SORBISTIN, A NEW AMINOGLYCOSIDE ANTIBIOTIC COMPLEX OF BACTERIAL ORIGIN

I. PRODUCTION, ISOLATION AND PROPERTIES

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A strain of a new *Pseudomonas* species produced the aminoglycoside antibiotic complex, sorbistin, which was separated by ion-exchange chromatography into three bio-active components A_1, A_2 and B, and two bio-inactive components C and D. Sorbistins A_1, A_2 and B showed moderate intrinsic activity against a wide range of bacterial species and inhibited most of the aminoglycoside-resistant organisms. Sorbistin A_1 exhibited the highest activity among the three bio-active components. Sorbistins showed low order of acute toxicity in mice.

A new antibiotic complex was isolated from the fermentation broth of a new bacterial strain, *Pseudomonas sorbicinii* sp. nov.[1] This antibiotic, originally designated Bu-2183 and now named sorbistin, is a complex of unique aminoglycoside components A_1, A_2 and B, along with two structurally-related, bio-inactive components C and D. The structure for each of these components has been determined and described in a companion paper[2]. This paper reports the production, isolation, physicochemical and biological properties of sorbistin.

Antbiiotic Production

A psychrotrophic bacterial strain No. D946-B83, which was isolated from an East Himalayan soil sample and found to be a new species of genus *Pseudomonas*, was grown at 20~30°C on agar slant. A well-grown agar slant of the sorbistin-producing organism was used to inoculate seed medium containing 3% glucose, 2% fish meal, 0.5% soybean meal, 0.2% peptone and 0.6% $CaCO_3$, the pH being adjusted to 7.0 before sterilization. The seed culture was incubated at 28°C for 48 hours on a rotary shaker (250 rpm), and 2 ml were transferred to a 500-ml Erlenmeyer flask containing 100 ml of fermentation medium composed of 2% glycerol, 2% linseed meal, 1% peanut meal, 2% fish meal, 0.3% $(NH_4)_2SO_4$ and 0.5% $CaCO_3$. Antibiotic production reached a maximum after 3~5 days shaking at 28°C. The antibiotic activity in the fermentation broth was determined by a paper disc-agar diffusion assay using *Bacillus subtilis* PCI 219 as the test organism.

Fermentation studies were also carried out in 10-liter jar fermentors and 300-liter pilot tanks. The seed culture (1~2%) was inoculated to tank fermentation medium which contained 2% glycerin, 1% Pharmamedia, 2% fish meal, 2% linseed meal, 0.3% $(NH_4)_2SO_4$ and 0.6% $CaCO_3$. The tanks were operated at 30°C with stirring at 140 rpm. The broth pH gradually rose with the progress of fermentation and reached 8.0~8.2 after 60~70 hours when the peak antibiotic potency of 1,500~2,000 mcg/ml was obtained.

Isolation and Purification

The fermentation broth was filtered with filter aid at pH 2, the filtrate was adjusted to pH 7 and applied on a column of Amberlite IRC-50 (NH_4^+ form). The column was washed with water and N/50 NH_4OH successively, and then developed with N/2 NH_4OH. The active eluates were combined, concentrated *in vacuo* and lyophilized. The crude solid thus obtained was shown by TLC to be a mixture of three bio-active components (designated as A_1, A_2 and B) and two inactive components (C and D).

The complex solid was separated into its components by ion-exchange column chromatography using Amberlite CG-50 resin (NH_4^+ form) developed with increasing concentration of aqueous ammonia. Component A (A_1+A_2) was eluted first with N/20 NH_4OH followed by component B, and successive elution with N/10 NH_4OH gave components C and D in order. Fractions containing each component were separated and rechromatographed on a CG-50 column. Component A was further separated into A_1 and A_2, the latter being a minor component eluted earlier than the major component A_1. An example of the relative yield of sorbistin components was approximately A_1: A_2: B: C: D=25: 2: 40: 25: 8 in weight. Two TLC systems, S-117 and S-122, were found suitable for differentiating the components (Table 1).

Table 1. TLC of sorbistin components

System	Rf*				
	A_1	A_2	B	C	D
S-117	0.46	0.55	0.36	0.27	0.28
S-122	0.38	0.48	0.27	0.15	0.03

S-117: silica gel, $CHCl_3$ - CH_3OH - 28% NH_4OH (1: 3: 2)
S-122: silica gel, $CHCl_3$ - CH_3OH - 2N NH_4OH - CH_3COOH (20: 65: 40: 5)
* detection by ninhydrin reagent

Physico-chemical Properties

Sorbistins A_1, A_2, B, C and D are basic substances and were isolated as a white amorphous carbonate salt. They are freely soluble in water, slightly soluble in methanol and ethanol, but practically

Fig. 1. IR spectrum of sorbistin A_1

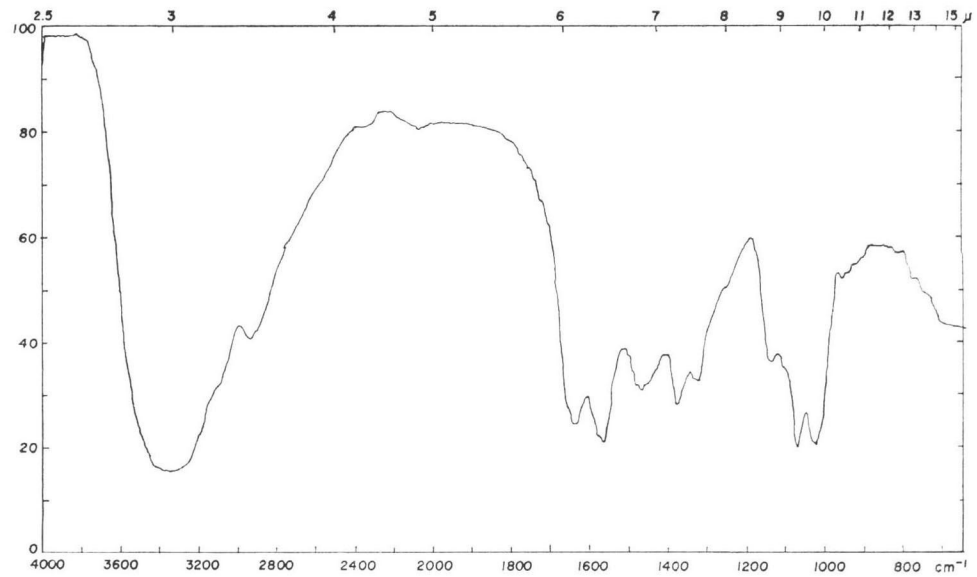
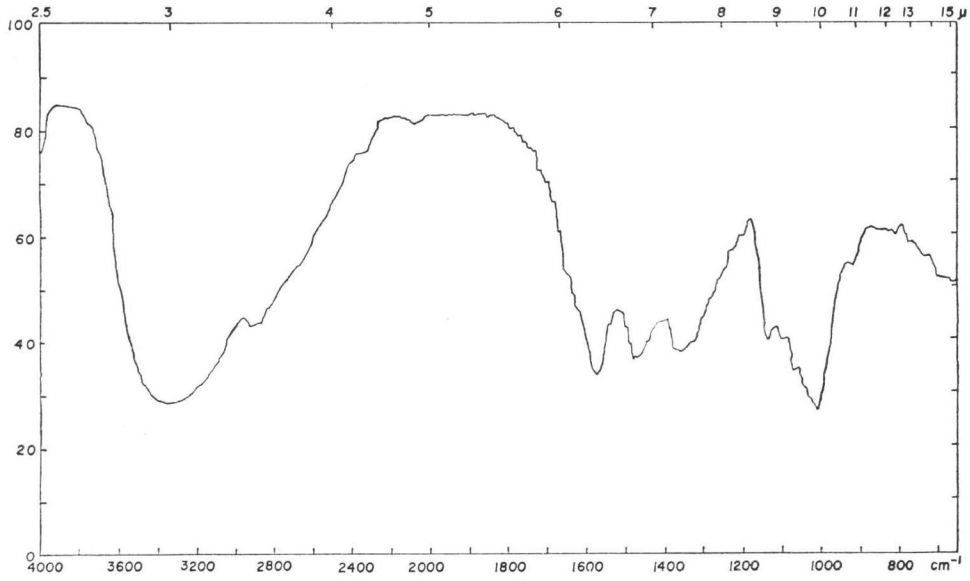


Table 2. Physico-chemical properties of sorbistin components

Component		A_1	A_2	B	C	D
$[\alpha]_D$ (*c* 1.0, H_2O)		+78.5	+79.1	+85.0	+81.5	+71.0
Microanalysis						
Found	C:	43.22	44.35	41.92	39.89	38.67
	H:	7.52	7.83	7.43	7.16	6.93
	N:	9.49	9.21	9.93	7.21	10.16
Calc'd for		$C_{15}H_{31}N_3O_9$ ·1/2 H_2CO_3	$C_{16}H_{33}N_3O_9$ ·1/2 H_2CO_3	$C_{14}H_{29}N_3O_9$ ·1/2 H_2CO_3	$C_{12}H_{26}N_2O_6$ ·1/2 H_2CO_3	$C_{12}H_{27}N_3O_8$ ·H_2CO_3
	C:	43.45	44.79	42.02	40.21	38.70
	H:	7.53	7.75	7.30	7.29	7.25
	N:	9.81	9.50	10.14	7.50	10.42
pKa (in H_2O)		6.8, 9.4	6.9, 9.4	6.8, 9.4	6.8, 9.4	6.6(2 eq.), 9.8
Titration equiv.		422	434	414	379	415

Fig. 2. IR spectrum of sorbistin C



insoluble in *n*-butanol, acetone and other organic solvents. All the components give positive reactions with ninhydrin and anthrone reagents but are negative to TOLLENS, FEHLING and SAKAGUCHI reactions. The microanalysis, titration and specific rotation data are shown in Table 2 for each of the sorbistin components.

Sorbistins exhibit only end absorption in the UV spectrum. The IR spectra of three bio-active components A_1 (Fig. 1), A_2 and B are nearly identical, showing amide carbonyl bands at 1635 and 1570 cm^{-1} which, however, are absent in the IR spectra of sorbistins C and D (Figs. 2 and 3). The NMR spectrum of sorbistin A_1 is shown in Fig. 4 which indicates the presence of an anomeric proton (δ 5.23 ppm) and signals for a propionyl group. Sorbistins A_2 and B differ from A_1 in that, instead of the pro-

pionyl group in sorbistin A_1, signals for *n*-butyryl group are present in the NMR spectrum of sorbistin A_2 (Fig. 5) and an acetyl group in sorbistin B (Fig. 6). The NMR spectra of components C and D (Figs. 7 and 8) show the presence of an anomeric proton but lack the signals for any acyl group.

Fig. 3. IR spectrum of sorbistin D

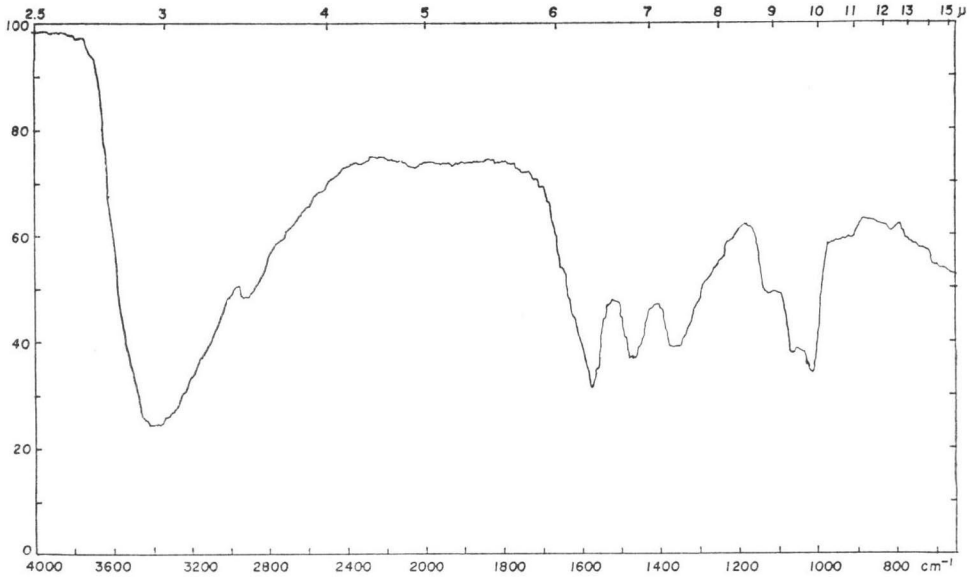
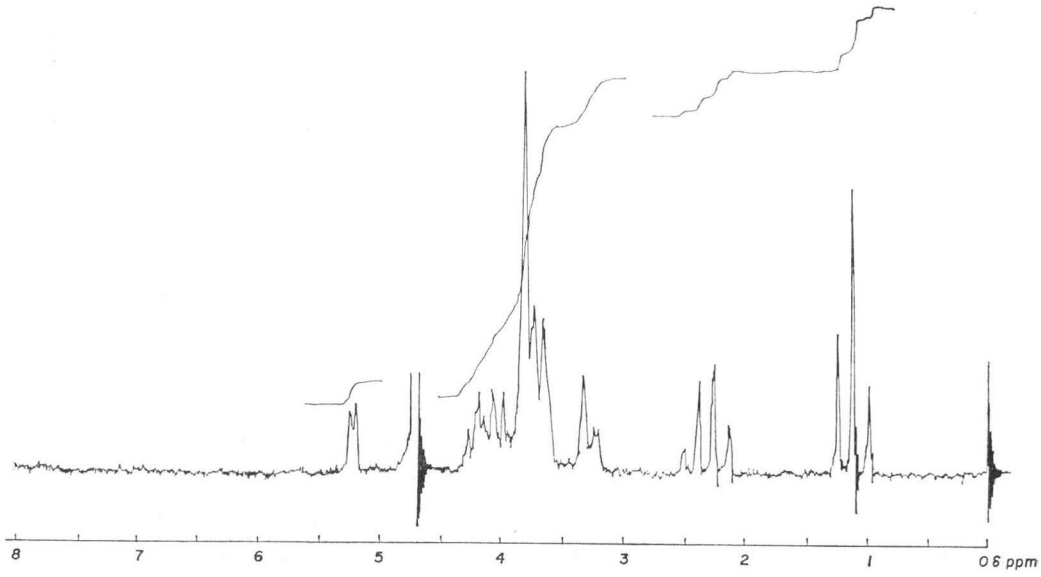


Fig. 4. NMR spectrum of sorbistin A_1 hydrochloride (60 MHz, in D_2O)



Sorbistins A_1, A_2, B and C gave crystalline di-N-acetates, while a tri-N-acetate was obtained from sorbistin D[2]. The di-acetate of sorbistin B was identified as the tri-acetate of sorbistin D, suggesting that sorbistin B is a mono-acetyl derivative of sorbistin D.

Fig. 5. NMR spectrum of sorbistin A_2 hydrochloride (60 MHz, in D_2O)

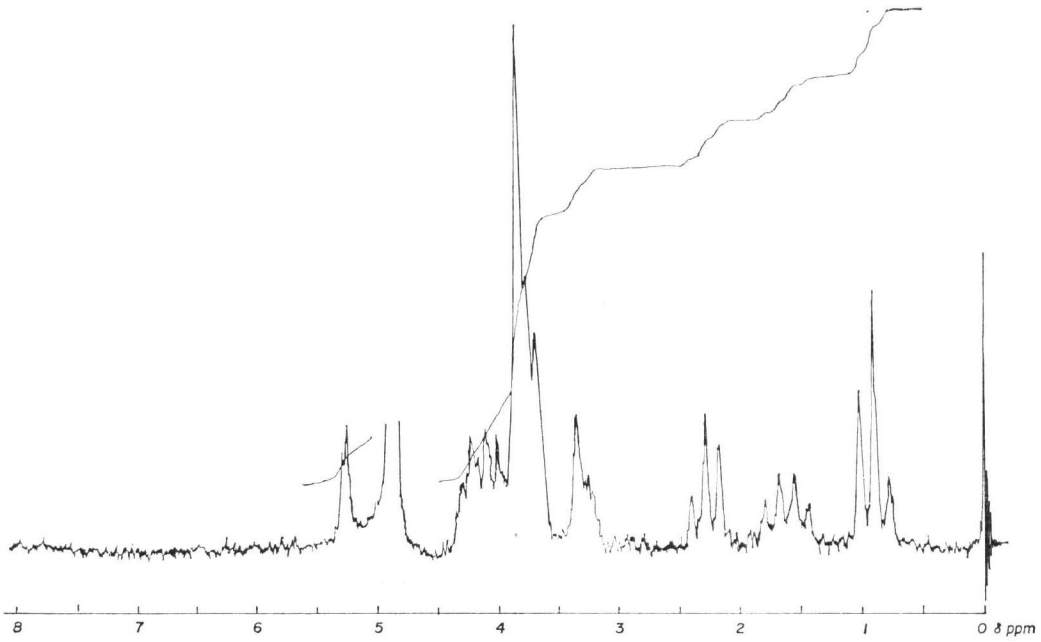


Fig. 6. NMR spectrum of sorbistin B hydrochloride (60 MHz, in D_2O)

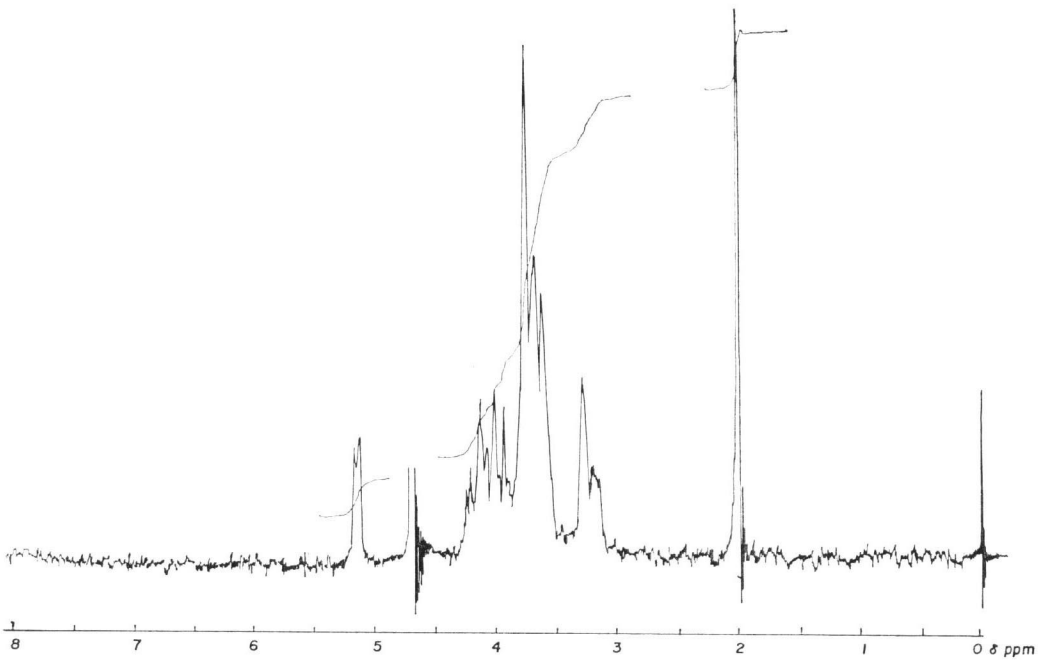


Fig. 7. NMR spectrum of sorbistin C hydrochloride (60 MHz, in D_2O)

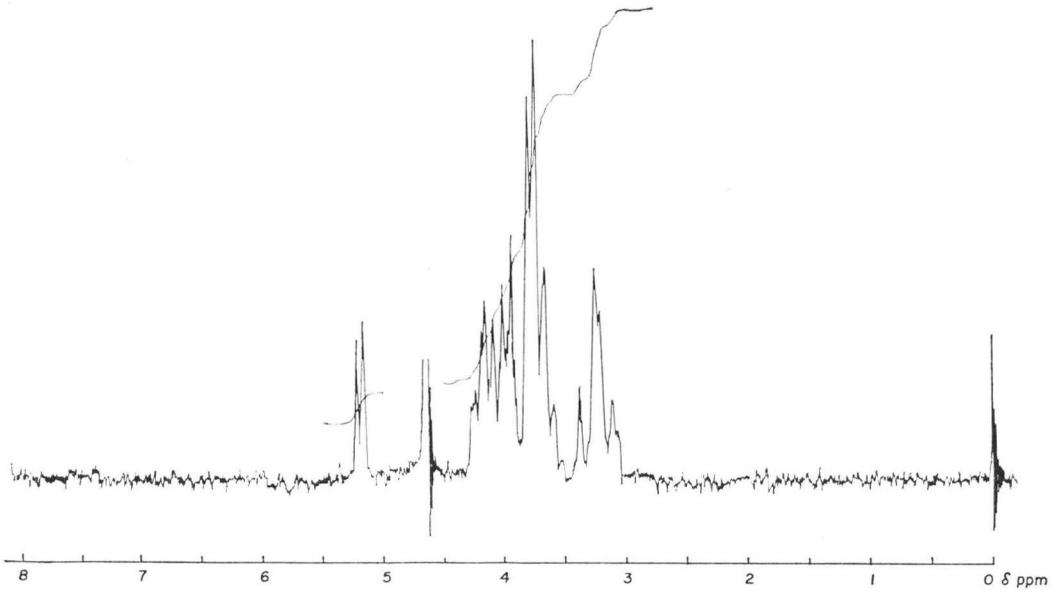
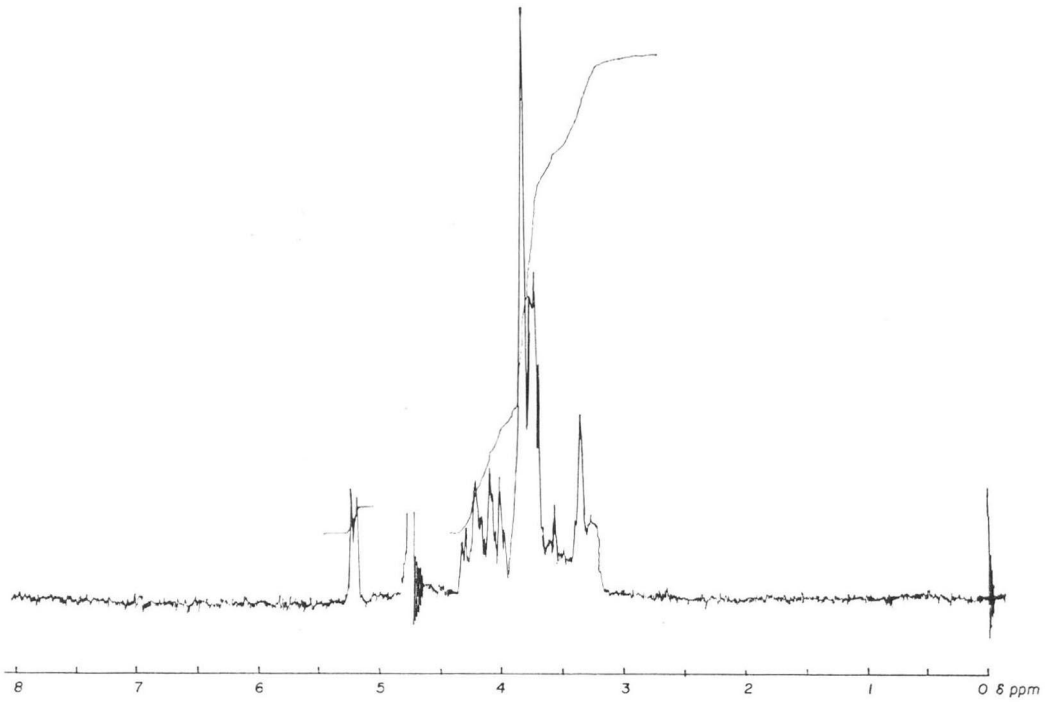


Fig. 8. NMR spectrum of sorbistin D hydrochloride (60 MHz, in D_2O)



Biological Activity *In Vitro*

The minimum inhibitory concentration (MIC) of sorbistins A_1, A_2 and B was determined by the serial two-fold agar dilution method (Nutrient Agar - Eiken) against gram-positive and gram-negative bac-

Table 3. Antibacterial spectra of sorbistins A_1, A_2 and B

Test organism	MIC (mcg/ml)*			
	Sorbistin A_1	Sorbistin A_2	Sorbistin B	Kanamycin A
Staphylococcus aureus 209P	12.5	—	50	0.2
Staphylococcus aureus Smith	12.5	25	50	0.1
Staphylococcus aureus #193	25	50	50	0.4
Staphylococcus aureus D133	25	100	100	0.8
Staphylococcus aureus A20239	50	—	100	50
Sarcina lutea PCI 1001	25	—	200	6.3
Micrococcus flavus D12	12.5	—	100	1.6
Bacillus anthracis Yale	25	—	100	0.8
Bacillus cereus ATCC 10702	100	—	200	0.8
Bacillus subtilis PCI 219	25	—	50	0.05
Streptococcus pyogenes S-23	50	—	200	12.5
Streptococcus pyogenes Dick	50	—	200	12.5
Streptococcus pyogenes A15041	25	—	50	6.3
Diplococcus pneumoniae type II	12.5	—	25	6.3
Diplococcus pneumoniae Neufeld	12.5	—	25	6.3
Diplococcus pneumoniae type III	12.5	—	25	6.3
Escherichia coli NIHJ	12.5	50	25	0.2
Escherichia coli P01495	25	100	50	0.4
Escherichia coli Juhl	50	—	100	0.8
Escherichia coli JR66/W677	50	—	100	100
Klebsiella pneumoniae D11	3.1	12.5	12.5	0.2
Klebsiella pneumoniae A9977	6.3	—	12.5	0.1
Enterobacter cloacae A20364	25	100	50	100
Enterobacter cloacae A21006	25	100	100	50
Proteus mirabilis A9554	12.5	50	25	0.4
Proteus morganii A20031	25	50	50	0.8
Proteus vulgaris A9436	12.5	100	50	0.2
Providencia stuartii A20644	100	—	400	1.6
Alcaligenes faecalis A9423	50	—	100	1.6
Serratia marcescens A20019	400	—	800	0.8
Serratia marcescens A21247	100	—	400	200
Serratia marcescens A21591**	50	—	200	400
Pseudomonas aeruginosa A9930	12.5	50	25	6.3
Pseudomonas aeruginosa D15	12.5	—	50	6.3
Pseudomonas aeruginosa A20653	25	100	100	800
Pseudomonas aeruginosa A21509**	100	—	200	100
Mycobacterium 607	>100	>100	>100	0.8
Mycobacterium phlei	50	50	50	0.8

* determined by nutrient agar medium (Eiken). Ten percent horse blood was incorporated to the medium for the testing of streptococci and diplococci.

** resistant to most aminoglycoside antibiotics by reduced cellular permeability (K. E. PRICE, personal communication).

teria including many aminoglycoside-resistant organisms. Kanamycin A was used as a reference in these *in vitro* tests. The MIC against acid-fast bacteria was determined in DUBOS liquid medium. As shown in Table 3, the intrinsic activity of sorbistins A_1, A_2 and B is moderate in terms of MIC values but their antibacterial spectra are broad, inhibiting most of the bacterial strains tested. Sorbistin A_1 showed the highest activity among the three bio-active components. Table 4 shows the activity of sorbistin A_1 against various types of aminoglycoside-resistant strains whose mechanisms of resistance

Table 4. Activity of sorbistin A_1 against aminoglycoside-resistant organisms

Organism*	Aminoglycoside-inactivating enzyme[7]	MIC (mcg/ml)**	
		Sorbistin A_1	Kanamycin A
Escherichia coli NIHJ	none	12.5	0.2
Escherichia coli ML-1630	APH (3′)-I	25	100
Escherichia coli A20107	APH (3′)-II	25	12.5
Escherichia coli R5	AAC (6′)-1	12.5	6.3
Escherichia coli JR88	AAC (3)-I	12.5	0.8
Escherichia coli A20732	ANT (2″)	12.5	12.5
Proteus rettgeri A20921	AAC (2′)	200	0.8
Pseudomonas aeruginosa A20741	AAC (3)-II	50	100
Pseudomonas aeruginosa GN4925	AAC (6′)-3	25	50
Pseudomonas aeruginosa GN315	AAC (6′)-4	50	100

* Resistant strains were obtained through the courtesy of Drs. J. DAVIES, H. KAWABE, S. MITSUHASHI, K. E. PRICE and H. UMEZAWA.

** determined in nutrient agar medium (Eiken).

Table 5. Effect of media pH on MIC of sorbistin A_1

Test organism	MIC (mcg/ml)*			
	pH 6	pH 7	pH 8	pH 9
E. coli NIHJ	50	12.5	6.3	6.3
K. pneumoniae D11	12.5	6.3	3.1	1.6
Ps. aeruginosa D15	50	12.5	6.3	3.1
S. aureus Smith	50	12.5	12.5	12.5
B. subtilis PCI 219	50	6.3	6.3	6.3

* Medium: Nutrient agar

Table 6. Effect of media on activity of sorbistin A_1

Test organism	MIC (mcg/ml)		
	Nutrient agar	Heart infusion agar	MUELLER-HINTON agar
E. coli NIHJ	12.5	50	100
K. pneumoniae D11	3.1	12.5	25
Ent. cloacae A20364	25	50	100
Pr. vulgaris A9436	12.5	50	100
Pr. mirabilis A9554	12.5	50	50
Ps. aeruginosa A9930	12.5	25	50
S. aureus Smith	12.5	50	50
S. aureus A20239	25	100	>100

have been reported. It is likely from the results that sorbistin is resistant to most of the aminoglycoside-inactivating emzymes identified to date. A few strains of multi-aminoglycoside-resistant mutants which are supposed to have a reduced cellular permeability were relatively sensitive to sorbistin A_1.

The *in vitro* antibacterial activity of sorbistin was greater at alkaline pH as shown in Table 5. The effect of media on the activity of sorbistin A_1 was compared in nutrient agar, heart infusion agar and MUELLER-HINTON agar. As shown in Table 6, the greatest activity was demonstrated when nutrient agar was used as the test medium.

Table 7. Activity of sorbistin A_1 against phytopathogenic organisms

Organism	MIC (mcg/ml)		
	Sorbistin A_1	Sorbistin B	Kasugamycin
Xanthomonas citri	6.3	6.3	12.5
Xanthomonas oryzae	6.3	12.5	12.5
Pseudomonas tabaci	12.5	25	12.5
Erwinia aroideae	12.5	25	100
Piricularia oryzae	>100	>100	1.6
Alternaria kikuchiana	50	>100	6.3

Table 8. *In vivo* activity of sorbistins A_1 and B

Infective organism	PD_{50} (mg/kg)		
	single dose		double doses
	Sorbistin A_1	Sorbistin B	Sorbistin A_1
S. aureus Smith	42	100	36×2
E. coli NIHJ	92	135	45×2
P. aeruginosa A9930	230	540	80×2

Sorbistins A_1 and B did not inhibit any of the anaerobic bacteria tested (*Bacteroides fragilis*, *Sphaerophorus necrophorus*, *Fusobacterium varium*, *Veillonella parvula*, *Acidaminococcus fermentans*, *Clostridium chauvoei*, *C. perfringens*, *Peptostreptococcus anaerobius* and *Peptococcus aerogenes*) at a concentration of 100 mcg/ml in Gifu anaerobic medium (GAM-Nissui).

Sorbistins were also tested for the activity against phytopathogenic organisms. The MICs were determined by agar dilution method using glucose-peptone agar for phytopathogenic bacteria (*Xanthomonas*, *Pseudomonas* and *Erwinia* species) and potato-dextrose agar for fungi (*Piricularia* and *Alternaria* species). Kasugamycin was included as a reference compound. As shown in Table 7, sorbistins A_1 and B inhibited phytopathogenic bacteria at concentrations comparable to those of kasugamycin. The two fungal strains tested were not susceptible to sorbistins.

In Vivo Activity and Toxicity

Sorbistins A_1 and B were evaluated *in vivo* in experimental infections of mice. The pathogenic bacteria employed were *S. aureus* Smith, *E. coli* NIHJ and *P. aeruginosa* A9930. Mice were challenged with a $100\times LD_{50}$ dose of the pathogens in a 5% suspension of hog gastric mucin. A single subcutaneous treatment with the antibiotic was made immediately after the bacterial challenge (0 hour), and in a double dose schedule the antibiotic was administered at 0 and 3 hours after the challenge. A group of 5 mice was used for each dosage level and the animals were observed for 5 days to determine the median protective dose (PD_{50}). As shown in Table 8, sorbistins A_1 and B demonstrated *in vivo* activity against all of the infections tested.

The acute toxicity of sorbistins A_1 and B was tested in mice by the subcutaneous and intravenous routes. The mice were observed for 15 days and the intravenous (iv) and subcutaneous (sc) LD_{50}'s of sorbistin A_1 were found to be 2,500 mg/kg and 1,000 mg/kg, respectively. Sorbistin B was less toxic than A_1 and no death occurred at doses up to 2,000 mg/kg by either iv and sc route during the observation period of 15 days.

Discussion

In recent years there have been several examples of aminoglycoside biosynthesis by bacterial strain, such as butirosins A and B[3], Bu-1709 E_1 and E_2[4], xylostatin[5] and 4′-deoxybutirosins[6]. These antibiotics are similar in that they all have a neamine moiety or paromamine in the structure and are produced by a species of *Bacillus* group. Sorbistin a is new bacterial aminoglycoside antibiotic produced by a strain of *Pseudomonas* species. It demonstrated a moderate but very broad antibacterial activity inhibiting a majority of aminoglycoside-resistant organisms, which might be due to its unique chemical structure as disclosed in a companion paper[2].

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