ANTIBIOTIC GE2270 A: A NOVEL INHIBITOR OF BACTERIAL PROTEIN SYNTHESIS

I. ISOLATION AND CHARACTERIZATION

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(Received for publication February 5, 1991)

A novel antibiotic, GE2270 A, was isolated from the fermentation broth of a strain of *Planobispora rosea*. The product was found to inhibit bacterial protein synthesis. Structural characteristics showed similarities between GE2270 A and thiazolyl peptides such as micrococcin which is known to inhibit protein synthesis by acting directly on the ribosome. Despite this similarity GE2270 A showed functional analogy to kirromycin-like antibiotics and pulvomycin, as its molecular target was found to be elongation factor Tu (EF-Tu).

GE2270 A is active against Gram-positive microorganism and anaerobes and differs from the other EF-Tu inhibitors in its spectrum of antimicrobial activity.

GE2270 A, a novel peptide antibiotic, emerged from a screening program designed to detect inhibitors of protein synthesis. The present paper deals with the discovery, isolation, initial physico-chemical and biological characterization of this antibiotic.

Materials and Methods

Cultural and Growth Characteristics of the Producing Strain

Colonial and morphological characters were determined with standard methods[1,2].

Color determination was made according to MAERZ and PAUL[3]. Growth on sole sources of carbon was determined after incubation at 28°C for 2 weeks[1].

Chemotaxonomic Characteristics of the Producing Strain

Freeze-dried biomass was examined to determine the major chemotaxonomic characteristics. Cell wall diamino acids were determined by TLC by a modification of the method of BECKER *et al.*[4,5]. Whole cell sugars were hydrolyzed, reduced and derivatized. The resultant alditol acetates were analyzed by GC[6]. Fatty acid methyl esters were similarly analyzed by GC[7]. Menaquinones and polar lipids were extracted and analyzed by HPLC and 2D TLC, respectively[8].

Fermentation of the Producing Strain

A 500-ml Erlenmeyer flask containing 100 ml of seed medium (Polypeptone 0.5%, yeast extract 0.3%, beef extract 0.2%, soybean meal 0.2%, starch 2%, calcium carbonate 1%, pH 7.0) was inoculated from an oatmeal slant of the producing strain. After incubation at 28°C for 96 hours on a rotary shaker (200 rpm), the biomass was transferred to a 10-liter jar fermenter containing 4 liters of the seed medium. This culture was grown for 72 hours at 28°C with 2 liters/minute air flow and stirring at 900 rpm, prior to inoculating a jar fermenter containing 50 liters of production medium (starch 2%, peptone 0.25%,

hydrolyzed casein 0.25%, yeast extract 0.3%, beef extract 0.2%, soybean meal 0.2%, calcium carbonate 0.1%, pH 7.4).

Isolation of Antibiotic GE2270 A

After 72 hours fermentation the antibiotic, GE2270 A, was found both in the supernatant and adsorbed on the mycelium. The filtrate was adjusted to pH 7.0 and extracted with 50 liters of ethyl acetate. The crude antibiotic (415 mg) was precipitated from the concentrated organic phase upon addition of petroleum ether. The mycelial cake was extracted twice with 20 liters of methanol and the pooled extracts were concentrated under vacuum to the water residue. This solution was extracted twice with ethyl acetate. The crude antibiotic (6.06 g) was precipitated from the concentrated organic phase upon addition of petroleum ether. Part of this crude preparation (3 g) was applied to a 300 g (230~400 mesh) silica gel column equilibrated with CH_2Cl_2. The column was developed sequentially with 2 liters CH_2Cl_2, and then with 1.5 liters of each of the following mixtures of CH_2Cl_2-methanol (98:2, 96:4, 94:6, 92:8, 90:10 and 88:12). The fractions containing the purified GE2270 A were pooled and concentrated to an oily residue. GE2270 A (600 mg) was precipitated upon addition of petroleum ether.

HPLC

HPLC analysis was performed on an Altex Ultrasphere (5 μm) ODS 4.6 × 250 mm column connected to a Brownlee Labs RP 18 (5 μm) precolumn eluted at 1.8 ml/minute flow rate with a linear gradient of 45% to 70% of eluent A in 20 minutes. The elution phases were: CH_3CN-18 mM sodium phosphate (70:30) (phase A) and CH_3CN-18 mM sodium phosphate (10:90) (phase B). The phases were brought to pH 7.0 with NaOH. UV detection was at 254 nm.

Potentiometric Analysis

Titration of the antibiotic was carried out in Methyl Cellosolve-water (4:1) with 0.1 N KOH and with 0.1 N HCl and in glacial acetic acid with 0.1 N $HClO_4$.

FAB-MS Studies

Positive ion FAB spectra were obtained on a Kratos MS-50 double focusing mass spectrometer, using 8 kV accelerating voltage. A saddle field atom gun was used with Xe gas (2×10^{-5} torr pressure indicated on the source ion gauge) at 6 kV voltage and 1 mA current. The sample, dissolved in 0.2 N acetic acid in ethanol-ethyl acetate (1:1) was mixed with thioglycerol matrix. Using HR peak matching methods the precision was about ± 0.02 dalton.

Acid Hydrolysis Followed by GC-MS Analysis

Acid hydrolysis was carried out at 105°C for 20 hours in the presence of 6 N HCl containing 1% phenol. The acid hydrolysate (2 mg) was derivatized at 90°C for 1 hour with 2 N HCl in anhydrous propanol and then with a 10% solution of pentafluoropropionic anhydride in anhydrous dichloromethane. The analysis was carried out on a Hewlett Packard HP5985B GC-MS system equipped with a *n*-propionyl-L-valine *tert*-butylamide polysiloxane-coated fused silica capillary column (25 m × 0.2 mm i.d.; OS6411, C.G.C. Analytic); temperature program: 80°C for 4 minutes, then increasing at 4°C/minute.

MIC Determinations

Efrotomycin was a gift of Merck Co. Pulvomycin was purified from fermented mycelium of *Streptoverticillium netropsis* and was characterized by spectroscopic analyses.

MIC were determined by microbroth dilution methodology, except for *Clostridium difficile*, *Propionibacterium acnes* and *Bacteroides fragilis* (agar dilution). Inocula were $10^4 \sim 10^5$ cfu per ml or per spot (10^4 color changing units (CCU)/ml for *Ureaplasma urealyticum*). With the exception of *Candida albicans* (grown at 30°C), all microorganisms were cultured at 37°C. MIC were read at 18~24 hours, except for *Neisseria gonorrhoeae*, *Moraxella catarrhalis*, *Haemophilus influenzae*, *C. difficile*, *B. fragilis*, and *P. acnes* (48 hours). *N. gonorrhoeae* and *H. influenzae* were incubated in a 5% CO_2 atmosphere; anaerobes were incubated in an anaerobic gas mixture. Media used were: Oxoid Iso-Sensitest broth (Staphylococci, *Enterococcus faecalis*, *Escherichia coli*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*);

Difco Todd-Hewitt broth (Streptococci); Difco GC base broth with 1% BBL IsoVitaleX for *N. gonorrhoeae*; Difco Brain-Heart Infusion broth with 1% Difco Supplement C for *H. influenzae*; BBL Mueller-Hinton broth for *M. catarrhalis*; Difco AC medium without agar for *Clostridium perfringens*; Difco Wilkins-Chalgren agar for the other anaerobes; Difco Evans and Taylor-Robinson Broth, with supplements, for *U. urealyticum*.

Chlamydia trachomatis was cultivated in microtiter plates on cycloheximide treated McCoy cell monolayers, in EAGLE's MEM medium (Gibco) with 10% fetal calf serum, in a 5% CO_2 atmosphere. Inocula were prepared so as to give 30~60 inclusions per 300 × microscope field. After 48 hours chlamydial inclusions were stained with fluorescein-labeled monoclonal antibody to the major outer membrane protein (Syva) and counted by fluorescence microscopy. The MIC was taken as the concentration at which inclusions were no longer seen and cellular morphology was normal.

Experimental Infections

CDF1 mice (Charles River) weighing 18~22 g were infected ip with 1.5×10^6 cells of *Staphylococcus aureus* Smith ATCC 19636 in 0.5 ml of 5% Difco bacteriological mucin. This challenge corresponded to about 80 LD_{50} of the infecting organism. GE2270 A was administered iv, immediately after infection. The ED_{50} was calculated by the Spearman and Kärber method[9] from the percentage of surviving animals (day 7th) at each dosage.

Mechanism of Action of GE2270 A

Bacillus subtilis 566/1 (*thy*A, *thy*B) was grown at 37°C in a rotary bath in Davis-Mingioli minimal medium with glucose 2%, asparagine 0.1 g/liter, Difco Casamino acids 5 g/liter, and thymine 1 mg/liter. When the optical density (590 nm) of the culture reached 0.4, it was divided in five parts. To four of these, precursors of DNA (2 mCi/liter [^{3}H]thymidine + 1 mg/liter unlabeled thymine), RNA (1 mCi/liter ^{3}H- + 10 mg/liter unlabeled uridine), protein (1 mCi/liter ^{3}H- + 1 mg/liter unlabeled tryptophan), and cell wall peptidoglycan (2 mCi/liter ^{3}H- + 3.5 mg/liter unlabeled *N*-acetylglucosamine) were added. The fifth culture was used to monitor cell density. After 15 minutes of incorporation, each culture was further divided into three parts: untreated control, 2 mg/liter GE2270 A, and positive control. The positive controls were 50 mg/liter nalidixic acid (DNA), 10 mg/liter rifampicin (RNA), 25 mg/liter chloramphenicol (protein), and 100 mg/liter ampicillin (cell wall). At various times, up to 60 minutes, 0.1 ml samples were added to 2 ml ice-cold 5% (w/v) TCA and filtered on glass fiber filters. Samples containing tritiated tryptophan were heated for 15 minutes at 75°C before filtering. The filters were placed in 15 ml of Insta-Fluor (Packard) and counted in a liquid scintillation spectrometer.

Activity of GE2270 A in Cell Free Eukaryotic and Procaryotic Systems

L-[^{14}C]Phenylalanine (513 mCi/mmol) and L-[^{14}C]leucine (348 mCi/mmol) were from the Radiochemical Centre, Amersham, Bucks., UK. Poly(*U*) was from Boehringer Mannheim, Germany. Nucleotides and all other reagents were from Sigma Chemical Company, St. Louis, MO, U.S.A. Ribosomes and the postribosomal enzymatic fractions were prepared from *E. coli* as described by WILHELM and HASELKORN[10]. Rabbit reticulocyte lysate was prepared as described by ALLEN and SCHWEET[11]. Protein synthesis assays were performed according to the method of HOSOKAWA *et al.*[12] for poly(*U*)-directed polyphenylalanine synthesis by *E. coli* ribosomes and according to SARGIACOMO *et al*[13] for endogenous RNA-directed protein synthesis by the rabbit reticulocyte lysate. Hot acid-insoluble radioactivity was collected as described by MONTANARO *et al.*[14].

Results

Physiological and Morphological Characteristics of the Producing Strain

The strain grew well on most standard media with the optimum growth temperature between 28°C and 37°C. No growth was observed at 15°C or 50°C. There was moderate growth at 20°C. The cultural and physiological characteristics and the carbon source utilization of the strain are reported in Tables 1, 2, and 3.

Table 1. Cultural characteristics of strain ATCC 53773.

Culture medium	Morphological characteristics
Oatmeal agar 6%	Abundant growth with smooth surface, coral pink (2-H-10) abundant production of light pink aerial mycelium (1-A-9)
ISP No. 2 (yeast extract-malt extract agar)	Abundant growth with wrinkled surface, light pink (2-E-9), trace of light aerial mycelium
ISP No. 3 (oatmeal agar 2%)	Moderate growth with smooth surface, light pink (2-E-8), trace of pinkish white aerial mycelium
ISP No. 4 (inorganic salts-starch agar)	Moderate growth with smooth surface, coral pink (2-E-10)
ISP No. 5 (glycerol-asparagine agar)	Moderate growth with smooth and flat surface, light pink (2-A-9), abundant production of white aerial mycelium
ISP No. 6 (peptone-yeast extract-iron agar)	Moderate growth, slightly wrinkled light coral pink (1-A-10)
ISP No. 7 (Tyrosine agar)	Moderate growth with smooth and thin surface light pink (1-A-9), abundant formation of light pink (1-C-9) aerial mycelium
HICKEY and TRESNER's agar	Abundant growth with thick and wrinkled surface light coral pink (1-A-10), moderate production of light pink aerial mycelium
CZAPEK glucose agar	Very scarce growth with smooth and thin surface, moderate production of light pink aerial mycelium
Glucose asparagine agar	Moderate growth with smooth and thin surface colorless, aerial mycelium absent
Nutrient agar	Good growth with smooth surface light orange with a pinkish tinge (9-A-7)
BENNETT's agar	Moderate growth with slightly wrinkled surface light amber pink (10-A-6)
Calcium malate agar	Poor growth with smooth and flat surface colorless
Skim milk agar	Moderate growth with smooth surface coral pink (2-F-9)
Egg albumin agar	Poor growth with smooth and thin surface colorless to light pink (2-A-8)
Glucose-Tryptose agar	No growth
Potato agar	Good growth with smooth surface light orange with a pinkish tinge (9-A-7)

Table 2. Carbohydrate utilization of ATCC 53773.

Carbon source	Growth	Carbon source	Growth
Arabinose	+	Sucrose	−
Xylose	+	Maltose	+
Ribose	−	Raffinose	−
Fructose	+/−	Cellulose	−
Galactose	−	Mannitol	−
Glucose	+	Salicin	+
Rhamnose	−	Inositol	+
Lactose	−	Cellobiose	−

+: Moderate growth, +/−: scant growth, −: no growth.

Table 3. Physiological characteristics of ATCC 53773.

Tests	Result
Starch hydrolysis	Positive
Hydrogen sulfide formation	Negative
Tyrosine reaction	Positive
Casein hydrolysis	Weakly positive
Calcium malate digestion	Negative
Gelatin liquefaction	Weakly positive
Milk coagulation	Negative
Milk peptonization	Negative
Nitrate reduction	Positive

The vegetative mycelium formed long and irregularly branched filaments (0.5 to 1.0 μm) penetrating the agar and forming a compact growth on its surface. Color ranged from light coral to pink coral on most media tested. The mycelium grown in liquid or in solid media remained unfragmented. The aerial mycelium was formed of long, wavy and thin hyphae with few lateral branches. The aerial mycelium had a white-pink color. The sporangia (6.0 to 8.0 μm long and 1.0 to 1.2 μm wide) were single or in groups along the hyphae of the aerial mycelium and were supported by short sporangiophore (1.0 to 2.0 μm long).

Each sporangium contained a pair of fusiform straight spores (3.0 to 3.5 × 1.0 to 1.2 μm) separated by a transverse septum. The spores became motile by means of peritrichous flagella when released from the sporangia. No relevant morphological differences were observed by direct comparison of the producing strain and *Planobispora rosea* strain ATCC 23866[15]. GE2270 is detectable also in cultures of strain ATCC 23866, when grown in proper conditions.

Chemotaxonomical Characteristics of the Producing Strain

The cell wall diamino acid of the GE2270 A producing strain was found to be *meso*-diaminopimelic acid. Madurose (3-*O*-methyl-D-galactose) was the only diagnostic sugar detected. The fatty acid pattern consisted of a complex mixture of saturated and unsaturated linear, as well as *iso* and 10-methyl branched fatty acids. Phosphatidylinositol-, phosphatidylethanolamine-, phosphatidylglycerol- and glucosamine-containing phospholipids were all found in the polar lipid extract. The sole isoprenoid quinones were di- and tetra-hydrogenated menaquinones with nine isoprene units.

These characteristics, in accordance with the literature[16], confirm the strain as a member of the genus *Planobispora*. This strain has been deposited as *P. rosea* ATCC 53773.

Structure and Physico-chemical Characteristics of GE2270 A

The purity of the isolated antibiotic was assessed by HPLC analysis (Rt 14.9 minutes) and TLC (Rf 0.36 on silica gel plates eluted with dichloromethane-methanol, 9:1), both with UV detection. GE2270 A has characteristic UV absorption maxima which are reported in Table 4. The antibiotic was obtained as a white precipitate sparingly soluble in water. No ionizable functions of strong or medium strength were detected from the titration in water. In acetic acid a weak basic function was revealed.

The antibiotic has a MW 1,289 daltons. The formula $C_{56}H_{55}N_{15}O_{10}S_6$ was determined by precise FAB-MS.

GC-MS of an acid hydrolysate of GE2270 factor A revealed the presence of three amino acids: Glycine, L-proline and L-serine. The enantiomers were distinguished by comparison of Rt's on the chiral GC column with those of standard amino acids[17]. The structure of GE2270 A, reported in Fig. 1, was elucidated by NMR and MS spectrometry studies which are reported in a companion paper[18].

Antibiotic GE2270 A belongs to the group of antibiotics classified by BÉRDY[19] as thiazolyl peptides. These modified peptides, which include

Fig. 1. Structure of GE2270 A.

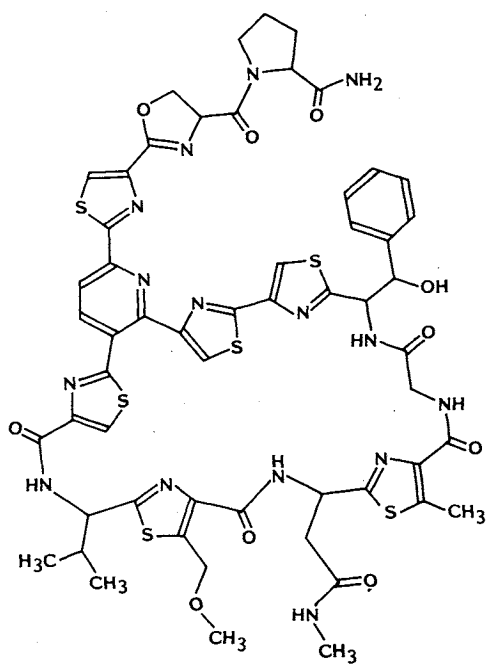


Table 4. UV absorption of GE2270 A.

	Max (nm)	$E^{1\%}_{1cm}$
0.1 N HCl	245 (sh), 310	203
0.1 N KOH	245 (sh), 313	210
Phosphate buffer pH 7.4	245 (sh), 314	206
MeOH	244 (sh), 310	237

thiostrepton[20], nosiheptide[21], micrococcin[22,23], thiocillin[24], sulfomycin[25], berninamycin[25] and thioxamycin[26], have a common framework characterized by a macrocycle containing thiazoles and one pyridine ring, or reduced pyridine ring, connected to a peptidic side chain. Some of these antibiotics, such as thiostrepton and nosiheptide, have an extra peptidic macrocycle which is not present in GE2270 A. Micrococcin and thiocillin show some similarities to GE2270 A in the chromophoric part centered on the pyridine ring which is conjugated to four thiazole rings although in a different sequence in GE2270 A. In comparison to thiazolyl peptides, the GE2270 A structure is original in many features such as the sequence of the peptidic backbone from which it derives, the number of amino acid units which are precursors of the macrocycle and the lack of diolehydroalanine and threonine which are frequently present in those antibiotics. GE2270 A is also unique in having methyl and methoxy-methylene substituents at the C-5 position of two out of the six thiazole rings present in the molecule.

Antibacterial Activity of GE2270 A *In Vitro* and in Experimental Infection

Table 5 shows the antibacterial spectrum of GE2270 A. It is active against all of the Gram-positive bacteria and particularly active against the anaerobe *P. acnes*. It is also active against *Mycobacterium tuberculosis*, with an MIC of 1 μg/ml. Among Gram-negative bacteria, it is quite active against *B. fragilis* (an anaerobe) and *M. catarrhalis*. It is not active against the yeast, *C. albicans*. In the murine model of *S. aureus* septicemia, GE2270 A had an ED_{50} of 1.13 mg/kg iv.

Mechanism of Action

GE2270 A inhibited bacterial growth by 80~90% at 2 μg/ml. At this concentration the antibiotic

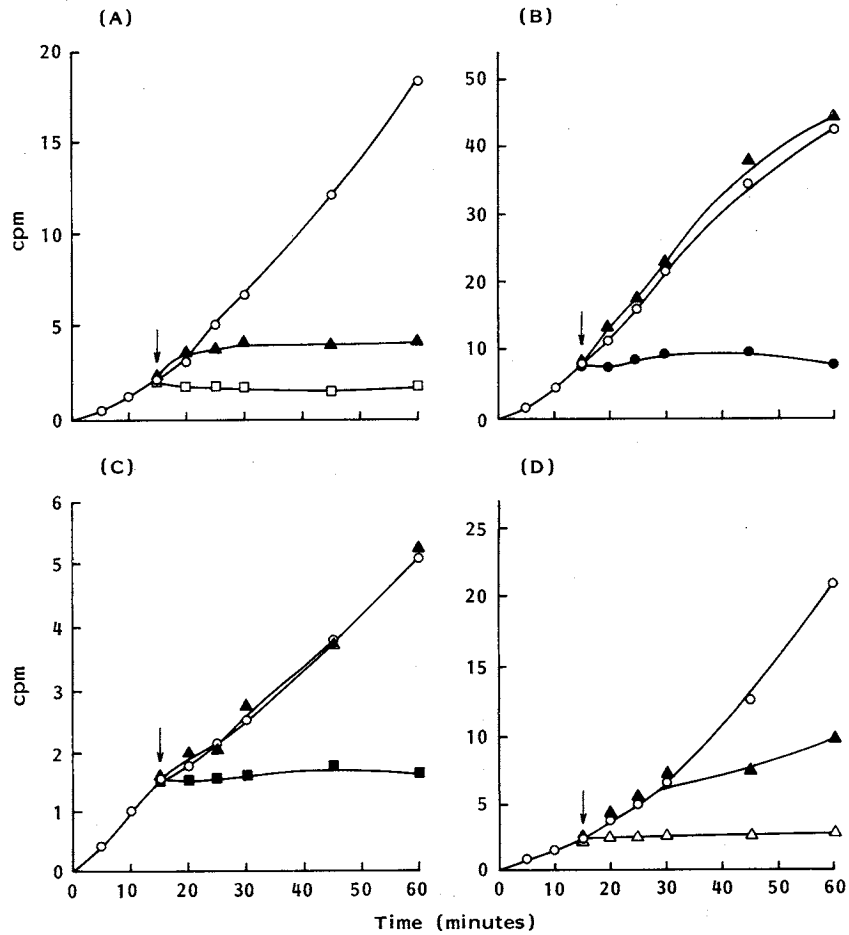
Table 5. Antibacterial spectrum of GE2270 A in comparison with known antibiotics acting on EF-Tu.

Strain	MIC (μg/ml)		
	GE2270 A	Pulvomycin	Efrotomycin[a]
Staphylococcus aureus L165 Tour	0.25	8	>128
S. aureus L165 Tour +30% bovine serum	0.25	32	>128
S. epidermidis L147 ATCC 12228	0.13	—	>128
S. haemolyticus L602 clinical isolate	0.5	4	>128
Streptococcus pyogenes L49 C203	0.25	128	8
S. pneumoniae L44 UC41	0.13	128	16
S. mitis L796 clinical isolate	0.13	>128	8
Enterococcus faecalis L149 ATCC 7080	0.13	8	>128
Clostridium perfringens L290 ISS30543	0.03	0.06	0.13
C. difficile L1363 ATCC 9689	0.03	—	—
Propionibacterium acnes L1014 ATCC 6919	<0.004	0.13	—
Bacteroides fragilis L1010 ATCC 23745	2	0.5	—
Neisseria gonorrhoeae L997 ISM68/126	32	0.13	0.25
Haemophilus influenzae L970 type b ATCC 19418	128	8	4
Moraxella catarrhalis L76 ATCC 8176	1	—	—
Ureaplasma urealyticum L1479 clinical isolate	32	0.5	2
Escherichia coli L47 SKF12140	>128	>128	>128
Proteus vulgaris L79 ATCC 881	>128	128	>128
Pseudomonas aeruginosa L4 ATCC 10145	>128	>128	>128
Klebsiella pneumoniae L142 ISM	>128	>128	>128
Chlamydia trachomatis	>128	[b]	0.5

[a] Kirromycin-like.
[b] Toxic to McCoy cells at 8 μg/ml.

Fig. 2. Mechanism of action of GE2270 A on *Bacillus subtilis*.

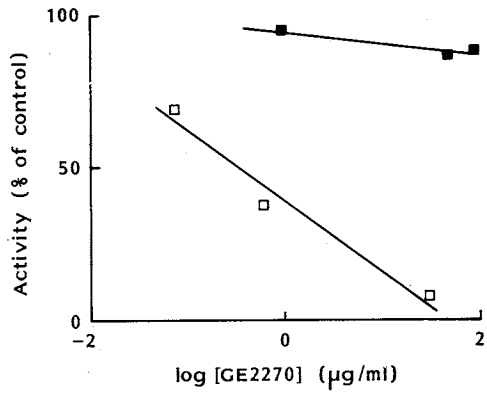
(A) Protein synthesis, (B) cell wall synthesis, (C) DNA synthesis, (D) RNA synthesis. ○ Control, □ chloramphenicol, ● ampicillin, ■ nalidixic acid, △ rifampicin, ▲ GE2270 A.



blocked protein synthesis within 5 minutes of addition as shown in Fig. 2. The other macromolecular syntheses were partially or totally blocked after 15 minutes or more. This indicates that the antibacterial action of GE2270 A is mediated by inhibition of protein synthesis. In cell free systems, the antibiotic inhibited poly(*U*)-directed prokaryotic protein synthesis (IC_{50} 0.34 μg/ml). This inhibitory concentration is in the range of MICs against sensitive bacteria. Eukaryotic protein synthesis was not inhibited up to the solubility limit of the antibiotic as shown in Fig. 3. In other studies, the molecular target of the antibiotic was shown to be protein synthesis elongation factor Tu (EF-Tu)[27].

Fig. 3. GE2270 A activity in cell-free protein synthesis systems.

■ Rabbit reticulocyte lysate system, □ *Escherichia coli* system.



Discussion and Conclusions

Antibiotic GE2270 A is produced by a strain which belongs to the *Planobispora* genus. Few species of this genus of rare Actinomycetales have been previously described[5~7] and none has yet been reported to produce antibacterial activity. Antibiotic GE2270 A is structurally related to thiazolyl peptides and has some structural similarity with micrococcin and thiocillin in the chromophoric part of the molecule. These antibiotics are produced by bacteria and are thus examples of closely related structures which are produced by microorganisms of different taxa.

The thiazolyl peptide antibiotics thiostrepton[28], nosiheptide[29], and micrococcin[30,31] are known inhibitors of bacterial protein synthesis and bind to the ribosome. GE2270 A has also been found to be an inhibitor of bacterial protein synthesis at the elongation step. However, its molecular target is EF-Tu which is the factor required for the binding of aminoacyl-tRNA to the A site of the ribosome[27]. Thus, although GE2270 A is structurally related to thiazolyl peptide antibiotics, it is functionally related to kirromycin-like antibiotics[32] and pulvomycin[33,34] for the EF-Tu target.

GE2270 A is different from the other EF-Tu inhibitors in its spectrum of antibacterial activity (Table 5). For example, efrotomycin, a kirromycin-like antibiotic, is highly active against *N. gonorrhoeae* but inactive against Staphylococci and most enterococcal isolates; pulvomycin is not active against Streptococci; GE2270 A is highly active against all Gram-positive tested, but not against *N. gonorrhoeae*.

GE2270 A, kirromycin and pulvomycin do not penetrate into *E. coli* but are active against EF-Tu extracted from this organism[31~33]. GE2270 A also inhibited cell free protein synthesis in a system derived from *E. coli*.

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