BBM-928, A NEW ANTITUMOR ANTIBIOTIC COMPLEX

I. PRODUCTION, ISOLATION, CHARACTERIZATION AND ANTITUMOR ACTIVITY

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A complex of the antitumor antibiotic BBM-928 was produced by an actinomycete strain No. G455–101. Four components, BBM-928 A, B, C and D, were isolated in crystalline form and characterized. They were shown to be cyclic depsipeptide antibiotics containing a quinoline nucleus as the chromophore. BBM-928 A is a monoacetyl derivative of BBM-928 B and a diacetyl derivative of BBM-928 C. BBM-928 components exhibit antimicrobial activity against Gram-positive and acid-fast bacteria. BBM-928 A is highly active in mice against various experimental tumors including leukemia P388, leukemia L1210, melanoma B16, LEWIS lung carcinoma and sarcoma 180. BBM-928 B is less active than BBM-928 A, and BBM-928 C has no antitumor activity.

In the course of an antitumor screening program for microbial fermentation products with weak or no antibacterial activity, an actinomycete strain No. G455–101 was found to produce a complex of antitumor antibiotics named BBM-928. The active principle was extracted from the fermentation broth and separated into six components, three major components (BBM-928 A, B and C) and three minor (D, E and F). BBM-928 A, B, C and D were isolated first in crystalline form and characterized in detail. This paper reports the production and isolation of the BBM-928 complex as well as the physico-chemical properties, antimicrobial and antitumor activities of BBM-928 A, B, C and D. The taxonomy of the BBM-928-producing organism, *Actinomadura luzonensis* nov. sp., and the structure determination of the BBM-928 components are reported in separate papers.[1,2]

Fermentation of BBM-928

A well-grown agar slant of strain G455–101 was used to inoculate vegetative medium containing 2% soluble starch, 1% glucose, 0.5% Pharmamedia, 0.5% yeast extract, 0.5% NZ-amine (Type A) and 0.1% $CaCO_3$, the pH being adjusted to 7.2 before sterilization. The seed culture was incubated at 32°C for 72 hours on a rotary shaker (250 rpm), and 5 ml of the growth was transferred to a 500-ml Erlenmeyer flask containing 100 ml of fermentation medium composed of 2% soluble starch, 1% Pharmamedia, 0.003% $ZnSO_4 \cdot 7H_2O$ and 0.4% $CaCO_3$. The production of BBM-928 complex generally reached a maximum after 5 days' shaking culture.

Fermentation studies were also carried out in tank fermentors. A seed culture was shaken for 4 days in Erlenmeyer flasks and used to inoculate 100 liters of germination medium composed of 0.2% oat meal (Quaker Products, Australia), 0.5% glucose, 0.2% dry yeast, 0.0008% $MnCl_2 \cdot 4H_2O$, 0.0007% $CuSO_4 \cdot 7H_2O$, 0.0002% $ZnSO_4 \cdot 7H_2O$ and 0.0001% $FeSO_4 \cdot 7H_2O$ in a 200-liter seed tank fermentor which was stirred at 200 rpm at 30°C for 54 hours. A 15-liter portion of the seed culture was then ino-

culated to 170 liters of fermentation medium containing 2% soluble starch, 1.0% Pharmamedia, 0.003% $ZnSO_4 \cdot 7H_2O$ and 0.4% $CaCO_3$ in a 400-liter tank fermentor which was operated at 30°C at 200 rpm with an aeration rate of 150 liters/min. The broth pH gradually increased with the progress of fermentation and reached 8.4~8.5 after 100~120 hours at which time the peak antibiotic potency was obtained.

Antibiotic levels in the fermentation broth and the extracts of BBM-928 complex were assayed by a paper disc - agar diffusion method using *Sarcina lutea* PCI-1001 as a test organism. Nutrient agar was used as the assay medium with the pH being adjusted to 9.0 for optimal sensitivity of the assay system. BBM-928 A, which was used as the assay standard, gave an inhibition zone of 22~23 mm (diameter) around an 8-mm paper disc soaked with 25 mcg/ml of the material.

Isolation and Purification

BBM-928 complex was isolated from the fermentation broth by a solvent extraction procedure. Harvested broth (170 liters, pH 8.5) was filtered with filter aid and the activity was found both in the mycelial cake and filtrate. The mycelial cake was extracted twice with a solvent mixture of acetone and methanol (1: 1, 30 liters×2). The extracts were combined and evaporated *in vacuo* to give an aqueous concentrate which was extracted with *n*-butanol. The broth filtrate was extracted twice with *n*-butanol (40 liters×2). The two *n*-butanol extracts were combined and concentrated *in vacuo*. The residue was lyophilized to give a crude solid (21.4 g), which was shown by TLC to be a complex consisting of three major components, A, B and C, and three minor components, D, E and F.

The crude complex was purified by a preparative counter current distribution apparatus (Mitamura, 100 ml/tube) using a solvent system of carbon tetrachloride - chloroform - methanol - water (5: 2: 5: 1). After 50 transfers, tube Nos. 5 through 20 were combined and concentrated to give a pale yellow powder (4.4 g) containing components A, B, D, E and F. This mixture was dissolved in a small amount of chloroform and charged on a column of silica gel C-200 (500 ml) which was pretreated by ethyl acetate. The column was developed by ethyl acetate with an increasing amount of methanol (2~5%, v/v) and fractions monitored by optical density at 345 nm.

Component D was eluted first with ethyl acetate followed by component A. Components E, B and F were eluted next in that order at 3% methanol concentration. Each fraction containing the appropriate component was evaporated *in vacuo* and the residue crystallized from chloroform-methanol. Likewise, a crude preparation of component C was obtained from tube Nos. 21 through 35 of the above-described counter current distribution. Component C was purified by silica gel chromatography and then crystallized by essentially the same method as used for component A. Yields for components A, B, C, D, E and F in the above experiment were, respectively, 988 mg, 420 mg, 848 mg, 130 mg, 119 mg and 114 mg.

Physico-chemical Properties

Each of the six BBM-928 components has been isolated as colorless crystals. The individual components of BBM-928 showed solubility and color reactions similar to each other. They are, however, readily differentiated from each other by two TLC systems, N-103 and N-118, as shown in Table 1.

BBM-928 components are readily soluble in chloroform and methylene chloride, slightly soluble in benzene, ethanol, methanol and *n*-butanol and insoluble in water and *n*-hexane. They give positive reactions with ferric chloride and EHRLICH reagents but are negative to TOLLENS, SAKAGUCHI and nin-

Table 1. Silica gel TLC of BBM-928 components.

	Rf values*	
	System N-118**	System N-103***
BBM-928 A	0.71	0.48
〃 B	0.53	0.26
〃 C	0.27	0.07
〃 D	0.73	0.53
〃 E	0.56	0.34
〃 F	0.39	0.17

* Detection by UV scanner (Shimadzu CS-910) at 345 nm
** *n*-Butanol - methanol - water (63:27:10)
*** Xylene - methylethyl ketone - methanol (5:5:1)

hydrin reactions.

The physico-chemical properties of four components, A, B, C and D, are shown in Table 2. The UV absorption maxima of BBM-928 components are observed at 235, 264 and 345 nm in neutral and acidic solutions, which undergo bathochromic shifts to 230, 256, 330 and 383 nm in alkaline solution, suggesting the presence of a phenolic hydroxyl group in the structure. The IR and proton NMR (PMR) spectra of BBM-928 A, B, C and D are shown in Figs. 1~4 and Figs. 5~8, respectively. The ^{13}C-NMR spectrum of component A is shown in Fig. 9. The PMR spectra of BBM-928 A, B and C are very similar to each other with the only difference being the presence of acetyl groups in component A (δ 2.03 ppm, 2 mol equivalent) and B (δ 2.05 ppm, 1 mol equivalent) but not in C. The difference in the number of acetyl function in the three components A, B and C is also indicated by ^{13}C-NMR spectra. Upon acetylation with acetic anhydride in pyridine, 2, 3 and 4 molar equivalents of acetyl group were introduced to BBM-928 A, B and C, respectively. The three acetylation products thus obtained showed identical properties in TLC, UV, IR and NMR spectra, indicating that BBM-928 A is a monoacetyl derivative of BBM-928 B and a diacetyl derivative of BBM-928 C.

Table 2. Physico-chemical properties of BBM-928 components A, B, C and D.

		BBM-928			
		A	B	C	D
Melting point		246~248°C	214~217°C	244~248°C	224~227°C
$[\alpha]_D^{25}$ (c 1, $CHCl_3$)		−27°	−74°	−91°	−13°
Anal. found C:		53.19	50.14	51.77	50.75
〃 H:		5.40	5.29	5.29	5.25
〃 N:		12.92	12.34	13.55	12.58
λ_{max} in nm ($E^{1\%}_{1cm}$)	in EtOH	235 (586)	235 (570)	235 (638)	235 (550)
		264 (415)	264 (400)	264 (442)	264 (380)
		345 (165)	345 (163)	345 (173)	345 (155)
	in EtOH-HCl	234 (610)	234 (556)	234 (650)	234 (565)
		264 (410)	264 (446)	264 (442)	264 (405)
		345 (165)	345 (188)	345 (173)	345 (165)
	in EtOH-NaOH	230 (564)	230 (530)	230 (580)	230 (650)
		256 (763)	256 (775)	256 (704)	256 (930)
		330 (118)	330 (116)	330 (117)	330 (140)
		383 (117)	383 (122)	383 (122)	383 (145)
Molecular weight (Osmometer, in $CHCl_3$)		1,450	—	1,470	—

Fig. 1. Infrared spectrum of BBM-928 A (KBr).

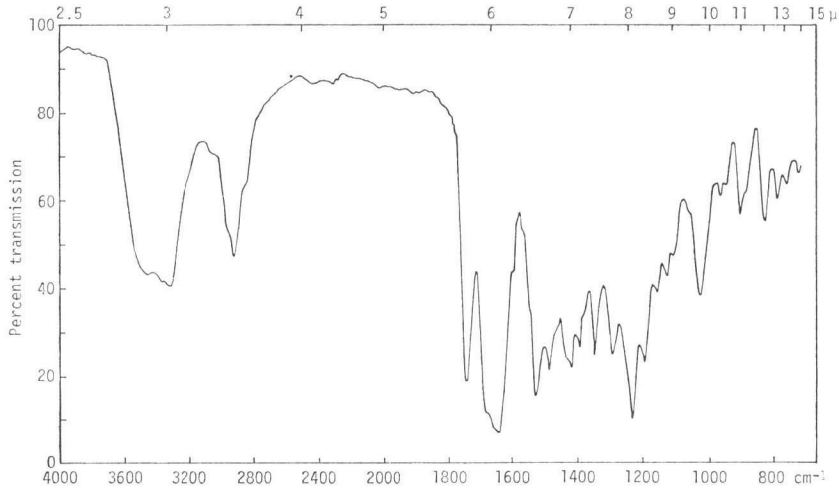
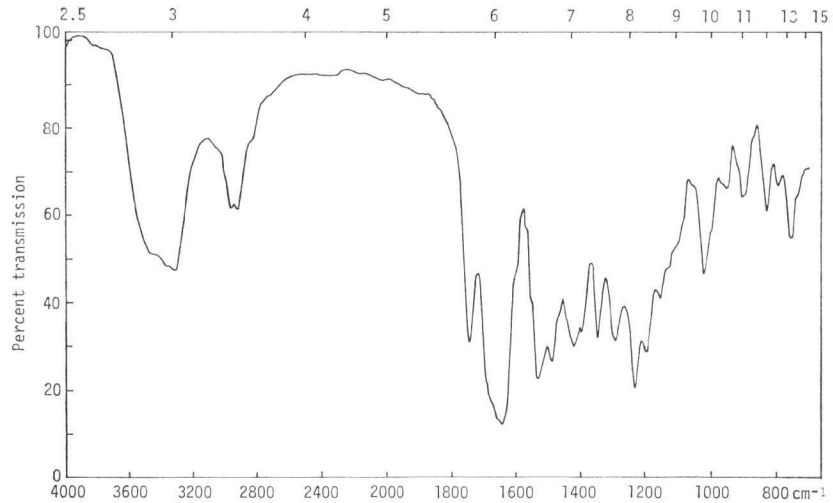


Fig. 2. Infrared spectrum of BBM-928 B (KBr).



Antimicrobial Activity

Antimicrobial activity of BBM-928 components (A, B, C and D) was determined against Gram-positive and Gram-negative bacteria by a serial agar dilution method in MUELLER-HINTON agar using Steer's multi-inoculating apparatus. The inoculum size was standardized to apply a 0.0025-ml aliquot of bacterial suspensions containing approximately 10^6 cells of a test organism per ml. For species of *Micrococcus, Sarcina* and *Streptococcus*, a 10^7 cell/ml suspension was used. Minimum inhibitory concentrations (MIC) were determined after overnight incubation at 37°C. For the test of acidfast bacteria, a 10^8 cell/ml suspension was inoculated on agar plates containing No. 1001 medium* and the MIC

* 3% Glycerol, 0.3% sodium L-glutamate, 0.2% peptone, 0.31% Na_2HPO_4, 0.1% KH_2PO_4, 0.005% ammonium citrate, 0.001% $MgSO_4$, 1.5% agar.

Fig. 3. Infrared spectrum of BBM-928 C (KBr).

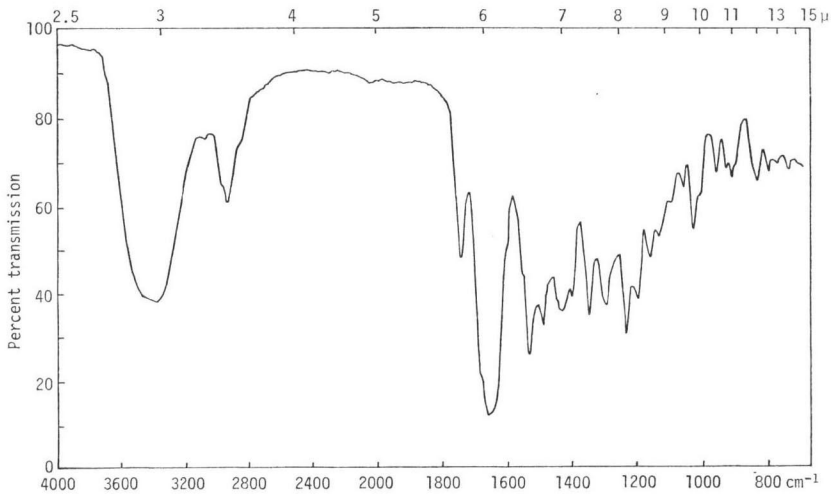
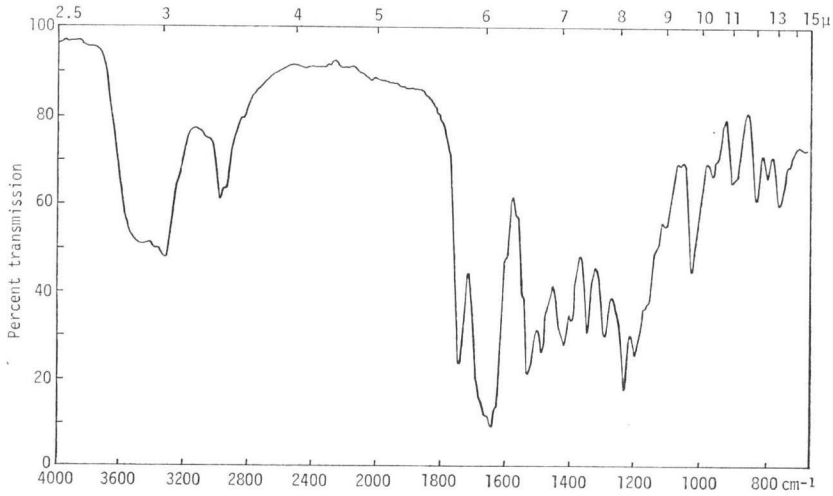


Fig. 4. Infrared spectrum of BBM-928 D (KBr).



determined after 42 hours' incubation at 37°C. The results are shown in Table 3 along with those of echinomycin which was tested comparatively as a reference antibiotic. BBM-928 components inhibited all the Gram-positive bacteria tested at low concentration. Component A was the most active followed by D and B, and component C was the least active. The antibacterial spectrum of BBM-928 is similar to that of echinomycin. However, the intrinsic antibacterial activity of BBM-928 was generally 16~64 times less potent than that of echinomycin.

The ability to induce prophage in lysogenic bacterium (ILB) was determined for BBM-928 components. No significant ILB activity was demonstrated with BBM-928 components A, B and C up to a concentration of 100 mcg/ml.

Fig. 5. NMR spectrum of BBM-928 A in $CDCl_3$ (60 MHz).

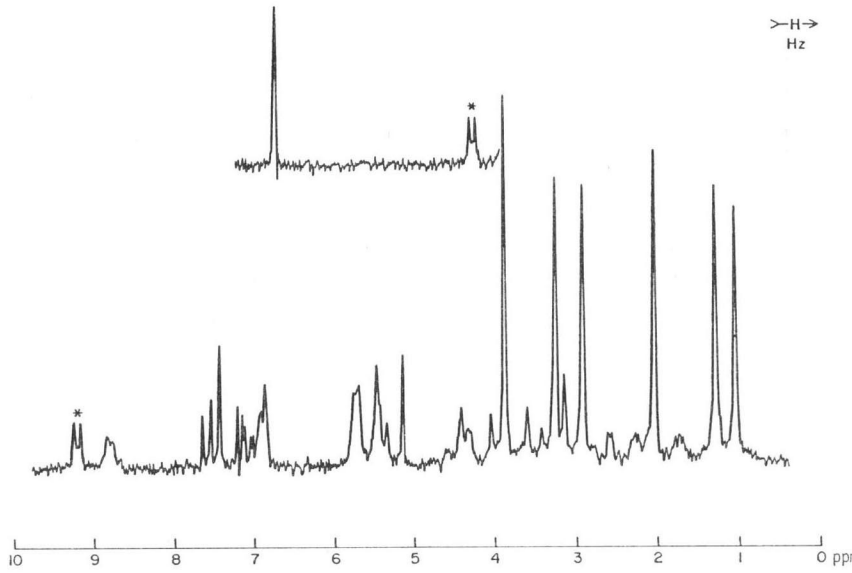
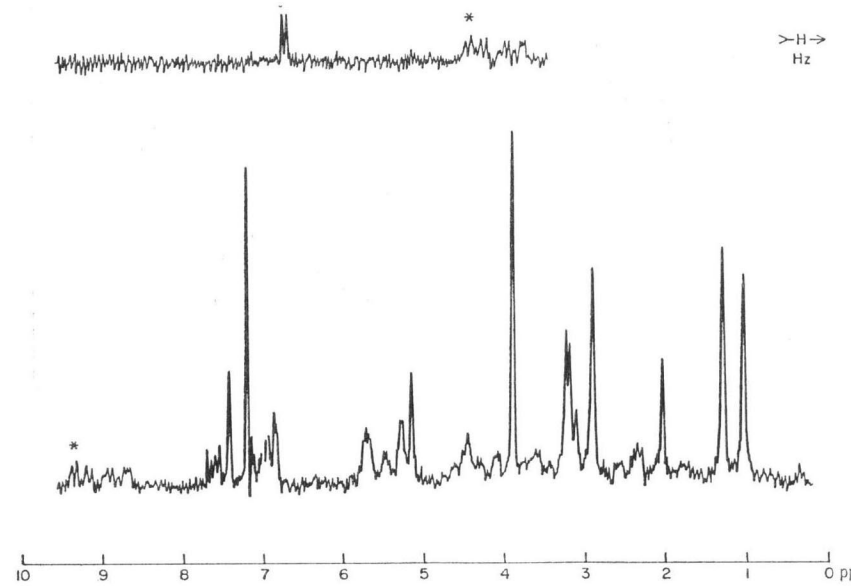


Fig. 6. NMR spectrum of BBM-928 B in $CDCl_3$ (60 MHz).



Antitumor activity and Toxicity

The antitumor activity of BBM-928 components was determined in experimental tumor systems in mice. Lymphocytic leukemia P388, lymphoid leukemia L1210, melanotic melanoma B16 and LEWIS lung carcinoma were implanted intraperitoneally into BDF_1 mice of either sex at an inoculum size of 10^6, 10^5, 5×10^5 and 5×10^5 cells per mouse, respectively. Sarcoma 180 ascites tumor was inoculated intraperitoneally into male ICR mice with 2.5×10^6 cells per mouse. Twenty-four hours after the implanta-

Fig. 7. NMR spectrum of BBM-928 C in $CDCl_3$ (60 MHz).

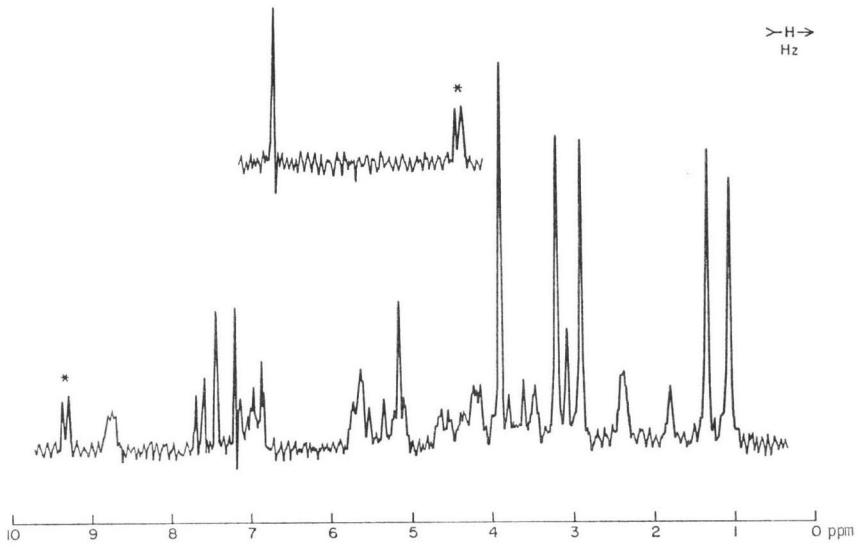
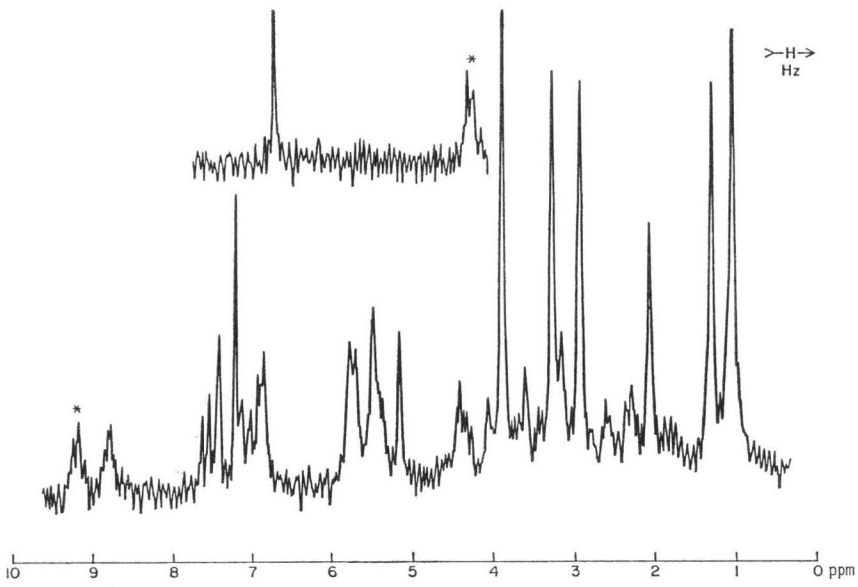


Fig. 8. NMR spectrum of BBM-928 D in $CDCl_3$ (60 MHz).



tion of tumor cells, graded doses of test compounds were administered to mice intraperitoneally in an injection volume of 0.02 ml per gram of body weight. A logarithmic dilution series consisting of one-half log unit increments of the antibiotics was used for the dose-response study. Treatments were given on day 1 only, day 1, 4 and 7 ($q3d\times3$) or once daily for 9 days ($qd\ 1\rightarrow9$) except for the mice inoculated with LEWIS lung carcinoma which were treated for 11 days ($qd1\rightarrow11$). Mitomycin C and echinomycin were comparatively tested as reference compounds. BBM-928 components and echinomycin were

Table 3. *In vitro* antimicrobial activity of BBM-928 components.

Test organism	MIC in mcg/ml				
	BBM-928 components				Echinomycin
	A	B	C	D	
Staphylococcus aureus FDA 209 P	0.2	0.8	6.3	0.4	0.0125
Staphylococcus aureus Smith	0.2	0.4	6.3	0.4	0.0125
Streptococcus pyogenes S-23	0.1	0.2	0.8	0.1	0.0063
Sarcina lutea PCI 1001	0.2	0.2	6.3	0.2	0.0063
Micrococcus flavus D 12	0.2	0.8	6.3	0.2	0.0063
Corynebacterium xerosis 53 K-1	0.4	1.6	6.3	6.3	0.2
Bacillus subtilis PCI 219	0.4	1.6	6.3	6.3	0.0031
Bacillus megaterium D 2	0.2	0.4	1.6	0.4	0.1
Bacillus anthracis A 9504	0.2	0.2	1.6	0.4	0.025
Escherichia coli NIHJ	>100	>100	>100	>100	1.6
Klebsiella pneumoniae D-11	>100	>100	>100	>100	6.3
Proteus vulgaris A 9436	>100	>100	>100	>100	>100
Pseudomonas aeruginosa A 9930	>100	>100	>100	>100	>100
Mycobacterium smegmatis 607 D 87	0.4	0.4	0.8	100	6.3
Mycobacterium phlei D 88	0.4	0.4	0.4	3.1	0.2

Table 4. Antitumor activity of BBM-928 A, B and C against leukemia P 388.

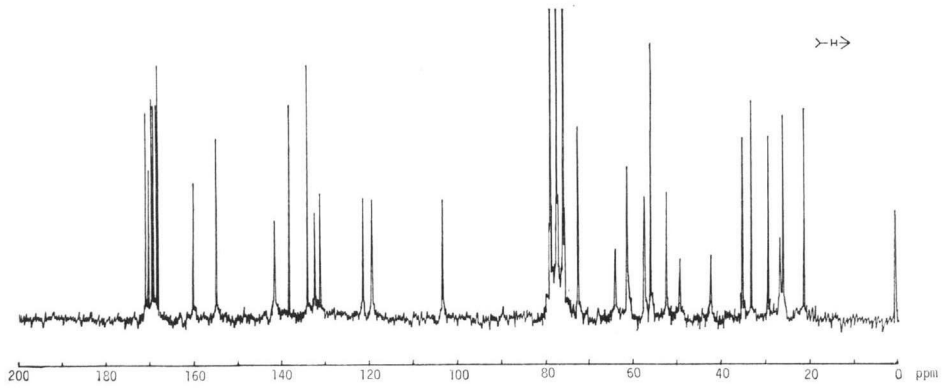
Compound	Dose (mg/kg/day)	Treatment schedule	T/C % in MST	Survivors on day 5	MED (mg/kg/day)
BBM-928 A	0.03	day 1 only	**163***	5/5**	0.01
	0.01	〃	**138**	5/5	
	0.003	〃	113	5/5	
	0.001	〃	113	5/5	
BBM-928 B	0.1	day 1 only	**156**	5/5	0.03
	0.03	〃	**133**	5/5	
	0.01	〃	110	5/5	
	0.003	〃	100	5/5	
BBM-928 C	1	day 1, 4 & 7	toxic	0/5	—
	0.3	〃	78	3/5	
	0.1	〃	100	5/5	
	0.03	〃	100	5/5	
	0.01	〃	100	5/5	

* Boldface type indicates significant antitumor effect.
** number of survivor/tested.

dissolved in 0.9% saline containing 10% dimethyl sulfoxide and mitomycin C was dissolved in 0.9% saline.

Death or survival of the treated and non-treated (control) animals was recorded daily during the observation period of 45 days after the implantation of tumor cells, and the median survival time (MST) was calculated for each of the test (T) and control (C) groups. A T/C value equal to or greater than 125% indicates that a significant antitumor effect was achieved. The lowest dose giving a T/C equal to or greater than 125% was defined as minimum effective dose (MED).

Fig. 9. Carbon-13 NMR spectrum of BBM-928 A in $CDCl_3$.



The antitumor activity of BBM-928 A, B and C was determined comparatively against leukemia P388. As shown in Table 4, components A and B inhibited the tumor with a single treatment on day 1 only, while component C was inactive even on a triple dosing schedule. Component A was approximately 3-times as potent as component B.

BBM-928 A was compared with echinomycin and mitomycin C for activity against leukemia P388 by a *qd* 1→9 dosing schedule. As shown in Table 5, the anti-P388 activity of BBM-928 was approximately 3-fold more potent than that of echinomycin and 100-fold greater than that of mitomycin C.

Table 5. Antitumor activity of BBM-928 A, echinomycin and mitomycin C against leukemia P 388.

Dose* (mg/kg/day)	BBM-928 A	Echino-mycin	Mito-mycin C
1.0	—	—	**200****
0.3	—	—	**163**
0.1	**175**	**212**	**125**
0.03	**200**	**225**	113
0.01	**188**	**175**	—
0.003	**175**	**150**	—
0.001	**150**	113	—
0.0003	113	100	—
MED in mg/kg/day	0.001	0.003	0.1

* Dosing schedule: *qd* 1 → 9
** Boldface type indicates significant effect.

The antitumor activity of BBM-928 A was further evaluated in several tumor systems including leukemia L1210, LEWIS lung carcinoma, melanoma B16 and sarcoma 180. Mitomycin C was used as a reference compound in these experiments. The results are summarized in Table 6. BBM-928 A was generally 100-times as active as mitomycin C in most tumor systems except for sarcoma 180 which was about 300 times more sensitive to BBM-928 A than mitomycin C. The results also indicate that the range of effective doses for BBM-928 A was wider than that of mitomycin C in most of the tumor systems examined.

The acute toxicity of BBM-928 components was determined in mice by intraperitoneal administration of graded doses of test compounds to groups of 5 ~ 10 normal male *ddY* mice. The LD_{50} was calculated according to the method of VAN DER WAERDEN[8] on day 30 in the case of a single administration, or on day 45 in the case of a multiple treatment schedule (*qd* 1→9). The results are shown in Table 7. BBM-928 A was somewhat less toxic than echinomycin but 55 times more toxic than mitomycin C in the LD_{50} determination by single administration. BBM-928 B and C were, respectively, 1.4 and 6.2 times less toxic than component A. BBM-928 A was 74 times more toxic than mitomycin C in the multiple treatment schedule.

Table 6. Antitumor activity of BBM-928 A and mitomycin C in various tumor systems.

Compound	Dose* (mg/kg/day)	Leukemia P388		Leukemia L1210		Lewis lung carcinoma		Melanoma B16		Sarcoma 180	
		T/C % in MST	Survivors (day 5)	T/C % in MST	Survivors (day 5)	T/C % in MST	Survivors (day 5)	T/C % in MST	Survivors (day 5)	T/C % in MST	Survivors (day 5)
BBM-928 A	0.1	—	—	—	—	—	—	—	—	toxic	4/10
	0.03	**200****	8/9	**150**	5/5	**233**	9/9	**214**	10/10	48	10/10
	0.01	**187**	10/10	**125**	5/5	**167**	10/10	**177**	10/10	**248**	10/10
	0.003	**153**	10/10	**125**	5/5	**139**	10/10	**157**	10/10	**234**	10/10
	0.001	**133**	10/10	100	5/5	106	10/10	123	10/10	**234**	10/10
	0.0003	107	10/10	100	5/5	106	10/10	109	9/9	**207**	10/10
	0.0001	107	10/10	—	—	94	10/10	103	10/10	**155**	10/10
	0.00003	—	—	—	—	—	—	—	—	103	10/10
MED in mg/kg/day		0.001		0.003		0.003		0.003		0.0001	
Mitomycin C	3	—	—	100	5/5	56	4/4	55	10/10	—	—
	1	**200**	5/5	**163**	5/5	**222**	4/4	**142**	10/10	—	—
	0.3	**147**	10/10	**125**	5/5	**159**	4/4	**127**	10/10	**185**	10/10
	0.1	**127**	10/10	100	5/5	119	4/4	103	10/10	**165**	10/10
	0.03	100	10/10	100	5/5	—	—	—	—	**170**	10/10
	0.01	107	10/10	—	—	—	—	—	—	118	10/10
MED in mg/kg/day		0.1		0.3		0.3		0.3		0.03	

* Treatment schedule: *qd* 1 → 9 (*qd* 1 → 11 for Lewis lung carcinoma).

** Boldface type indicates significant effect.

Table 7. Acute toxicity of components A, B and C of BBM-928 in mice.

	Intraperitoneal LD_{50} in mg/kg/day	
	Single dose (day 1)	Multiple dose (day 1 → 9)
BBM-928 A	0.13	0.019
〃 B	0.18	—
〃 C	0.81	—
Echinomycin	0.11	—
Mitomycin C	7.1	1.4

Discussion

The physico-chemical properties of BBM-928 components are in part similar to those of the quinoxaline group of antibiotics which include echinomycin[4], actinoleukin[5], quinomycins[6] and triostins[7]. The quinoxaline antibiotics are potent antitumor agents characterized by having UV spectra with absorptions at 243 and 320 nm due to a common chromophore, quinoxaline-2-carboxylic acid. The UV absorption maxima of BBM-928 components were observed at 235, 264 and 345 nm, suggesting a different chromophore structure. All the quinoxaline antibiotics have a sulfur-containing bridge in the cyclic depsipeptide structure, while BBM-928 does not contain sulfur in the molecule.

When compared biologically with echinomycin, a representative quinoxaline antibiotic, BBM-928 A was relatively less potent than echinomycin in terms of antibacterial activity. However, BBM-928 A was about 3 times more active than echinomycin in regard to antitumor activity against leukemia P388. BBM-928 B and C are mono- and di-desacetyl analogs of BBM-928 A, respectively. It is interesting that antibacterial and antitumor activities of BBM-928 components parallel the degree of acetylation in the molecular structure.

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