotic which prevented turbidity after 18 hours of incubation at 37°C.

Bactericidal Activity

B. subtilis was grown overnight in minimal medium (25 ml in a 250 ml Erlenmeyer flask) at 37°C, 200 rpm to stationary phase. The stationary-phase culture was washed and resuspended in phosphate buffer (0.03 M, pH 7.2) to obtain 2×10^7 cfu/ml. For an exponential-phase culture, the overnight culture was diluted in fresh medium (1 : 1000) and incubated at 37°C, 200 rpm until an A_{450} of 0.5 was reached. The cells in stationary and exponential phases (10 ml in a 250 ml Erlenmeyer flask at 37°C, 200 rpm) were separately exposed to different concentrations of pyrroindomycin B-Ac_2. At the indicated times, aliquots (100 μl) were removed, serially diluted in saline (0.9%) and plated onto MH agar[13]. After 24 hours at 37°C, the colonies were enumerated to determine viable cell counts.

Incorporation of Radiolabeled Precursors

Macromolecular synthesis in *B. subtilis* was studied by measuring the incorporation of appropriate radiolabeled precursors into TCA-precipitable material. *B. subtilis* was grown at 37°C, 200 rpm in modified minimal medium (50 ml medium/250-ml Erlenmeyer flask) to an A_{450} of 0.20. This exponential-phase culture was dispensed as 125 μl aliquots into microtiter wells containing the test sample and either ^{3}H-Tdr (0.5 μCi/ml), ^{3}H-Udr (0.5 μCi with 0.5 μg unlabeled uridine/ml), or ^{3}H-AA (10 μCi/ml). After various periods of exposure, 100 μl from each well were removed and precipitated with chilled 10% TCA supplemented with 0.5 mg/ml of unlabeled precursor. After refrigeration for 1 hour, the precipitate was collected onto a glass fiber filter (Wallac filtermat B, Wallac 1205-404) using the Skatron 96-well cell harvester (Model 11050). The harvester was programmed to prewet for 3 seconds with chilled DI water, wash for 10 seconds with 5% chilled TCA, wash for 10 seconds with chilled DI water, and finally air dry for 10 seconds[14]. After the filter mat was dried for 7 minutes at high power in a microwave oven (Quasar, 700 Watts), solid scintillant (MeltilexB, Pharmacia 1205-402) was applied, and the filter was counted for 1 minute in a LKB Betaplate scintillation counter (Wallac 1205). Incorporation of ^{3}H-Tdr, ^{3}H-Udr, and ^{3}H-AA are expressed as percent of untreated control.

Morphological Effects on *B. subtilis*

An overnight culture of *B. subtilis* was diluted 1 : 1000 into fresh minimal medium, which was incubated at 37°C, 200 rpm until an A_{450} of 0.20 was reached. The log-phase culture was treated with different concentrations of pyrroindomycin or water (untreated control). After 10, 20, 30, and 60 minutes, the cells were examined by phase-contrast microscopy (1000× under oil) for any morphological change.

Potassium Leakage Study

B. subtilis was grown in minimal medium at 37°C, 200 rpm, to exponential-phase (A_{450}=0.2) and was then treated with drug or water. At 5 minutes, 10 ml aliquots were filtered onto an 0.45 μm membrane. The retentate was washed with chilled 0.25 M sucrose solution (15 ml) and was then resuspended in HPLC grade water (10 ml). Four milliliters (4 ml) were added to a tube containing 4 ml of 1 M sulfuric acid. After heating at 100°C for 30 minutes and cooling, the mixture was centrifuged, and the potassium in the supernatant was measured using an atomic absorption spectrophotometer (Instrumentation Laboratories 551)[15]. The amount of intracellular potassium was determined from the difference in potassium levels between the supernatants of untreated and drug treated cells and is expressed as a percentage of untreated cells.

To study the effect of drug on exponential-phase cells (A_{450}=0.2) resuspended in an osmotically protective buffer, the cells were washed, resuspended in warm (37°C) 0.1 M sucrose-phosphate (pH 7.2), and then treated with drug. After 5 minutes, the cells were filtered onto a 0.45 μm membrane, and the amount of potassium released during exposure to the drug was determined by measuring the level of potassium in the filtrate using atomic absorption spectrophotometry. The amount of intracellular potassium released is expressed as a percentage of untreated control cells. Total intracellular potassium was determined by completely hydrolyzing the cells before filtration.

In Vivo Studies

The antibacterial activity of various drugs against an acute, lethal *S. aureus* Smith infection in mice was determined. Female mice, strain CD-1 (Charles River Laboratories, NY), weighing 20±2 g each were challenged by intraperitoneal injection of 0.5 ml of the bacterial suspension in broth containing 5% hog gastric mucin (10 to 100 LD_{50}). Six dose levels of the antibiotic in phosphate-buffered saline (pH 7.4, 0.01 M) were administered intravenously (0.2 ml), subcutaneously (0.5 ml), or orally (0.5 ml) 30 minutes post-infection. Each dose group had five animals. All untreated animals died within 48 hours of infection. The median effective dose (ED_{50}) was estimated from the survival ratios by computerized Probit Analysis[16]. The LD_{50} of the antibiotic represents the dose (mg/kg body weight) which killed ≥50% of a population of uninfected animals (six groups of five mice) after 7 days.

Table 1. Minimum inhibitory concentration (μg/ml) of antibiotics[a].

Organism (Number)	PYR-A	PYR-B	PYR-B-Ac_2	Vancomycin	Erythromycin	Piperacillin
MSSA (4)	0.06~0.12	0.12~0.50	0.06~0.25	0.50~1	0.25~>128	1~4
MRSA (3)	0.06~0.12	0.12~0.50	0.06~0.25	0.50~1	4~>128	128~>128
CNS (5)	0.06~0.12	0.12~0.50	0.25~0.50	0.50~2	0.12~>128	1~8
CNS-MR (1)	0.06~0.12	0.12~0.50	0.25~0.50	0.50~2	>128	>128
S. hemolyticus (1)	0.06	0.25	0.50	1	>128	128
E. faecalis VS (4)	0.25~0.50	0.25~1	2~4	0.50~2	2~>128	2~>128
E. faecalis VR (1)	0.25~0.50	0.25~1	2	>128	>128	8
E. faecium VS (2)	0.25~0.50	0.25~1	4~8	0.50~1	≤0.06~>128	0.12~2
E. faecium VR (2)	0.25~0.50	0.25~1	4	>128	>128	>128
B. cereus (1)	0.06	0.50	0.06	0.50	0.12	2
G-negative rods (4)	>64	>64	>64	>128	64~>128	2~64

[a] Agar dilution method in MHA (BBL); inoculum, 10^4 cfu/spot; incubation, 35°C for 18 hours. PYR=pyrroindomycin. MSSA, methicillin-sensitive *S. aureus*; MRSA, methicillin-resistant *S. aureus*; CNS, coagulase-negative staphylococci; CNS-MR, methicillin-resistant CNS; *S., Staphylococcus*; E., *Enterococcus*; VS, vancomycin-sensitive; VR, vancomycin-resistant; *B., Bacillus*; G-negative rods, *Escherichia coli* (2), *Pseudomonas aeruginosa* (1) and *Morganella morganii* (1).

Results

In Vitro Antibacterial Activity

The pyrroindomycins exhibited good to excellent *in vitro* activity against Gram-positive bacteria and poor activity against Gram-negative bacteria (Table 1). Of the three pyrroindomycins tested, pyrroindomycin A exhibited the best overall activity, while pyrroindomycin B was the least active. Although the activity of pyrroindomycin B-Ac_2 was comparable against most isolates, it was slightly less effective against coagulase-negative staphylococci and *Enterococcus* species. Antibacterial activity of all of the pyrroindomycins decreased 8~10 fold when 5% sheep blood was added to the medium (data not shown). Although pyrroindomycin B-Ac_2 did not show any activity against wild-type Gram-negative bacteria, it exhibited good activity against an *E. coli* (*imp*) permeability mutant[11], similar to that observed against *B. subtilis* (Table 2). Pyrroindomycin B-Ac_2 was bactericidal against exponential-phase cells of *B. subtilis*, but not against stationary-phase cells (Fig. 2).

Table 2. Broth MIC (μg/ml) of antimicrobial agents.

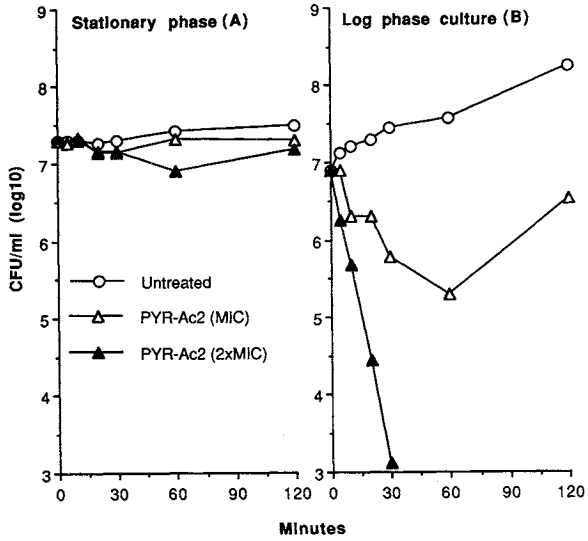
Compound	*B. subtilis* BGSC1A1	*E. coli* BAS849 (*imp*)
Pyrroindomycin B-Ac_2	0.50	1
Ciprofloxacin	0.12	≤0.015
Rifampin	0.25	≤0.015
Chloramphenicol	4	4
Polymyxin B	16	2
Penicillin G	0.015	2

Microbroth Dilution Method in minimal medium; Inoculum, $1\sim5\times10^5$ cfu/ml; Incubation, 37°C for 18 hours.

Mechanistic Studies

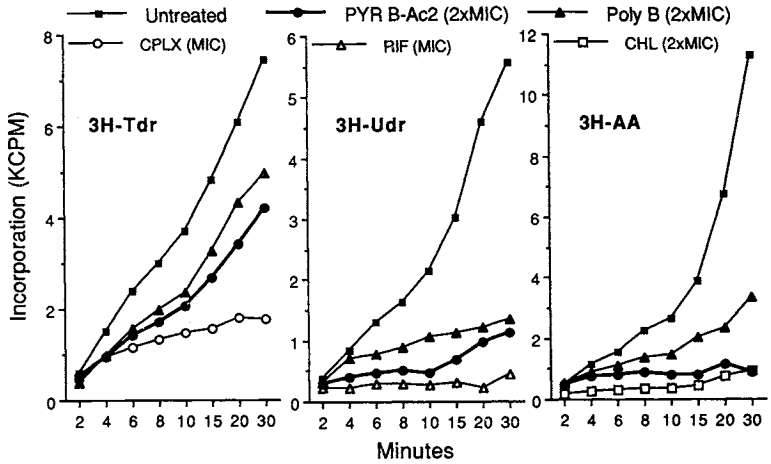
Incorporation of ^{3}H-Tdr, ^{3}H-Udr, and ^{3}H-AA into TCA precipitable material was used to assess the effect of pyrroindomycin B-Ac_2 on DNA, RNA, and protein syntheses, respectively. As expected, ciprofloxacin, rifampin, and chloramphenicol predominantly inhibited DNA, RNA and protein synthesis, respectively (Fig. 3). Although pyrroindomycin B-Ac_2 rapidly inhibited incorporation of radiolabeled precursors into all three macromolecules, DNA synthesis was somewhat less affected. Polymyxin B, which disrupts bacterial membranes, also inhibited incorporation of all three precursors. However, DNA synthesis was less affected, similar to the pattern observed with pyrroindomycin B-Ac_2.

Fig. 2. Bactericidal activity of pyrroindomycin B-Ac_2.



(A) *B. subtilis* was grown overnight in minimal medium to stationary phase, cells were washed, resuspended in phosphate buffer, and then treated with pyrroindomycin B-Ac_2 (PYR-Ac_2). Aliquots were removed at the indicated times for enumeration by serial dilution and plating. (B) *B. subtilis* was grown to log-phase and then exposed to PYR B-Ac_2. Viable counts were determined as in A.

Fig. 3. Effect of antimicrobial agents on macromolecular synthesis in *B. subtilis*.



DNA, RNA, and protein syntheses are expressed as incorporated KCPM for ^{3}H-Tdr, ^{3}H-Udr, and ^{3}H-AA , respectively. PYR B-Ac_2, pyrroindomycin B-Ac_2; CPLX, ciprofloxacin; RIF, rifampin; CHL, chloramphenicol; and Poly B, polymyxin B.

Effect on Cellular Integrity

The effects of pyrroindomycin B-Ac_2 on the morphology of an exponential-phase culture of *B. subtilis* were examined (data not shown). After 10 minutes of exposure to pyrroindomycin B-Ac_2 at 2× MIC, 70~80% of the cells lysed. Although lysis was not observed after 10 minutes at 1/2×MIC, 40~50% of the cells lysed after 30 minutes of exposure to pyrroindomycin B-Ac_2. After 30 minutes at 1/4×MIC,

Table 3. Effect of pyrroindomycin B-Ac_2 on intracellular potassium in *B. subtilis*.

Compound	Conc. (μg/ml)	Solution	Potassium released (% untreated control)
Pyrroindomicin B-Ac_2	2	Medium	51
	2	Buffer	24
Polymyxin B	32	Medium	22
	32	Buffer	68

Table 4. *In vivo* antibacterial activity of pyrroindomycins.

Compound	Route	ED_{50} (mg/kg)	LD_{50} (mg/kg)
Pyrroindomycin B-Ac_2	SOD	>32	>32
	SSC	4~8	>16
	SIV	> 2	2~4
Pyrroindomycin A	SSC	>16	>16
	SIV	> 2	2~4
Vancomycin	SSC	1~2	N.T.
	SIV	1~2	N.T.

SSC, single subcutaneous dose; SIV, single intravenous dose; SOD, single oral dose; N.T., not tested.

approximately 15% of the cells appeared swollen, and none lysed even after 60 minutes of exposure to pyrroindomycin B-Ac_2.

Pyrroindomycin B-Ac_2 was compared to polymyxin B for effects on the intracellular potassium level in *B. subtilis*. Exponential-phase cells suspended in growth medium released 51% of their intracellular potassium when treated with pyrroindomycin B-Ac_2 for 5 minutes (Table 3). In contrast, exponential-phase cells resuspended in sucrose-phosphate buffer released only 24% of their intracellular potassium. Polymyxin B caused more leakage of the intracellular potassium from exponential-phase cells resuspended in buffer than those in growth medium. Very little additional intracellular potassium was released even after 15 minutes' exposure to either drug (data not shown).

In Vivo Efficacy against Murine Infection

Pyrroindomycins A and B-Ac_2 were compared to vancomycin with respect to toxicity and ability to protect mice from a lethal challenge of *S. aureus* Smith (Table 4). Although both pyrroindomycins were ineffective when administered by the oral route (data not shown), pyrroindomycin B-Ac_2 was protective at 4~8 mg/kg when given subcutaneously. Intravenous administration of the drug increased toxicity to a point that an effective dose could not be determined.

Discussion

The pyrroindomycins are novel antibiotics with good to excellent activity against Gram-positive bacteria, including strains resistant to other classes of antibacterial agents such as β-lactams, macrolides, and aminoglycosides. These compounds demonstrate poor activity against Gram-negative bacteria, which is likely to be the result of the permeability barrier provided by the outer membrane. When tested against an *E. coli imp* strain, pyrroindomycin B-Ac_2 exhibited excellent activity comparable to that observed against Gram-positive bacteria. The *imp* mutation increases the permeability of the cell membrane and renders the bacteria much more susceptible to many antibacterial agents, especially larger molecules[11].

Interestingly, the bactericidal action of pyrroindomycin B-Ac_2 was only observed with exponential-phase, metabolically active cells. Exponential-phase cells resuspended in buffer or stationary-phase cells resuspended in either spent-medium or buffer were unaffected by the drug. However, when stationary-phase cells were resuspended in fresh medium containing pyrroindomycin B-Ac_2, viability decreased after 30 minutes around the same time that the lag-phase ended and growth resumed (data not shown). It is not clear from these data if the bactericidal activity of pyrroindomycin B-Ac_2 requires an active transport system or a metabolic transformation to an active form.

Mechanistic studies of pyrroindomycin B-Ac_2 in *B. subtilis* support an effect on the bacterial membrane.

Observations of nonspecific inhibition of macromolecular syntheses, cell lysis, and potassium leakage are consistent with membrane damage. Furthermore, the effects of pyrroindomycin B-Ac_2 on these processes were very similar to those observed for polymyxin B. In both instances, RNA and protein synthesis ceased within 5 minutes, followed by the cessation of DNA synthesis. These data are consistent with our observations for other compounds affecting the bacterial membrane[17]. In addition, release of intracellular potassium has been observed with other antibacterial agents causing membrane disruption[15]. This effect on the bacterial membrane most likely accounts for the observed bactericidal activity.

The decreased activity of pyrroindomycins in the presence of blood suggests a strong interaction with blood components, such as serum. Poor diffusion in an agar assay (data not shown) and the need for a very basic pH or DMSO to solubilize these compounds may explain the lack of *in vivo* efficacy. Among the two pyrroindomycins tested, only pyrroindomycin B-Ac_2 protected mice against an infection with *S. aureus* Smith. However, the therapeutic index was too low for clinical application.

Although the pyrroindomycins are active *in vitro* against clinical isolates, they demonstrate limited *in vivo* activity and a low therapeutic index. Further studies on additional derivatives may lead to a compound with better clinical potential.

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References

1) SILVER, L. L. & K. A. BOSTIAN: Discovery and development of new antibiotics: the problems of resistance. Antimicrob. Agents Chemother. 37: 337~383, 1993
2) OMURA, S.: Trends in the search for bioactive microbial metabolites. J. Ind. Microbiol. 10: 135~156, 1992
3) BULL, A. T.; M. GOODFELLOW & J. W. SLATER: Biodiversity as a source of innovation in biotechnology. Annu. Rev. Microbio. 46: 219~252, 1992
4) STEINBERG, D. A.; M. P. SINGH, N. V. JACOBUS, P. J. PETERSEN, M. J. MROCZENSKI-WILDEY, W. M. MAIESE & M. GREENSTEIN: Pyrroindomycins, novel antibiotics produced by *Kitasatosporia* sp. LL-42D005 II. Biological activities. Program and Abstracts of 33rd Intersci. Conf. on Antimicrob. Agents Chemother., No. 450, p. 201, New Orleans, Oct. 17~20, 1993
5) BERNAN, V. S.; D. A. MONTENEGRO, J. D. KORSHALLA, M. R. ALLURI, G. T. CARTER, W. M. MAIESE, D. A. STEINBERG, & M. GREENSTEIN: Pyrroindomycins, novel antibiotics produced by *Streptomyces rugosporus* sp. LL-42D005. III. Taxonomy and fermentation. J. Antibiotics, in preparation
6) DING, W. D.; D. WILLIAMS, P. NORTHCOTE, M. SIEGEL, R. TSAO, J. S. ASHCROFT, G. O. MORTEN, M. ALLURI, D. ABBANAT, W. M. MAIESE & G. A. ELLESTAD: Pyrroindomycins, novel antibiotics produced by *Streptomyces rugosporus* sp. LL-42D005. I. Isolation and structure determination. J. Antibiotics 47: 1250~1257, 1994
7) TAMAOKI, T.; M. KASAI, K. SHIRAHARA, S. OHKUBO, M. MORIMOTO, K. MINEURA, S. ISHII & F. TOMITA: Tetrocarcins, novel antitumor antibiotics. II. Isolation, characterization and antitumor activity. J. Antibiotics 33: 946~951, 1980
8) WAITZ, J. A.; A. C. HORAN, M. KALYANPUR, B. K. LEE, D. LOEBENBERG, J. A. MARQUEZ, G. MILLER & M. G. PATEL: Kijanimicin (Sch 25663), a novel antibiotic produced by Actinomadura kijaniata SCC 1256. Fermentation, isolation, characterization and biological activities. J. Antibiotics 34: 1101~1106, 1981
9) KOBINATA, K.; M. URAMOTO, T. MIZUNO & K. ISONO: Antlermicins B and C, new member of the antlermicin family. J. Antibiotics 33: 772~775, 1980
10) MATSUMOTO, M.; Y. KAWAMURA, Y. YOSHIMURA, Y. TERUI, H. NAKAI, T. YOSHIDA & J. SHOJI: Isolation, characterization and structure of PA-46101 A and B. J. Antibiotics 43: 739~747, 1990
11) SAMPSON, B. A.; R. MISRA & S. A. BENSON: Identification and characterization of a new gene of *Escherichia coli* K-12 involved in outer membrane permeability. Genetics 122: 491~501, 1989
12) National Committee for Clincal Laboratory Standards 1991. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically: approved standard M7-A. National Committee for Clincal Laboratory Standards, Villanova, Pa
13) KROGSTAD, D. J. & R. C. MOELLERING: Antimicrobial combinations. *In* Antibiotics in Laboratory Medicine, 2nd edition. *Ed.*, V. LORIAN, pp. 546~547, Baltimore, Williams and Wilkins, 1986
14) User Manual. Skatron 96 well cell harvester, model 11050, Skatron Instruments, Inc., Norway, pp. 1~28, 1989
15) GALVEZ, A.; E. VALDIVIA, M. MARTINEZ & M. MAQUEDA: Effect of peptide AS-48 on *E. faecalis* subsp. *liquefaciens*

S-47. Antimicrob. Agents Chemother. 33: 641～645, 1989
16) FINNEY, D. J.; Probit Analysis, 3rd ed. Cambridge University Press, London.
17) BERNAN, V. S.; D. M. ROLL, C. M. IRELAND, M. GREENSTEIN, W. M. MAIESE, & D. A. STEINBERG: A study on the mechanism of action of sceptrin, an antimicrobial agent isolated from the South Pacific sponge, *Agelas mauritiana*. J. Antimicrob. Chemother. 32: 539～550, 1993