GLYCOCINNAMOYLSPERMIDINES, A NEW CLASS OF ANTIBIOTICS

II. ISOLATION, PHYSICOCHEMICAL AND BIOLOGICAL PROPERTIES OF LL-BM123β, γ_1 AND γ_2

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LL-BM123β, γ_1, and γ_2 are three new antibiotics produced by fermentation of an unidentified species of *Nocardia*. These strongly basic, water-soluble compounds were isolated from the culture filtrate by CM-Sephadex ion-exchange and carbon chromatography. All three antibiotics are active against both gram-positive and gram-negative bacteria. A mixture of LL-BM123 γ_1 and γ_2 is more active than the β component but generally less active than gentamicin.

A previous paper from these laboratories described the structure of a new *myo*-inosamine-2 containing antibiotic called LL-BM123α isolated from the fermentation broth of an unidentified species of *Nocardia*.[1] In this report we describe the isolation and biological properties of three new antibiotics designated LL-BM123β, γ_1, and γ_2, from the same organism. The taxonomy of the organism and the fermentation conditions for producing these new metabolites were discussed in the first paper of this series.[2] Results from hydrolytic experiments and spectral studies have shown these antibiotics to have the structures shown in Fig. 1.[3] They are the first examples of a new class of antibiotics which we call the glycocinnamoylspermidines.

Isolation of LL-BM123β, γ_1, and γ_2

LL-BM123β, γ_1 and γ_2, were isolated from the fermentation broth by CM-Sephadex ion-exchange and carbon chromatography. The pH of the whole broth was adjusted from 4.3 to 7.0 with NaOH and the neutralized broth filtered using 5% diatomaceous earth as a filter aid. The filtrate was charged onto a column of CM-Sephadex C-25 ion-exchange resin in the Na^+ form. The column was washed with water and developed first with 1% NaCl and then 5% NaCl solutions. Active fractions in the 5% NaCl eluate were desalted

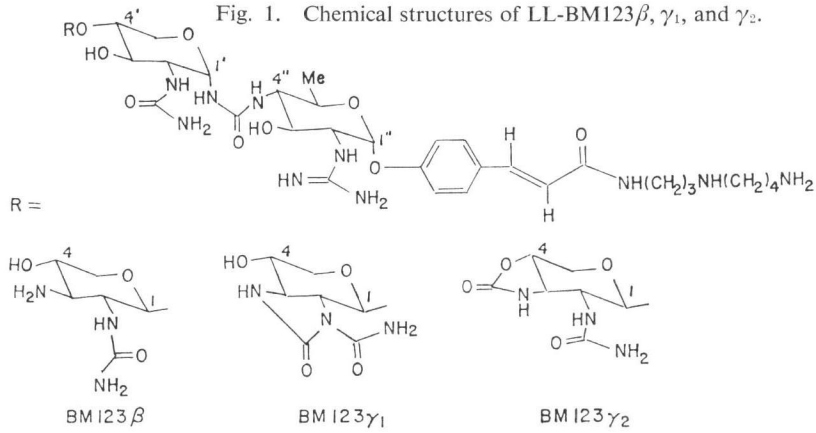


Fig. 1. Chemical structures of LL-BM123β, γ_1, and γ_2.

by passage through a granular carbon column. The charged column was washed with water and developed with 15% followed by 50% aqueous methanol and finally 50% aqueous acetone. The 15% aqueous methanol eluate was concentrated to a small volume and the pH adjusted from 4.5 to 6.0 with Amberlite IR-45 (OH^-) ion-exchange resin. The resin was removed by filtration and the filtrate concentrated and lyophilized to give a mixture consisting primarily of LL-BM123β and a small amount of LL-BM123γ_2 as indicated by cellulose thin-layer chromatography.

The 50% aqueous methanol and the 50% aqueous acetone eluates were processed in the same manner as above to give a mixture of crude LL-BM123γ_1 and γ_2.

Purification of LL-BM123β

LL-BM123β was further purified by chromatography of the crude β preparation over CM-Sephadex (NH_4^+) equilibrated with 3% NH_4Cl. The column was developed with a 3~6% NH_4Cl gradient and the elution monitored by UV at 286 nm and bioautography against *Klebsiella pneumoniae* AD. Fractions containing LL-BM123β were desalted over granular carbon and the antibiotic eluted with 20% aqueous methanol. The methanol was removed *in vacuo* and the aqueous concentrate lyophilized to yield LL-BM123β as the amorphous hydrochloride as indicated by cellulose thin-layer chromatography.

Purification of LL-BM123γ_1

A crude preparation of primarily γ_1 containing a little γ_2 was chromatographed over CM-Sephadex ion-exchange resin in the Na^+ form equilibrated with a 2% NaCl solution. The column was developed with a 2~4% NaCl gradient and monitored as described above. The initial antibiotic fractions were a mixture of LL-BM123γ_1 and γ_2 whereas the later fractions contained essentially pure LL-BM123γ_1. These later fractions were desalted with granular carbon and the LL-BM123γ_1 hydrochloride recovered by elution of the carbon with 50% aqueous methanol, concentration of the eluate to a small volume, and lyophilization.

Purification of LL-BM123γ_2

A crude mixture of LL-BM123β and γ_2 was chromatographed over a CM-Sephadex (Na^+) column equilibrated with 2% NaCl. The column was eluted with a gradient between 2~4% NaCl and monitored by UV at 286 nm. Fractions containing LL-BM123γ_2 were combined and desalted over granular carbon. The carbon column was eluted first with 10% and then with 50% aqueous methanol. Both eluates were worked up as described above. The 10% aqueous methanol eluate yielded fairly pure LL-BM123γ_2 hydrochloride whereas the 50% eluate yielded slightly less pure LL-BM123γ_2 as shown by cellulose thin-layer chromatography.

Physicochemical Properties

The physicochemical properties of LL-BM123β, γ_1, and γ_2 are given in Table 1. These antibiotics are amorphous, strongly basic compounds (strong SAKAGUCHI and ninhydrin reactions) and are soluble in aqueous solutions and slightly soluble in methanol. They undergo gradual decomposition starting in the vicinity of 200°C. The microanalytical data only approximate the values necessitated by the formulae for the structures depicted in Fig. 1. This is presumably due to the

Table 1. Physicochemical properties of LL-BM123β, γ_1 and γ_2.

	LL-BM123β·HCl	LL-BM123γ_1·HCl	LL-BM123γ_2·HCl
Physical state	Amorphous white powder	Amorphous white powder	Amorphous white powder
Decomposition point	~200°C	~200°C	~200°C
$[\alpha]_D^{25}$	+67° (*c* 1.0, H_2O)	+55° (*c* 0.80, H_2O)	+60° (*c* 0.85, H_2O)
UV (MeOH)	286 nm	286 nm	286 nm
	$E_{1cm}^{1\%}$ 260	$E_{1cm}^{1\%}$ 225	$E_{1cm}^{1\%}$ 220
Elem. analysis			
C	39.29%	37.84%	36.14%
H	6.33	5.73	5.67
N	16.58	15.58	15.1
Cl (ionic)	13.28	10.01	11.11
Loss on drying	6.90%	10.45%	10.87%

Fig. 2. IR spectrum of LL-BM123β in a KBr disc.

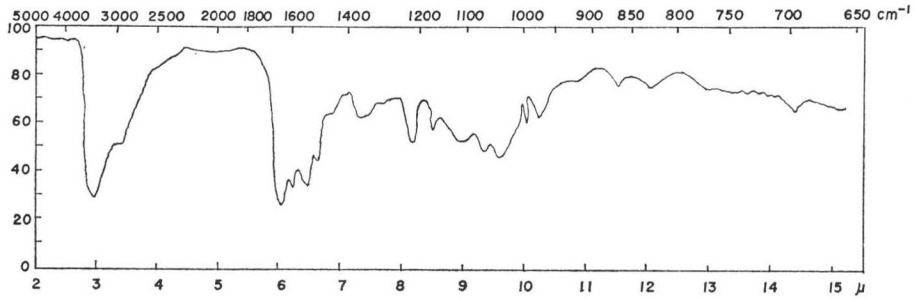


Fig. 3. IR spectrum of LL-BM123γ_1 in a KBr disc.

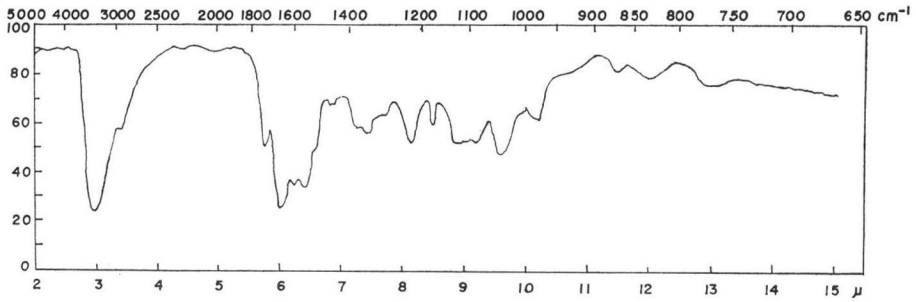


Fig. 4. IR spectrum of LL-BM123γ_2 in a KBr disc.

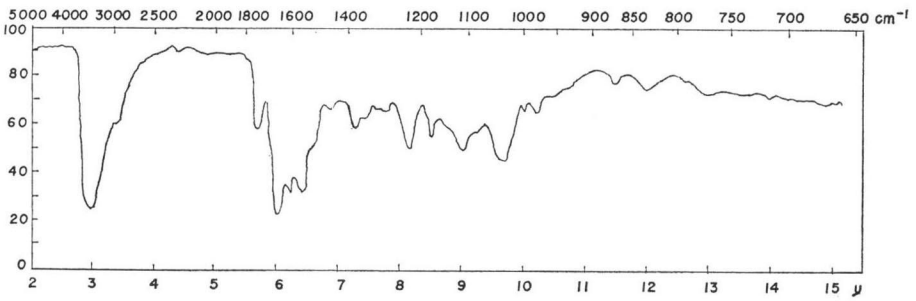
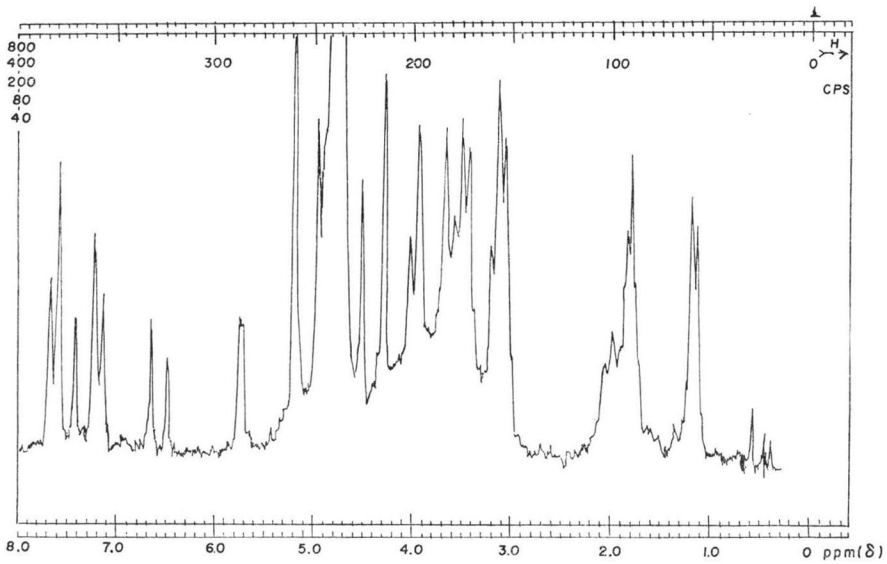


Fig. 5. 100 MHz PMR spectrum of LL-BM123γ_1 in D_2O.



failure to obtain absolutely pure analytical samples in conjunction with the extreme hygroscopicity of these antibiotics. Because of this difficulty in obtaining good elemental analyses, accurate knowledge of the molecular formulae was not obtained until structural studies were almost completed by the use of ^{13}C nmr and analysis of hydrolysis fragments. Mass spectral studies, including attempts at field desorption, were also unsuccessful on the intact antibiotics.

A UV maximum at 286 nm is common to all three components. As seen from Figs. 2, 3 and 4, the IR spectra are essentially the same for all three antibiotics except for the characteristic differences in the carbonyl frequencies. The H nmr spectra (100 MHz, run in D_2O with TMS as reference standard) of β, and γ_2 are almost identical with that of LL-BM123γ_1 which is shown in Fig. 5.

The γ_1 and γ_2 components have a very narrow pH stability range between 4.0 and 6.0. Alkaline pH's, especially, cause rapid loss of biological activity. LL-BM123β, however, is relatively more stable than the γ compounds at the same pH's. All three antibiotics are deactivated in boiling water.

These antibacterial agents can be distinguished by paper and thin-layer chromatography. For the former, Whatman No. 1 strips were spotted with a water or aqueous methanol solution of the antibiotics and equilibrated for 1~2 hours in the presence of both upper and lower phases from the system 90% phenol - *m*-cresol - acetic acid - pyridine - water (100: 25: 4: 4: 75 by volume). The strips were developed overnight with the lower (organic) phase of the above system. The strips were then air-dried for 1~2 hours, washed with ether, and bioautographed on agar plates seeded with *Klebsiella pneumoniae* AD. Representative Rf values are listed in Table 2. In this system, LL-BM123β was composed of a major antibiotic of Rf of 0.70 and a very minor component of Rf 0.50.

For thin-layer chromatographic analysis, cellulose plates (0.1-mm thick, supplied by EM Laboratories, Inc., Elmsford, N.Y.) were spotted with a water solution of the antibiotics and developed overnight with the system 1-butanol - water - pyridine - acetic acid (15: 12: 10: 1 by volume). The plates were air-dried for 1 hour and then visualized with either SAKAGUCHI or ninhydrin-spray reagents. Rf values are given in Table 3. LL-BM123β again showed up as a major component with

Table 2. Rf Values of LL-BM123 antibiotics on paper chromatograms developed with 90% phenol-*m*-cresol - AcOH - pyridine - H_2O (100:25:4:4:75)

Component	Rf
LL-BM123β	0.50 (minor), 0.70 (major)
LL-BM123γ_1 & γ_2	0.85

Table 3. Rf Values of LL-BM123 antibiotics on cellulose TLC plates developed with BuOH - H_2O - pyridine - AcOH (15: 12: 10: 1)

Component	Rf
LL-BM123β	0.80 (major), 0.14 (minor)
LL-BM123γ_2	0.17
LL-BM123γ_1	0.23

an Rf of 0.08 and a very minor component with an Rf of 0.14. The γ_1 and γ_2 components were resolved by this method whereas in the above paper chromatographic system they moved as one spot.

Antibacterial Properties

Methods

Because of the difficulty in obtaining quantities of LL-BM123γ_2 completely free of γ_1 other than for spectral and analytical data, these biological studies were carried out on a 50: 50 mixture of γ_1 and γ_2 which is called LL-BM123γ. This mixture was compared with LL-BM123β and gentamicin. Minimal inhibitory concentrations of the agents were determined by means of a standard two-fold serial dilution method in MUELLER-HINTON agar. The agar surfaces in Petri plates were inoculated with approximately 10^4 CFU (colony forming units) of bacteria by means of a Steers multiple inocula replicator. The minimal inhibitory concentration (MIC) was defined as the lowest concentration of antibiotic inhibiting growth of the organism after 18~22 hours incubation at 37°C.

Therapeutic and toxic effects were determined in 18~20 g female mice of the CF-1 strain (Carworth Farms). In the therapeutic studies, mice were challenged intraperitoneally with sufficient organism suspended in 0.5 ml of trypticase-soy broth to kill 90~100% of non-treated mice within 72 hours. The antibiotic doses were contained in 0.5 ml of 0.2% aqueous agar and administered in a single subcutaneous dose approximately 30 minutes after infection. Acute lethal toxic effects were determined in non-infected mice. In each test 5~10 mice were treated at each dose level and survival ratios determined 7~14 days after infection. The results from two to four separate tests were pooled for the estimation of median-effective doses (ED_{50}) or median-lethal doses (LD_{50}) by the method of LITCHFIELD and WILCOXON.[4]

Results

The LL-BM123 antibiotics were active against gram-negative and gram-positive bacteria. The

Table 4. Antibacterial activities of LL-BM123β and LL-BM123γ (Agar dilution method—MUELLER-HINTON agar)

Organism	No. of strains tested	Range of minimal inhibitory concentration mcg/ml	
		LL-BM123β	LL-BM123γ
Escherichia coli	5	0.5~ 2	0.25~ 1
Proteus spp.	2	1	0.25~ 0.5
Enterobacter-Klebsiella spp.	4	1 ~ 2	0.25~ 1
Salmonella spp.	3	0.5~ 1	0.25
Shigella spp.	3	1 ~ 2	0.5 ~ 1
Serratia spp.	3	0.5~ 1	0.25~ 0.5
Acinetobacter calcoaceticus	3	2 ~32	0.5 ~ 8
Pseudomonas aeruginosa	3	32 ~64	4 ~ 8
Staphylococcus aureus	3	1 ~ 2	0.25~ 1
Enterococcus spp.	3	>128	32

γ antibiotic ($\gamma_1+\gamma_2$) was two to four fold more potent than LL-BM123β in both *in vitro* and *in vivo* tests (Tables 4 and 5).

LL-BM123γ was also compared with gentamicin. *In vitro* against clinical isolates of bacteria, LL-BM123γ was generally 1/4 to 1/2 as potent as gentamicin except against *Pseudomonas* and *Acinetobacter* where gentamicin exceeded the potency of LL-BM123γ by 8~32 fold. Both LL-BM123γ and gentamicin were ineffective against *Bacteroides* (see Table 6).

LL-BM123γ was effective at relatively low doses against infections produced by gram-negative and gram-positive bacteria in mice (Table 7). On a dosage basis, γ was less potent than gentamicin.

Table 5. Therapeutic effects of LL-BM123β and LL-BM123γ against experimental infections in mice

Infection[b]	Estimated ED_{50}, mg/kg[a]	
	BM123β	BM123γ
Escherichia coli #311	2~8	0.5~2
Klebsiella pneumoniae AD	4~8	1 ~2
Proteus mirabilis #4671	4~16	2 ~4

(a) Single subcutaneous dose administered approximately 30 minutes after infection. Five to 25 mice were tested at each dose level. $ED_{50's}$ (median effective doses) were estimated from the 7th day survival ratios.

(b) 90~100% of non-treated infected mice died within 3 days after infection.

Table 6. Antibacterial activity of LL-BM123γ compared with gentamicin (Agar dilution method—MUELLER-HINTON agar)

Organism	No. of strains tested	Range of minimal inhibitory concentration mcg/ml	
		LL-BM123γ	Gentamicin
Escherichia coli	20	1 ~ 4	1 ~ 4
Proteus spp.	24	1 ~ 8	0.5 ~16
Enterobacter-Klebsiella spp.	20	2 ~ 8	0.5 ~ 4
Salmonella spp.	21	0.12~ 1	0.12~ 1
Shigella spp.	14	1 ~ 4	0.12~ 1
Serratia spp.	21	1 ~ 8	0.5 ~ 4
Acinetobacter calcoaceticus	20	0.06~16	0.5 ~ 2
Pseudomonas aeruginosa	22	4 ~64	0.5 ~ 2
Staphylococcus aureus	24	0.5 ~ 4	0.03~ 1
Bacteroides fragilis	10	>128	>128

Table 7. Therapeutic effects of LL-BM123γ and gentamicin against experimental infections in mice

Infection	Median effective dose, mg/kg[a], (95% confidence limits)	
	LL-BM123γ	Gentamicin
Escherichia coli	1.0 (0.7~ 1.5)	0.2 (0.15~0.28)
Klebsiella pneumoniae AD	1.6 (1.2~ 2.1)	0.5 (0.3 ~0.7)
Proteus mirabilis #4671	2.0 (1.4~ 2.9)	0.3 (0.2 ~0.4)
Enterobacter aerogenes #75	6.4 (4.8~ 8.8)	0.3 (0.2 ~0.4)
Acinetobacter calcoaceticus #10	7.0 (5.0~ 9.0)	0.4 (0.3 ~0.6)
Salmonella typhi #6539	8.9 (6.0~ 13)	0.18 (0.14~0.23)
Staphylococcus aureus, Smith	1.5 (1.1~ 1.9)	0.2 (0.06~ 0.6)
Staphylococcus aureus, Rose	20 (14 ~ 29)	2.0 (1.6 ~ 2.8)
Streptococcus pyogenes C203	43 (15 ~120)	11 (10 ~13)

(a) Single subcutaneous dose administered approximately 30 minutes after infection.

micin. Also it was not as well tolerated in mice as gentamicin. The median lethal subcutaneous dose of LL-BM123γ was 88 (68~100) mg/kg and for gentamicin 350 (300~410) mg/kg. However, the antibacterial effects of LL-BM123γ are sufficiently interesting to warrant further investigation. Indeed, chemical modification studies[5] on LL-BM123γ_1 and γ_2 have resulted in a derivative with greatly enhanced activity[6] over that of the natural products.

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