Bu-2313, A NEW ANTIBIOTIC COMPLEX ACTIVE AGAINST ANAEROBES

I. PRODUCTION, ISOLATION AND PROPERTIES OF Bu-2313 A AND B

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An unidentified oligosporic actinomycete strain, No. E864-61, produced two new antibiotics, Bu-2313 A ($C_{27}H_{35}NO_9$) and Bu-2313 B ($C_{26}H_{33}NO_9$). Bu-2313 A and B each exhibited a broad antibiotic spectrum against Gram-positive and Gram-negative anaerobic bacteria, and showed *in vivo* activity against experimental infections produced by *B. fragilis* and *C. perfringens*. Bu-2313 also inhibited some aerobic bacteria such as streptococci. Bu-2313 B was approximately two-fold more active than Bu-2313 A.

With the increasing awareness of the role of anaerobic infections in clinical medicine, effective and nontoxic agents active against anaerobic organisms are needed. Benzylpenicillin is considered to be the drug of choice for many anaerobic infections[1] except for those caused by *Bacteroides fragilis*, the single most common anaerobic organism found in clinical specimens[2]. As compared with other anaerobes, members of this species are relatively resistant to many antibiotics.

In our antibiotic screening program using *B. fragilis* as one of the assay test organisms, an oligosporic actinomycete strain was found to produce a new antibiotic complex designated as Bu-2313. It was extracted from the fermentation broth and separated into two components A and B. Both components showed antibiotic activity against a variety of anaerobic bacteria as well as some aerobic microorganisms. The structures of Bu-2313 A and B have been determined[3], indicating that they belong to the acyltetramic acid group of antibiotics which also includes streptolydigin[4] and tirandamycin[5].

This paper reports on the production, isolation, physico-chemical and biological properties of Bu-2313 A and B.

Producing Organism

The aerobic actinomycete strain, No. E864-61, produced aerial spore chains consisting of a varied number of spores (1~8). The color of the aerial mycelium was initially white, later turning to dull bluish green or light greyish green with abundant sporulation. Two types of pigment, violet and dark green, were produced in agar media. The spores were spherical to oval in shape, 0.6~1.2 μm in size, and had a smooth surface. Aerial hyphae were occasionally spiralled and coiled in several to ten turns. The substrate mycelium did not become fragmented within a week at 37°C. The growth of strain E864-61 was inhibited by sodium chloride. It produced no melanoid pigment and grew at 20~50°C but not at 55°C.

The cell-wall of strain E864-61 was found to contain *meso*-diaminopimelic acid, glutamic acid and alanine. Galactose and a small amount of rhamnose were contained in the whole cell hydrolyzate.

Strain E864-61 resembles the actinomycetes genera of *Microtetraspora, Micropolyspora, Saccharomonospora, Thermomonospora* and *Actinomadura* in some of the morphological and biochemical properties including the presence of *meso*-diaminopimelic acid in the cell wall. Results of further taxonomic studies on strain E864-61 will be published elsewhere.

Antibiotic Production

A well-grown agar slant culture of strain E864-61 was used to inoculate vegetative medium containing 3% glucose, 3% soybean meal, 1% corn steep liquor and 0.5% $CaCO_3$. The media pH was adjusted to 7.0 before sterilization. The seed culture was incubated at 34°C for 3 days on a rotary shaker (250 rpm) and 2~3 ml of the growth transferred to a 500-ml Erlenmeyer flask containing 100 ml of fermentation medium composed of 3% sucrose, 3% linseed meal, 0.3% $(NH_4)_2SO_4$, 0.003% $ZnSO_4 \cdot 7H_2O$ and 0.5% $CaCO_3$. The broth pH gradually rose over the course of shaking fermentation at 28°C and reached 8.1~8.8 after 4~7 days, when the peak of antibiotic production was attained. The antibiotic activity in the fermentation broth was determined by a paper disc-agar plate method using *Bacteroides fragilis* A20928 as a test organism. The organism was grown on GAM agar plate (Gifu anaerobe medium, Nissui, Tokyo) under anaerobic condition using GasPak generators (BBL, Cockeysville, Md.).

Fermentation studies were also carried out in stir jar fermentors and tanks. In one of the examples, 110 liters of seed culture was inoculated to 1,500 liters of fermentation medium in a 2,500-liter tank containing 3% glucose, 3% soybean meal, 1% corn steep liquor and 0.5% $CaCO_3$. The tank was operated at 31°C with stirring at 200 rpm and an aeration rate at 1,000 liters/min. The fermentation was continued for 72 hours, at which time the broth pH was 8.2 and the antibiotic potency maximal.

Isolation and Purification

The fermentation broth was filtered with filter aid and the mycelial cake washed with water. The filtrate was combined with the wash, adjusted to pH 8.3 and applied to a column of high-porous adsorption resin (Diaion HP-20). The column was washed successively with water and 40% aqueous methanol, and then developed with 95% aqueous methanol. Active fractions were combined and concentrated *in vacuo* to an aqueous concentrate. The wet mycelial cake obtained above was suspended in methanol and extracted with stirring. This was repeated twice and the combined methanol extract was concentrated *in vacuo*. The two aqueous concentrates obtained from the HP-20 eluate and the mycelial cake extract were combined and further evaporated *in vacuo*, and the activity in the concentrate was extracted twice with ethyl acetate. The extract was concentrated *in vacuo* to dryness to leave an oily solid (*ca.* 20% purity), which was dissolved in a mixture of ethyl acetate and methanol (20: 1) and applied to a column of activated carbon. The column was developed with the same solvent mixture. The active eluates were combined and concentrated *in vacuo* to give a dark brown solid, which was crystallized from hot methanol to yield a brownish yellow crystalline powder (60~70% purity).

The crystalline material thus obtained was a complex of Bu-2313 A and B, whose separation was achieved by Diaion HP-20 chromatography: The solid was dissolved in 90% aqueous methanol and the solution applied on a column of HP-20 resin. The column was developed with 80% aqueous methanol to give two active fractions. From the concentrate of the first active fraction, Bu-2313 B was

obtained as pale yellow needle-like crystals which were recrystallized twice from methanol to give an analytically pure preparation. The second active eluate gave a mixture of components A and B which was separated by silica-gel column chromatography developed with chloroform. Pure crystals of Bu-2313 A were obtained, after recrystallization from methanol, from the fast-moving fractions, and an additional amount of Bu-2313 B recovered from the latter part of the eluates. Approximately 11 g of Bu-2313 A and 110 g of Bu-2313 B were isolated from 1,500 liters of fermentation broth.

Table 1. UV spectra of Bu-2313 A and B.

Solvent	λ_{max} in nm ($E^{1\%}_{1cm}$)	
	Bu-2313 A	Bu-2313 B
Ethanol	245 (170) 295 (212, sh*) 355 (509) 375 (390, sh)	239 (170) 295 (225, sh) 351 (525) 370 (420, sh)
0.01 N HCl - 95% EtOH	242 (170) 358 (645) 375 (555, sh)	237 (170) 353 (668) 370 (580, sh)
0.01 N NaOH - 95% EtOH	262 (375) 286 (393) 337 (430)	253 (345) 286 (411) 331 (467)

* shoulder

Physico-chemical Properties

Bu-2313 A and B are acidic substances isolated as pale yellow crystals. They are readily soluble in most organic solvents such as lower alcohols, ethyl acetate, chloroform and benzene, slightly soluble in hexane and alkaline water, and practically insoluble in water. They are stable in acidic solution but less so in alkaline solution, losing activity in 0.1 N NaOH solution when held overnight at room temperature. Both components react positively with ferric chloride but give negative reactions in ninhydrin, anthrone, SAKAGUCHI and TOLLEN's tests. Bu-2313 A and B can be differentiated by TLC, IR and NMR spectra as described below.

Bu-2313 A melts at 116~118°C and is optically active: $[\alpha]_D^{26}$ −58° (*c* 0.5, MeOH). It showed a pKa' of 5.2 in 50% aqueous ethanol solution with a titration equivalent of 519. The

Fig. 1. Ultraviolet absorption spectra of Bu-2313 A.

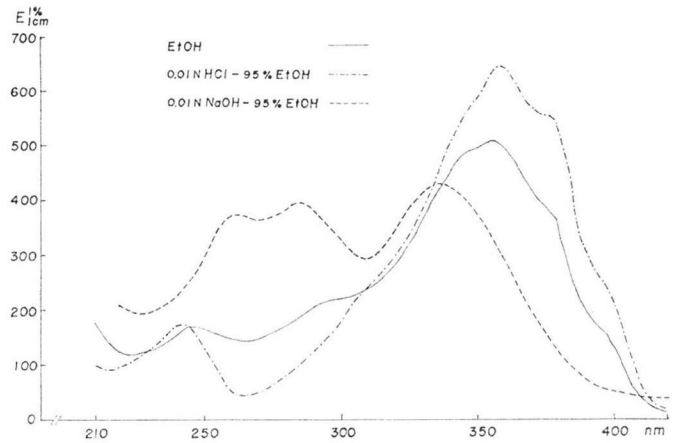


Fig. 2. Ultraviolet absorption spectra of Bu-2313 B.

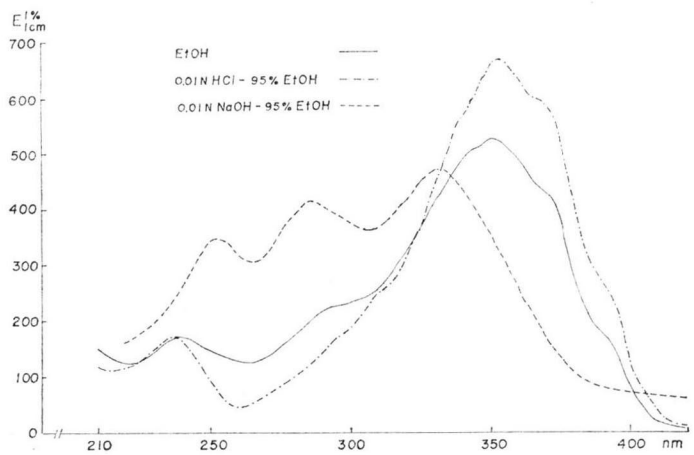


Fig. 3. Infrared absorption spectrum of Bu-2313 A.

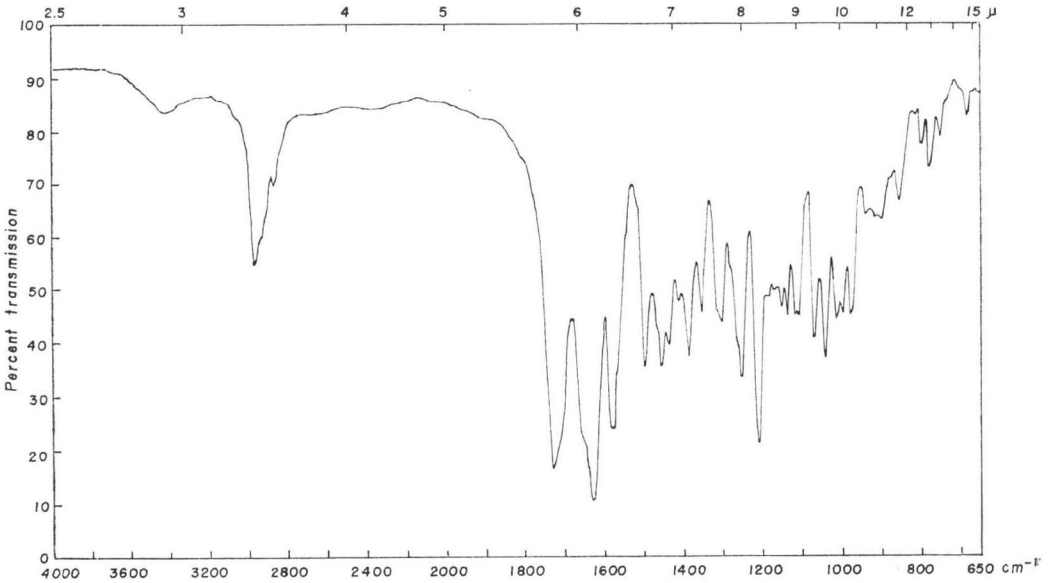
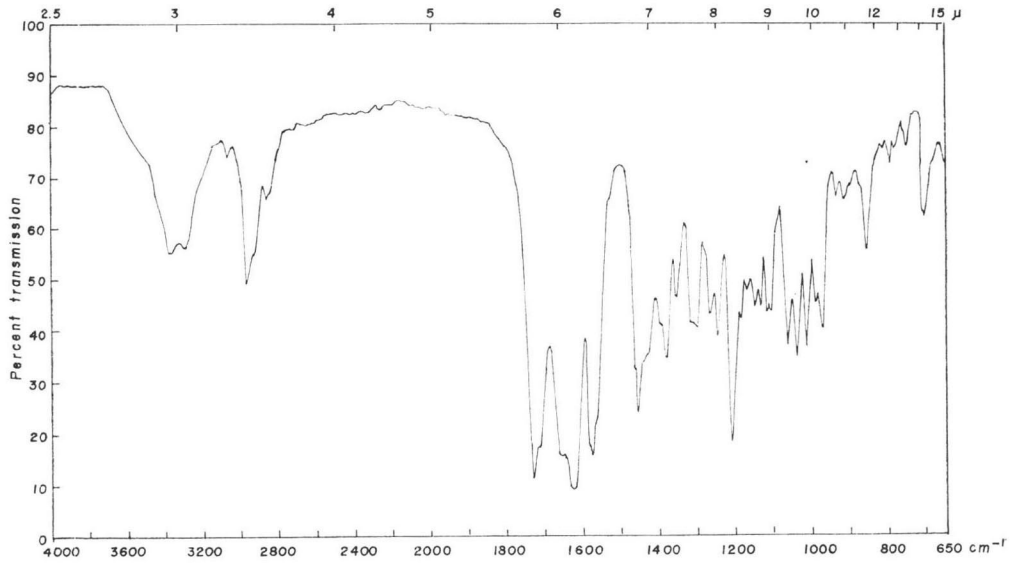


Fig. 4. Infrared absorption spectrum of Bu-2313 B.



mass spectrum of Bu-2313 A gave a molecular ion peak at *m/e* 517.

Anal. Calc'd for $C_{27}H_{35}NO_9$: C 62.65, H 6.82, N 2.71, O 27.82.
Found: C 62.57, H 6.64, N 2.60, O 28.19 (by difference).

Bu-2313 B melts at 160~162°C and its optical rotation is $[\alpha]_D^{26}$ −69.9° (*c* 0.3, MeOH) and −34.9° (*c* 0.93, $CHCl_3$). It showed a pKa′ of 4.9 in 50% aqueous ethanol solution with a titration equivalent of 509 and gave a molecular ion peak at *m/e* 503 in the mass spectrum.

Anal. Calc'd for $C_{26}H_{33}NO_9$: C 62.03, H 6.56, N 2.78, O 28.63.
Found: C 61.77, H 6.80, N 2.65, O 28.78 (by difference).

Fig. 5. NMR Spectrum of Bu-2313 A (60 MHz in $CDCl_3$).

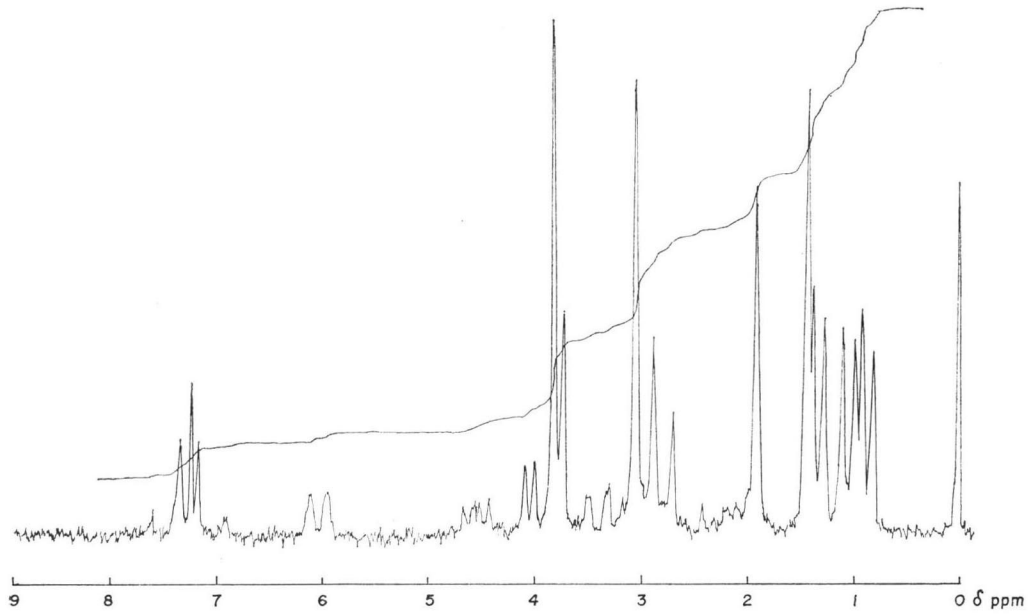
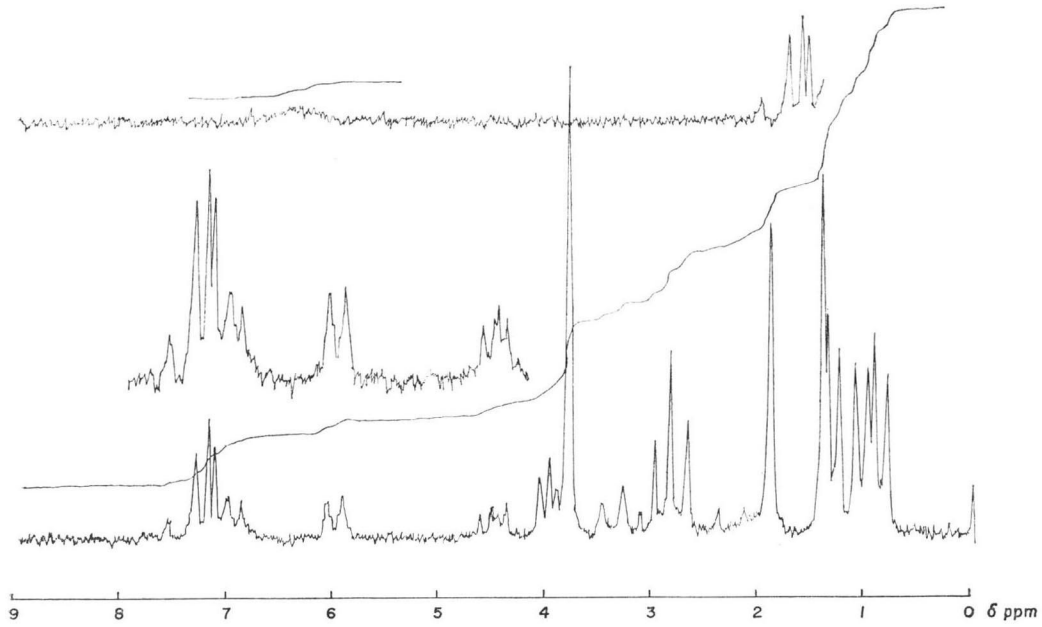


Fig. 6. NMR Spectrum of Bu-2313 B (60 MHz in $CDCl_3$).



The UV spectra of Bu-2313 A and B taken in neutral, acidic and basic conditions are shown in Figs. 1 and 2, and the intensities of the absorption maxima are shown in Table 1. The hypsochromic shift of the UV maximum at 350~360 nm to 330~340 nm in going from acidic to alkaline pH is characteristic to that reported for the dienoyltetramic acid antibiotics[6,7].

The infrared spectra of Bu-2313 A and B in KBr pellet (Figs. 3 and 4) are similar to each other showing strong absorption bands at 1730, 1660, 1630, 1580 and 1210 cm^{-1}. A sharp band at 1500 cm^{-1}

is present in component A but not B, while component B shows characteristic N-H absorptions at 3200~3400 cm^{-1} which are absent in component A. The proton NMR spectra of Bu-2313 A and B (in $CDCl_3$) are shown in Figs. 5 and 6. The NMR spectrum of component A showed an N-methyl signal at δ 3.03 ppm which is absent in component B.

Biological Properties

In Vitro Antibacterial Activity

The minimum inhibitory concentrations (MIC) of Bu-2313 A and B were determined for a variety of aerobic and anaerobic bacteria by the serial two-fold agar dilution method using a multi-inoculating apparatus. Nutrient agar medium (Eiken) was generally used for aerobic bacteria, GC medium (Eiken) for fastidious aerobic organisms such as streptococci, *Neisseria* and *Hemophilus* species, and GAM agar medium (Nissui) for anaerobic bacteria.

The *in vitro* antibacterial spectra of Bu-2313 A and B for aerobic organisms are shown in Table 2 and for anaerobic bacteria in Table 3. Against aerobic organisms Bu-2313 A and B inhibited the growth of various species of streptococci, *Bacillus* species, *Neisseria meningitidis*, *N. gonorrhoeae* and *Hemophilus influenzae* but showed only moderate to weak activity against aerobic organisms such as staphylococci and strains of *Enterobacteriaceae*. Bu-2313 A and B showed strong inhibitory activity against a wide range of anaerobic bacteria including Gram-positive and Gram-negative rods and cocci. The intrinsic *in vitro* activity of Bu-2313 B was approximately two times greater than that of Bu-2313 A.

The inhibitory potential of Bu-2313 A and B for two pathogenic species of *Trichomonas*, *T. vaginalis* and *T. foetus*, which are known to be anaerobic microorganisms, was also determined. The protozoa were grown at 37°C overnight in DIAMOND's medium containing 10% horse serum, 50 mcg/ml of streptomycin and 150 mcg/ml of phenethicillin. A series of tubes containing two-fold dilutions of the test compounds in the above medium were inoculated with the protozoa (inoculum size: 10%) and incubated overnight at 37°C to determine the MIC. Metronidazole (Flagyl, Shionogi) was tested as a reference compound. As shown in Table 4, Bu-2313 showed weak inhibitory activity against trichomonads.

Table 2. *In vitro* activity against aerobic bacteria.

Test organism	Strain No.	Test medium*	MIC (mcg/ml)	
			Bu-2313 A	Bu-2313 B
Staphylococcus aureus	209P	A	25	25
" "	Smith	A	12.5	12.5
Sarcina lutea	PCI-1001	A	25	12.5
Streptococcus pyogenes	A9604	B	0.8	0.4
Streptococcus viridans	A21354	B	0.8	0.4
Streptococcus pneumoniae	A9585	B	0.8	0.4
Bacillus subtilis	PCI-219	A	1.6	1.6
Bacillus cereus	ATCC 10702 A	A	0.8	0.8
Escherichia coli	Juhl	A	>100	>100
Klebsiella pneumoniae	A9678	A	>100	>100
Proteus morganii	A9553	A	>100	>100
Neisseria meningitidis	A20049	B	1.6	0.8
Neisseria gonorrhoeae	A15112	B	1.6	0.8
Hemophilus influenzae	A9832	B	0.8	0.4

* A: Nutrient agar (Eiken). B: GC medium (Eiken).

Table 3. *In vitro* activity against anaerobic bacteria.

Test organism	Strain No.	MIC (mcg/ml) in GAM agar medium	
		Bu-2313 A	Bu-2313 B
Bacteroides fragilis	A20926	0.2	0.1
" "	A20928-1	0.1	0.1
" "	A20929	0.2	0.1
" "	A20930	0.2	0.1
" "	A20932	0.2	0.1
" "	A20935	0.2	0.1
Sphaerophorus necrophorus	A15202	0.2	0.1
Sphaerophorus pseudonecrophorus	A20013	0.2	0.1
Fusobacterium mortiferum	ATCC 9817	0.2	0.1
Fusobacterium varium	ATCC 8501	0.1	0.1
Acidoaminococcus fermentans	ATCC 25085	0.2	0.1
Veillonella parvula	ATCC 17745	0.2	0.1
Clostridium acetobutylicum	IAM 19011	0.4	0.2
Clostridium caproicum	IAM 19228	0.4	0.2
Clostridium chavoei	A9561	0.2	0.2
Clostridium perfringens	A9635	0.4	0.2
" "	A21284	0.4	0.2
Peptococcus prevotii	ATCC 9321	0.2	0.1
Peptococcus aerogenes	ATCC 14963	0.2	0.2
Peptostreptococcus anaerobius	B43	0.4	0.4

Table 4. Antitrichomonas activity of Bu-2313 A and B.

Organism	MIC (mcg/ml)		
	Bu-2313 A	Bu-2313 B	Metro-nidazole
T. vaginalis 1099	12.5	50	0.8
T. foetus Inui	12.5	25	0.8

In Vivo Experiments

The effectiveness of Bu-2313 A and B *in vivo* was assessed in experimental infections of mice produced by two anaerobic bacteria, *Bacteroides fragilis* and *Clostridium perfringens*, and by one aerobic pathogen, *Streptococcus pyogenes*.

A localized infection of *B. fragilis* A20926 was established[8] by subcutaneous injection into the dorsal area of the neck of mice with 0.5 ml of the bacterial suspension containing $2\sim5\times10^6$ cells and 10 mg of microcrystalline cellulose. Treatment was given subcutaneously at the lower back or orally once daily for 5 consecutive days starting 30 minutes after the challenge. A group of five mice was used at each dosage level and the animals were dissected on the 6th day to measure the size of the subcutaneous abscess. The response in each animal was scored as $0\sim5$ according to the size of lesion, and the sum of the lesion scores was divided by the number of mice used per group to obtain the mean score for each of the treatment and control groups. Taking the mean score of the control group as the 100% infectivity level, the relative infection level was calculated for each of the treatment groups and a PD_{50} (protective dose, 50%) was then estimated by means of log-probit plot. As shown in Table 5, Bu-2313 B and metronidazole were effective in this infection model with somewhat greater activity

demonstrated by the latter compound.

The systemic infection of *C. perfringens* A9635 was produced in mice by intraperitoneal challenge with a lethal dose of the pathogen given in a 5% suspension of hog gastric mucin (Americal Laboratories, Omaha, Neb.). The antibiotic was administered subcutaneously or orally just before the bacterial challenge. The PD_{50} was determined after 5 days. As shown in Table 6, Bu-2313 B gave fairly good protection against the infection by both subcutaneous and oral routes, while Bu-2313 A was comparably active by the subcutaneous route but less effective than Bu-2313 B by the oral route. Metronidazole was less active than Bu-2313 in this anaerobic infection model.

Bu-2313 A and B were also tested in the *Streptococcus pyogenes* A20201 systemic infection by a procedure similar to that used to produce the above-described *Clostridium perfringens* infection. As shown in Table 7, Bu-2313 B showed activity by both subcutaneous and oral routes, while Bu-2313 A was only active parenterally.

Blood levels were determined in mice following subcutaneous or oral administration of Bu-2313 B. Blood samples were collected from orbital sinuses and assayed by the paper disc-agar plate method using *B. fragilis* A20926 as the test organism. As shown in Table 8, Bu-2313 B was fairly well absorbed in mice after both subcutaneous and oral dosing.

The acute toxicity of Bu-2313 A and B was determined in mice by the subcutaneous route. Bu-2313 B was found to be less toxic than Bu-2313 A: The subcutaneous LD_{50} was 90 mg/kg for Bu-2313 A and 320 mg/kg for Bu-2313 B.

Table 5. *In vivo* activity against localized infection with *B. fragilis*.

Subcutaneous dose (mg/kg/day)	Infectivity level (%)	
	Bu-2313 B	Metronidazole
50 ×5	52.4	47.6
12.5×5	66.7	57.1
3.1×5	81.0	81.0
0.8×5	95.2	95.2
PD_{50} (sc)	60 mg/kg	35 mg/kg

Oral dose (mg/kg/day)	Infectivity level (%)	
	Bu-2313 B	Metronidazole
100 ×5	57.1	38.1
25 ×5	71.4	61.9
6.3×5	81.0	81.0
1.6×5	95.2	95.2
PD_{50} (po)	*ca.* 200 mg/kg	50 mg/kg

Table 6. *In vivo* activity against systemic infection with *C. perfringens*.

Route of administration	PD_{50} (mg/kg)*		
	Bu-2313 A	Bu-2313 B	Metronidazole
Subcutaneous	6.3	6.3	31
Oral	>50	30	60

* single treatment

Table 7. *In vivo* activity against *S. pyogenes* infection.

Route of administration	PD_{50} (mg/kg)*	
	Bu-2313 A	Bu-2313 B
Subcutaneous	7.6	9.0
Oral	NA**	25

* single treatment
** no activity at 25 mg/kg

Table 8. Blood levels in mice.

Time after administration	Blood levels of Bu-2313 B (mcg/ml)	
	Subcutaneous dose of 25 mg/kg	Oral dose of 100 mg/kg
15 min.	12	
30 min.	10	12
1 hr.	4.6	5.5
2 hrs.	0.9	
3 hrs.		0.4
5 hrs.		0.3

Discussion

Bu-2313 A and B showed characteristic UV spectra which are similar to those of the dienoyltetramic acid antibiotics, streptolydigin and tirandamycin. A companion paper[3] indicates that Bu-2313 A and B do in fact have an acyltetramic acid structure. Bu-2313 A and B, streptolydigin and tirandamycin were differentiated from each other using three TLC systems as shown in Table 9. Although the antianaerobic activity of streptolydigin and tirandamycin against *Clostridium* species has been described, these two antibiotics were also detected in our anti-anaerobic screening program and found to be active against many anaerobic bacteria including *B. fragilis*. Recently, a new member of this group of antibiotics was reported under the name of nocamycin from Russian scientists[9]. The physico-chemical properties and spectral data of nocamycin[10] are very close to those of Bu-2313 B, but the structure assigned to nocamycin[11] is different from that of Bu-2313 B[3].

Table 9. TLC comparison of Bu-2313 A and B with related antibiotics.

Antibiotic	Rf values (silica gel TLC)*		
	System 1	System 2	System 3
Bu-2313 A	0.71	0.32	0.63
Bu-2313 B	0.49	0.12	0.28
Streptolydigin	0.71	0.30	0.55
Tirandamycin	0.44	0.17	0.28

* Detection by bioautography on *B. fragilis* plate.
System 1: benzene - ethanol - 20% aq. ammonia (65: 40: 9)
System 2: chloroform - methanol (4: 1)
System 3: ethyl acetate - methanol (4: 1)

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