Bu-2470, A NEW PEPTIDE ANTIBIOTIC COMPLEX

I. PRODUCTION, ISOLATION AND PROPERTIES OF Bu-2470 A, B_1 AND B_2

MASATAKA KONISHI, KOKO SUGAWARA, KOJI TOMITA, KIYOSHI MATSUMOTO,
TAKEO MIYAKI, KEI-ICHI FUJISAWA, HIROSHI TSUKIURA
and HIROSHI KAWAGUCHI

Bristol-Banyu Research Institute, Ltd.
Meguro, Tokyo, Japan

(Received for publication February 24, 1983)

A strain of *Bacillus circulans* produced a complex of basic peptide antibiotics designated Bu-2470, which was found to contain four active components, A, B_1, B_{2a} and B_{2b}.

Bu-2470 A specifically inhibited various *Pseudomonas* species including *P. aeruginosa*, *P. maltophilia* and *P. putida*, but otherwise its antibacterial spectrum was limited to certain Gram-negative organisms. Bu-2470 B_1 and B_2 ($B_{2a}+B_{2b}$) showed broad antibiotic activity against Gram-positive and Gram-negative bacteria including *Pseudomonas* species. The physico-chemical and biological properties of Bu-2470 B_1 and B_2 are very similar to those of the octapeptin group of antibiotics.

In the course of our screening of new antibiotics active against *Pseudomonas* species, a bacterial strain, No. G493-B6, identified as *Bacillus circulans* was found to produce a new antibiotic complex Bu-2470 which inhibited various Gram-positive and Gram-negative bacteria including *P. aeruginosa*. The antibiotic complex was separated into Bu-2470 A and B by extraction with 1-butanol at different pH's. Bu-2470 B was further separated into components B_1 and B_2 by chromatography. As reported in a companion paper[1], Bu-2470 B_2 was shown to be a mixture of subcomponents B_{2a} and B_{2b}, which are collectively called component B_2 in the present paper. This paper reports information on the producing organism, as well as the production, isolation and physico-chemical and biological properties of Bu-2470 A, B_1 and B_2.

Producing Organism

Strain No. G493-B6 which produces the antibiotic Bu-2470 was isolated from a soil sample collected in India in 1974. It is an aerobic, Gram-negative, spore-forming rod bacterium, and considered to belong to the genus Bacillus.

The morphological, cultural and physiological characteristics of strain G493-B6 are shown in Tables 1, 2 and 3, respectively. The diagnostic features of strain G493-B6 can be summarized as follows: (1) negative by Gram-stain; (2) sporangia swollen at the endospore site; (3) spores formed at terminal or subterminal sites; (4) elliptical spores; (5) acid but no gas produced from glucose, arabinose, xylose or mannitol; (6) starch is hydrolyzed; (7) acetoin is not produced; (8) indole is not produced; and (9) moderate growth occurs in ordinary media such as nutrient agar.

Among the 22 species of genus Bacillus described in BERGEY's Manual (8th Ed., 1974), 5 species do not grow in ordinary media and thus can be differentiated from strain G493-B6. Of the remaining 17 species, 8 species (*B. alvei, B. brevis, B. circulans, B. coagulans, B. laterosporus, B. macerans, B. polymyxa* and *B. stearothermophilus*) have some morphological similarity to strain G493-B6. Therefore, strain G493-B6 was compared with each of the eight species. The microbiological characteristics of strain

Table 1. Morphological characteristics of strain G493-B6.

Vegetative cells	
Shape	Rods. Rounded end.
Size	$0.5\sim0.7\times2.0\sim4.0$ μm
Motility	Positive
Spores	
Shape and size	Elliptical, 0.8×1.6 μm
Distension of sporangia	Swollen at spore site
Position	Terminal or subterminal, rarely central
Gram-stain	Negative

Table 2. Cultural characteristics of strain G493-B6.

Sabouraud dextrose broth	Poor growth.
Glucose peptone broth	Turbid with viscous sediment. No pellicle. pH 5.5.
Nutrient agar slant	Moderate growth. Thin, opaque, smooth, slightly viscous and creamy.
Colony on nutrient agar	Circular or slightly irregular. Raised with irregular margin. Opaque density and smooth surface. $2\sim4$mm in diameter. Slightly viscous and creamy white. No satellite colony.

Incubation at 37°C for 24 hours.

Table 3. Physiological characteristics of strain G493-B6.

Temperature for growth	
Growth	$20\sim45$°C
No growth	10°C and 50°C
Gas from glucose, arabinose, xylose or mannitol	Negative
Acid from arabinose, xylose or mannitol	Positive
Acetoin from glucose	Negative
Hydrolysis of starch	Positive
Indole production	Negative
Nitrite from nitrate	Positive
Liquefaction of gelatin	Positive
Catalase	Positive
Deamination of phenylalanine	Negative
Growth in 0.001% lysozyme	Positive
Growth on anaerobic agar (Hugh-Leifson medium)	Positive
Citrate utilization	Positive
Reaction in milk	Peptonized and coagulated
Decomposition of urea	Positive
NaCl tolerance	Growth at 3%, and no growth at 4%

G493-B6 are very similar to those of *B. circulans*. However, strain G493-B6 differs from *B. polymyxa* or *B. macerans* because of its inability to evoke gas production from glucose; from *B. stearothermophilus* or *B. coagulans* because of its sporangia distension at endospore site and its lack of growth at 50°C; from *B. alvei* in regard to its formation of acid from arabinose, xylose or mannitol, as well as its lack of indole production; and from *B. laterosporus* or *B. brevis* in respect to its formation of acid from arabinose or xylose, and its hydrolysis of starch. Consequently, the organism producing the antibiotic Bu-2470 was determined to be a strain of *Bacillus circulans*.

Antibiotic Production

An agar slant with well-established growth of *Bacillus circulans* strain G493-B6 was used to inoculate vegetative medium containing 2% glycerol, 1% corn steep liquor, 1% Pharmamedia, 0.3% $(NH_4)_2SO_4$, 0.003% $ZnSO_4\cdot7H_2O$ and 0.4% $CaCO_3$, the pH being adjusted to 7.0 before sterilization. The seed culture was incubated at 28°C for 72 hours on a rotary shaker (250 rpm), and 5 ml of the culture was transferred to a 500-ml Erlenmeyer flask containing 100 ml of fermentation medium composed of 3% glycerol, 3% soybean meal, 0.3% $(NH_4)_2SO_4$, 0.003% $ZnSO_4\cdot7H_2O$ and 0.4% $CaCO_3$ (pH 7.0). The fermentation was carried out on a rotary shaker at 28°C for 5 to 7 days. Antibiotic activity in the fermentation broth was determined by paper disc-agar diffusion assay using *Bacillus subtilis* PCI219 and *Escherichia coli* NIHJ as the test organisms. Antibiotic production reached a maximum in 4 to 6 days, at a time when the fermentation broth generally became viscous.

Isolation and Purification

The viscous fermentation broth (10 liters, pH 7.5) was diluted with an equal volume of water and

centrifuged to remove mycelial cake. Clear supernatant was stirred with Amberlite IRC-50 (NH_4^+, 2 liters) to adsorb the activity. The resin was washed with water (20 liters) and then eluted batchwise with 0.5 N HCl (2.5 liters, 3 times). The eluates were combined, adjusted to pH 7.0 and stirred with active charcoal (180 g). The carbon was separated and eluted with a mixture of 1-butanol and water (1: 1, 2 liters), the pH of the eluant being adjusted to 2.0. The 1-butanol layer was separated and concentrated *in vacuo* to yield a crude mixture of Bu-2470 B (1.35 g). The acidic aqueous layer was neutralized with Amberlite IR-45 (OH^-) and extracted with two 1-liter portions of 1-butanol. Evaporation of the 1-butanol extract afforded the second crop of crude Bu-2470 B (440 mg) containing a small amount of component A. The aqueous layer was then made alkaline (pH 10.0) with conc. NH_4OH and extracted repeatedly (3 times) with 1 liter each of 1-butanol. The latter 1-butanol extracts were combined, concentrated *in vacuo* and lyophilized to afford a crude solid of Bu-2470 A (4.65 g).

The crude preparation of Bu-2470 A (5.5 g) was applied on a column of Diaion HP-20 (800 ml) which was washed with 0.1 N NH_4OH before use. The column was developed with water (3.5 liters), 50% methanol (5.5 liters) and acidic 50% methanol (pH 2.0, 3.5 liters), successively, and the elution was monitored by bioassay* and TLC**. The first bioactive fractions eluted with 50% methanol were pooled, evaporated *in vacuo* and lyophilized to give a pure preparation of Bu-2470 A (788 mg). The following active fractions which contained a trace amount of impurities were rechromatographed to afford an additional quantity of pure Bu-2470 A (1.0 g).

The crude mixture of Bu-2470 B (500 mg) was loaded on a column of CM-Cellulose C-25 (400 ml) which was pretreated with aqueous 0.1 M NaCl solution, and the column developed with aq. 0.1 M NaCl

Table 4. TLC and PPC of Bu-2470 components.

	TLC (Rf)		PPC (moving distance in cm)
	PL-111*	BT-101**	PL-1***
Bu-2470 A	0.01	0.09	0
Bu-2470 B_1	0.07	0.14	16.7
Bu-2470 B_2	0.07	0.14	10.7
EM-49 mixture	0.11	0.17	16.7, 13.7, 10.5 and 6.9
EM-49 δ	0.11	0.17	16.8
Octapeptin C_1 (333-25)	0.07	0.14	5.6
Bu-1880	0.07	0.14	16.8

* PL-111 : $CHCl_3$ - EtOH - 14% NH_4OH (4: 7: 2).

** BT-101 : 1-PrOH - 28% NH_4OH - H_2O (8: 1: 1).

*** PL-1[7] : Developed with upper layer of *n*-amyl alcohol - *n*-amyl acetate - propionic acid - H_2O (6: 9: 5: 15) by descending for 16 hours at 27°C; paper was pretreated with a 1: 1 mixture of acetone and lower phase of the developing solvent system.

Table 5. HPLC of Bu-2470 components.

	HPLC (Finepak SIL C_{18}, Rt in minutes)*	
	System I	System II
Bu-2470 A	25.9	—
Bu-2470 B_1	—	36.1
B_{2a}	—	21.0
B_{2b}	—	23.1
Octapeptin C_1	—	11.9
B_1	—	28.7
B_2	—	17.0
B_3	—	18.7

System I: CH_3CN - 0.005 M tartrate buffer (pH 3.0), 19: 81 containing 0.05 M Na_2SO_4 and 0.005 M 1-butanesulfonic acid.

System II: CH_3CN - 0.005 M tartrate buffer (pH 3.0), 31: 69 containing 0.05 M Na_2SO_4 and 0.005 M 1-butanesulfonic acid.

* Detection of the antibiotics was performed by UV absorption at 220 nm.

* Paper disc-agar diffusion assay *vs. P. aeruginosa* Pss-1.

** Silica gel plate, $CHCl_3$ - MeOH - 28% NH_4OH, 1: 2: 1.

solution. The fractions showing activity against *B. subtilis* PCI 219 were pooled and extracted with 1-butanol. Bu-2470 B_2 (75 mg) was eluted first followed by a mixture of Bu-2470 B_1 and B_2 (89 mg), and finally by Bu-2470 B_1 (89 mg). Although Bu-2470 B_2 was first shown to be a single antibiotic by PPC and TLC (Table 4), it was separated into two subcomponents B_{2a} and B_{2b} by high performance liquid chromatography (Finepak SIL C_{18}) developed with tartrate buffer - acetonitrile containing sodium 1-butansulfonate and sodium sulfate (Table 5)[2]. This was confirmed by structural studies as discussed in a separate paper[1]. The preparative separation of Bu-2470 B_{2a} and B_{2b} has not yet been accomplished. In the following description, component B_2 means a mixture of subcomponents B_{2a} and B_{2b}, while components B_1 and B_2 are collectively called Bu-2470 B.

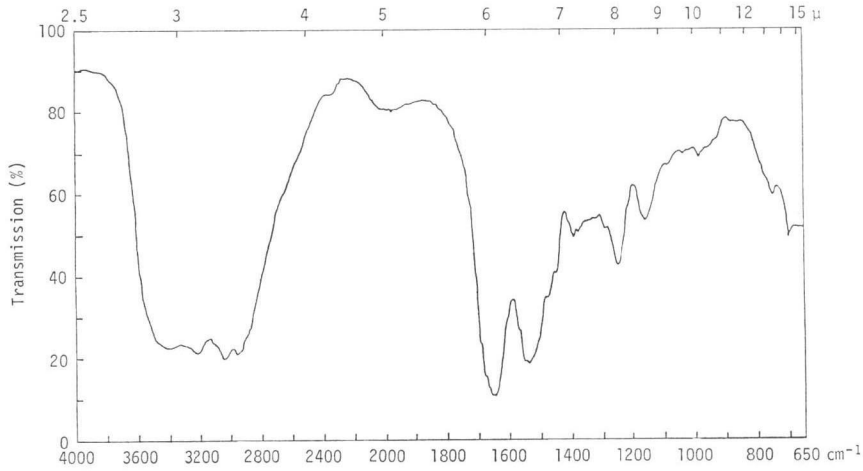
Physico-chemical Properties

Bu-2470 A, B_1 and B_2 are white amorphous solids isolated as hydrochlorides or free bases. They were differentiated from each other or from related antibiotics by TLC, PPC and HPLC as shown in Tables 4 and 5. Bu-2470 A is readily soluble in water over a wide pH range, aqueous lower alcohols, aqueous dioxane, dimethylsulfoxide (DMSO) and dimethylformamide (DMF), slightly soluble in lower alcohols but practically insoluble in other organic solvents. Bu-2470 B is soluble in acidic water, aqueous lower alcohols, aqueous dioxane, DMSO and DMF, slightly soluble in neutral and alkaline water and lower alcohols, but insoluble in other organic solvents. Bu-2470 A and B gave a positive response to ninhydrin reagent but were negative to anthrone, Fehling, Sakaguchi and $FeCl_3$ reactions.

Table 6. Microanalysis and optical rotation of Bu-2470 components.

		Bu-2470 A		Bu-2470 B_1		Bu-2470 B_2	
Formula		$C_{41}H_{71}N_{13}O_8 \cdot 5HCl \cdot 4H_2O$		$C_{52}H_{91}N_{13}O_{10} \cdot 4HCl \cdot 6H_2O$		$C_{51}H_{89}N_{13}O_{10} \cdot 4HCl \cdot 6H_2O$	
Microanalysis		Calcd.	Found	Calcd.	Found	Calcd.	Found
	C	43.64	42.94	47.59	47.52	47.18	46.65
	H	7.50	7.26	8.21	7.65	8.15	7.23
	N	16.14	16.00	13.88	13.78	14.03	13.66
	Cl	15.71	16.08	10.81	11.77	10.92	11.60
$[\alpha]_D^{29}$ in 0.5 N HCl		−76°		−53°		−69°	

Fig. 1. IR Spectrum of Bu-2470 A hydrochloride.



The free base and hydrochloride of Bu-2470 A did not show a definite melting point and decomposed above 230°C. Bu-2470 B_1 and B_2 gradually decomposed above 220°C. The microanalytical data and optical rotations for the hydrochlorides of Bu-2470 components are shown in Table 6. Bu-2470 A and B showed only end absorption in the UV spectra. The IR spectra of Bu-2470 A, B_1 and B_2 are shown in Figs. 1, 2 and 3, respectively. The proton NMR spectra of Bu-2470 A, B_1 and B_2, which are presented in Figs. 4, 5 and 6, respectively, all contain a characteristic five-proton singlet at δ: 7.23 ppm, suggesting the presence of a phenyl group in all of the Bu-2470 components.

Biological Properties

In Vitro Antibacterial Activity

Minimal inhibitory concentrations (MICs) of Bu-2470 A, B_1 and B_2 were determined by a two-fold serial dilution method using Mueller-Hinton agar medium with overnight incubation at 37°C. A 10^{-4} dilution of an overnight culture was used as the inoculum.

Fig. 2. IR Spectrum of Bu-2470 B_1 hydrochloride.

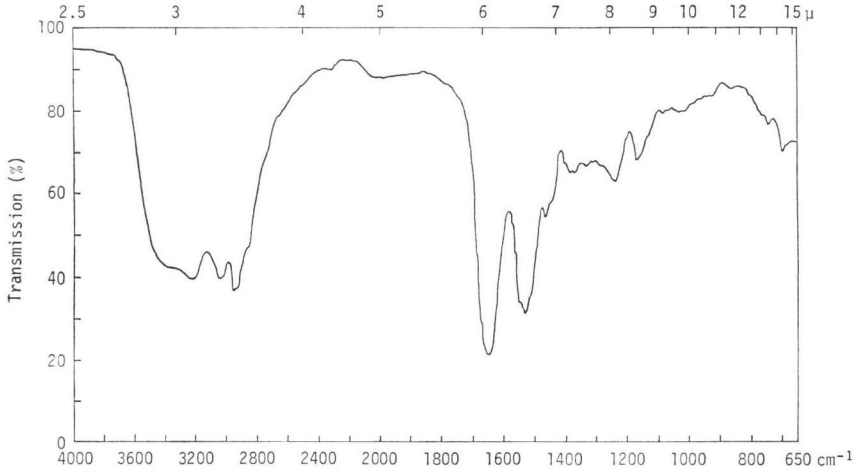


Fig. 3. IR Spectrum of Bu-2470 B_2 hydrochloride.

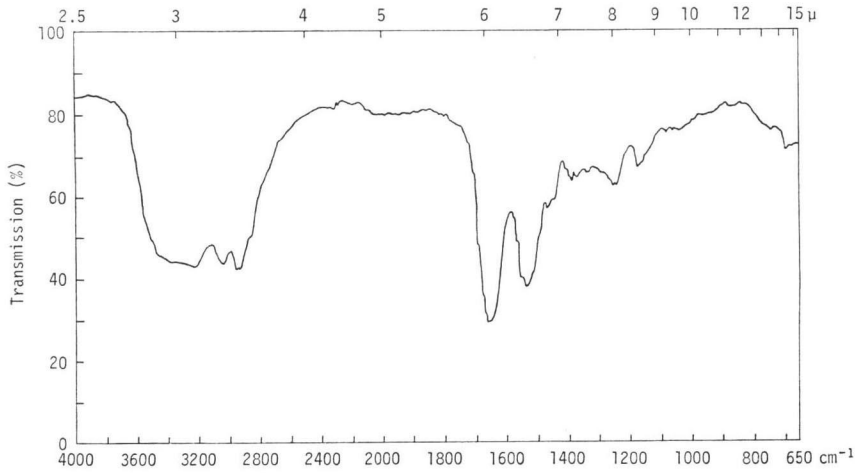


Fig. 4. NMR Spectrum of Bu-2470 A hydrochloride (60 MHz in D_2O).

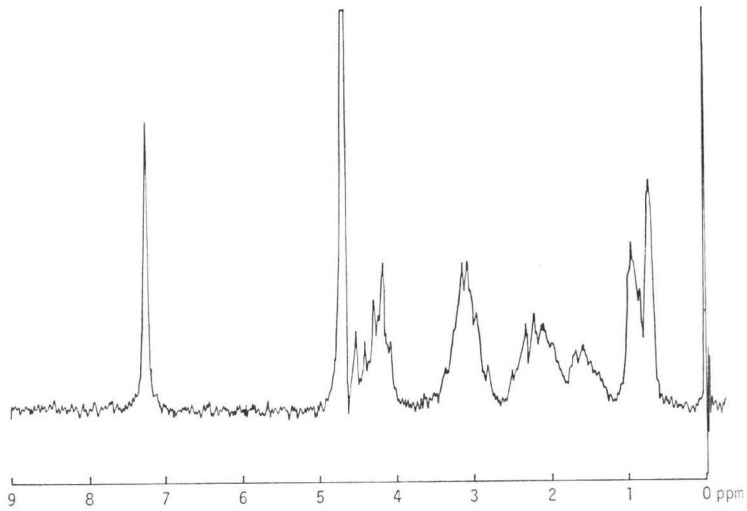
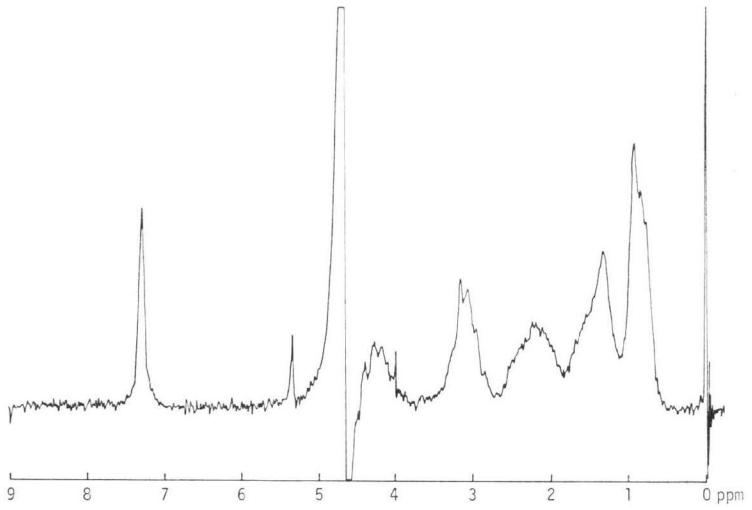


Fig. 5. NMR Spectrum of Bu-2470 B_1 hydrochloride (60 MHz in D_2O).



The antibacterial spectra of Bu-2470 A, B_1 and B_2 are shown in Table 7. Bu-2470 B_1 and B_2 were active against various Gram-positive and Gram-negative organisms but did not inhibit *Proteus* species. Bu-2470 A was generally less active than components B_1 and B_2 against the bacterial species shown in Table 7 except for *E. coli* and *Salmonella* species which were about equally susceptible to the three Bu-2470 components.

As shown in Table 8, Bu-2470 A, B_1 and B_2 were active against various *Pseudomonas* species, including *P. aeruginosa, P. cepacia, P. maltophilia, P. melanogenum* and *P. putida.* Bu-2470 A was shown to be equally or somewhat more active than Bu-2470 B_1 and B_2 against *Pseudomonas* species.

In Vivo Activity

The *in vivo* antibacterial activity of Bu-2470 A, B_1 and B_2 was evaluated in experimental infections of mice caused by *E. coli, Klebsiella pneumoniae* and *P. aeruginosa.* Mice were challenged intraperito-

Fig. 6. NMR Spectrum of Bu-2470 B_2 hydrochloride (60 MHz in D_2O).

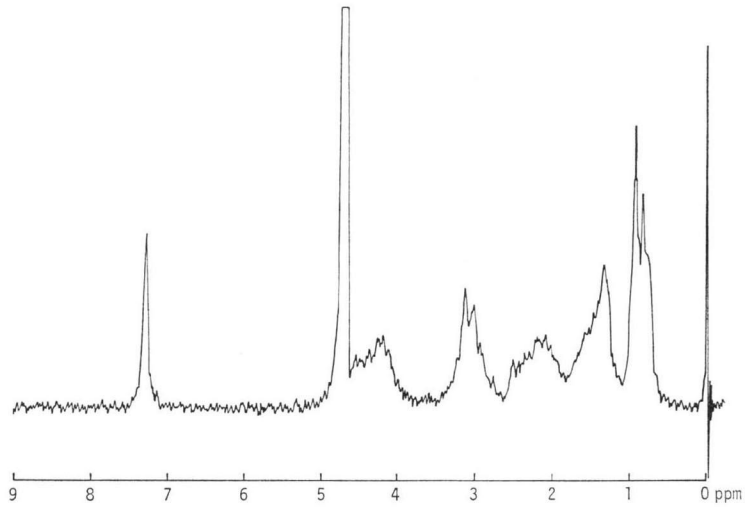


Table 7. Antibacterial activity of Bu-2470 A, B_1 and B_2.

Test organism	MIC (μg/ml)		
	Bu-2470 A	Bu-2470 B_1	Bu-2470 B_2
Staphylococcus aureus 209P	>100	12.5	12.5
〃 〃 Smith	>100	12.5	12.5
Streptococcus faecalis B402-3	50	3.1	3.1
Micrococcus luteus #1001	50	3.1	1.6
M. flavus D-12	50	3.1	3.1
Bacillus anthracis IID-115	100	6.3	3.1
B. subtilis PCI 219	50	3.1	3.1
Escherichia coli NIHJ	3.1	3.1	3.1
〃 〃 Juhl	6.3	6.3	3.1
Salmonella enteritidis #4432	12.5	6.3	3.1
S. typhosa A9498	6.3	6.3	3.1
Shigella dysenteriae D-163	100	3.1	3.1
S. sonnei Yale	25	3.1	3.1
Klebsiella pneumoniae D-11	50	6.3	3.1
Enterobacter cloacae A9654	>100	25	25
Proteus mirabilis A9554	>100	>100	>100
P. vulgalis A9526	>100	>100	100
P. morganii A9553	>100	>100	>100

neally with a 100 LD_{50} dose of the pathogens in a 5% suspension of hog gastric mucin (American Laboratories, Omaha, Neb.). Bu-2470 was administered by the intramuscular route immediately after the bacterial challenge. A group of 5 mice was used for each dosage level with animals being observed for 5 days to determine the median protective dose (PD_{50}). The results are shown in Table 9. Bu-2470 A was found to be relatively more active *in vivo* than *in vitro* in comparison with components B_1 and B_2.

The acute toxicities of Bu-2470 A and B_1 were determined in mice by intravenous (iv) and subcutaneous (sc) routes. Colistin was tested comparatively as a reference compound. As shown in Table 10,

Table 8. Activity against *Pseudomonas* species.

Test organism	MIC (μg/ml)		
	Bu-2470 A	Bu-2470 B_1	Bu-2470 B_2
Pseudomonas aeruginosa D15	6.3	12.5	12.5
〃 〃 A15150	12.5	12.5	6.3
〃 〃 A15194	6.3	6.3	3.1
〃 〃 GN 4925	6.3	12.5	12.5
〃 〃 A21428	6.3	12.5	6.3
P. cepacia SCH-15	50	6.3	1.6
P. maltophilia A20620	1.6	6.3	6.3
〃 〃 A21384	0.4	1.6	0.8
〃 〃 A21550	0.8	3.1	1.6
〃 〃 AKH-36	0.4	0.8	1.6
〃 〃 AKH-81	0.4	1.6	1.6
P. melanogenum A20817	1.6	3.1	6.3
P. putida AKH-15	6.3	6.3	6.3
〃 〃 AKH-66	12.5	6.3	3.1
〃 〃 KUH-11	6.3	6.3	6.3

Table 9. *In vivo* activity of Bu-2470.

Challenge organism	PD_{50} (mg/kg, im)		
	Bu-2470 A	Bu-2470 B_1	Bu-2470 B_2
E. coli Juhl	7.6	50	—
K. pneumoniae A9977	25	43	—
P. aeruginosa A9843	25	100	22

Table 10. Acute toxicity of Bu-2470.

Route of administration	LD_{50} (mg/kg)		
	Bu-2470 A	Bu-2470 B_1	Colistin
Intravenous	35	35	8.8
Subcutaneous	56	280	115

Bu-2470 B_1 was less toxic than colistin by both iv and sc routes, while Bu-2470 A was less toxic than colistin by the iv route but more toxic by the sc route.

Discussion

The physico-chemical properties and antibacterial activity of Bu-2470 B_1 and B_2 are similar to those of the octapeptin group of antibiotics[3] which include EM-49[4], 333-25 (octapeptin C_1)[5] and Bu-1880[6]. The octapeptin antibiotics are octapeptides acylated with a fatty acid residue and inhibit Gram-positive and Gram-negative bacteria including *P. aeruginosa*. As shown in Tables 4 and 5, Bu-2470 B_1 and B_2 can be differentiated from EM-49 and 333-25 by thin layer, paper and high performance liquid chromatographies. Bu-2470 B_1 and Bu-1880 could not be differentiated by the chromatographic system employed.

In contrast to the B components of Bu-2470 and octapeptin antibiotics, the antibiotic spectrum of Bu-2470 A is limited to certain Gram-negative organisms, in particular, *Pseudomonas* species including *P. aeruginosa*, *P. maltophilia*, *P. melanogenum* and *P. putida*. Bu-2470 A showed better *in vivo* activity than Bu-2470 B_1, suggesting a superior bioavailability of Bu-2470 A in animals relative to the latter.

Acknowledgment

The authors are indebted to Dr. E. MEYER of Squibb Institute of Medical Research for the sample of EM-49 and Dr. J. SHOJI of Shionogi Research Laboratory for the sample of 333-25 (octapeptin C_1).

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