# GLYCOCINNAMOYLSPERMIDINES, A NEW CLASS OF ANTIBIOTICS

## I. DESCRIPTION AND FERMENTATION OF THE ORGANISM PRODUCING THE LL-BM123 ANTIBIOTICS

H. D. TRESNER, J. H. KORSHALLA, A. A. FANTINI, J. D. KORSHALLA, J. P. KIRBY, J. J. GOODMAN, R. A. KELE, A. J. SHAY and D. B. BORDERS*

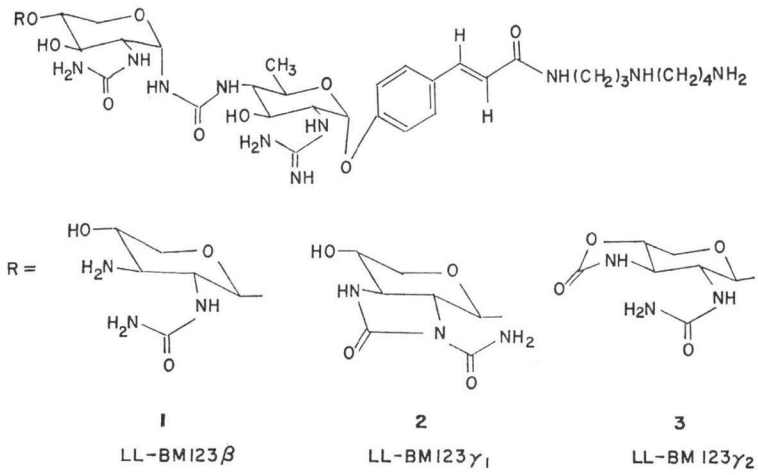
Lederle Laboratories, Division of American Cyanamid Company
Pearl River, New York 10965, U.S.A.

(Received for publication December 19, 1977)

The producing organism for the new broad spectrum glycocinnamoylspermidine antibiotics designated LL-BM123$\beta$, $\gamma_1$ and $\gamma_2$ was characterized as a *Nocardia* sp. by chemical analysis of the cell wall, growth requirements, morphology and physiological reactions. Fermentation conditions to elaborate and analytical methods to characterize these antibiotics in fermentation filtrates are described.

LL-BM123$\beta$, $\gamma_1$, and $\gamma_2$ (**1**, **2**, and **3**) are new, potent broad-spectrum antibiotics produced by an unidentified species of *Nocardia*.[1,2] These compounds represent a new structural class of antibiotics referred to as glycocinnamoylspermidines. They are very basic, water-soluble compounds which contain subunits of sugars, *p*-hydroxycinnamic acid, and spermidine. Chemical modification of LL-BM123$\gamma_1$ and $\gamma_2$ has resulted in a product[3] which is one of the most potent antibiotics that we have encountered in our laboratories.[4]

The characterization and fermentation of the organism to produce LL-BM123$\beta$, $\gamma_1$ and $\gamma_2$ are described in the following study. This same organism also produces the novel antibiotic LL-BM123$\alpha$ which was reported previously.[5]




**1** LL-BM123$\beta$ **2** LL-BM123$\gamma_1$ **3** LL-BM123$\gamma_2$

* Author to whom correspondence should be directed.

## Description of Organism

Culture BM123, NRRL 5646, was isolated from a soil sample collected at Osceola, Iowa, U.S.A. Taxonomic classification of the organism was based on observations of its cultural, physiological and morphological features in accordance with the methods detailed by SHIRLING and GOTTLIEB.[6] Chemical composition of the culture cell wall was determined by established procedures.[7,8,9]

### Cultural Characteristics

BM123 was cultivated on a variety of media. Growth was moderate on yeast extract, asparagine-dextrose, BENEDICT's, BENNETT's, and potato-dextrose agars; light on HICKEY and TRESNER's, tomato-paste-oatmeal and Pablum agars; and very light on inorganic salts-starch, KUSTER's oatflake, CZAPEK's solution, and rice agars.

Aerial mycelium was whitish and appeared only on yeast extract, asparagine-dextrose, BENEDICT's, BENNETT's and potato-dextrose agars. No soluble pigments were formed and reverse color ranged from colorless to yellowish shades.

### Micromorphology

Aerial mycelium arises from substrate mycelium as sparingly-branched, moderately long flexuous elements that commonly terminate in elongated primitive spirals. The flexuous elements are irregularly segmented into short, elliptical to cylindrical sections which disarticulate readily. The spiral terminal portions are less conspicuously segmented. Segments generally range $0.8 \sim 1.7\ \mu \times 0.3 \sim 0.5\ \mu$, averaging $0.4\ \mu \times 1.2\ \mu$.

### Physiological Reactions

Gelatin was not liquefied; nitrates were reduced to nitrites in 7 days; melanoid pigments were not formed on peptone-iron agar; there was no peptonization or curd formation in purple milk; NaCl tolerance in yeast extract agar was $\geqq 4\%$, but $< 7\%$; optimal growth temperature was 32°C. Carbon source utilization, according to the PRIDHAM and GOTTLIEB method[10] was as follows: good utilization of glycerol, salicin, *d*-trehalose and dextrose; fair utilization of *i*-inositol, and poor to non-utilization of *d*-fructose, maltose, adonitol, *l*-arabinose, lactose, *d*-mannitol, *d*-melibiose, *d*-raffinose, *l*-rhamnose, sucrose and *d*-xylose.

### Chemical Composition

An analysis of whole-cell hydrolysates of culture BM123 with paper chromatography by previously described procedures[7,8] resulted in the detection of *meso*-2,6-diaminopimelic acid, arabinose, and galactose. This indicated that the organism belonged to cell wall Type IV with a Type A sugar pattern[11] and taxonomically was most likely a *Nocardia* or *Mycobacterium*. When the whole-cells were extracted with methylene chloride, the extract methylated with boron trichloride-methanol, and the product analyzed by gas chromatography as described by LECHEVALIER[9], the resulting fatty acid patterns resembled *Nocardia* rather than *Mycobacteria*.

The morphological characteristics of BM123 were difficult to interpret and define because of the poor development of aerial mycelium on most media. Consequently, considerable importance is attached to the chemical analysis of its cells in determining its generic relationships. In accordance with the system proposed by LECHEVALIER *et al.*[11], BM123 can best be placed in the genus *Nocardia*. Additional characteristics that relate it to *Nocardia* are its fragmenting aerial growth on some media and its total absence of aerial growth on most media.

In view of the lack of adequate criteria for the characterization of the organism to the species level, this determination was not attempted. Until such a diagnosis is feasible, BM123 will remain an undetermined species of *Nocardia*.

## Fermentation

Culture BM123 was maintained on agar slants composed of (g/liter): yeast extract 4.0, malt extract 10.0, dextrose 4.0, and agar 20.0 (pH adjusted to 7.3 prior to sterilization). Spore suspensions from the above slants were used to inoculate 500-ml flasks containing 100 ml of a seed medium consisting of (g/liter): beef extract 3.0, yeast extract 5.0, Tryptose (Difco) 5.0, dextrose 10.0, and agar 1.5 (pH adjusted to 6.8~7.0 prior to sterilization). Seed flasks were incubated on a rotary shaker at 180~200 rpm for 2 days at 28°C.

The fermentation medium (100 ml/500-ml flask) was inoculated with 5.0 ml of the seed culture, and was prepared as follows (g/liter): glucose monohydrate 20.0, meat solubles (Hormel) 30.0, $(NH_4)_2SO_4$ 6.0, $MgSO_4 \cdot 7H_2O$ 3.0, $K_2HPO_4$ 6.0, $CaCO_3$ 2.0 (no pH adjustment). Flasks were incubated on a rotary shaker at 28°C and sampled at various time intervals for potency and thin-layer chromatography. Peak titers were observed in a 3~5-day incubation period. Production of the antibiotics was monitored by tlc of fermentation samples and detected by bioautography against a sensitive strain of *Pseudomonas aeruginosa* (Table 1).

## Antibiotic Characterization in Fermentations

LL-BM123$\beta$ and $\gamma$ can be identified in fermentation filtrates by characteristic mobilities on paper chromatography, paper electrophoresis, or thin-layer chromatography (Table 1). However, in these systems the individual $\gamma$ components, $\gamma_1$ and $\gamma_2$, have the same mobility and cannot be readily differentiated when detected by bioautography. Methods by which the $\gamma_1$ and $\gamma_2$ components were resolved by hplc and other techniques will be discussed in subsequent papers.

By paper electrophoresis the LL-BM123 antibiotics behave as strong bases. At 600 volts for 3.0 hours with a pH 2.0 buffer consisting of 2% HOAc, the LL-BM123 antibiotics migrated approximately 13.3 cm towards the cathode whereas a neomycin reference sample migrated 20 cm. Antibiotic zones were detected by bioautography against *Klebsiella pneumoniae* strain AD.

Table 1. Paper and thin-layer chromatography of LL-BM123 antibiotics and reference compounds

| Antibiotic | Rf[a] | | | |
|---|---|---|---|---|
| | 1 | 2 | 3 | 4 |
| LL-BM123$\gamma$[b] | 0.6 | 0.9 | 0.1 | 0.3 |
| LL-BM123$\beta$ | 0.6 | 0.5 | 0.1 | 0.5 |
| LL-BM123$\alpha$ | | 0.2 | | 0.9 |
| Netropsin | 0.1 | | | |
| Capreomycin | | 0.2 | | |
| Streptomycin | | | 0.0 | |

[a] Detection by bioautography *vs. Klebsiella pneumoniae* strain AD.
Paper chromatography solvent systems:
1) 5% ammonium chloride in water
2) 90% phenol-*m*-cresol-acetic acid-pyridine-water (100: 25: 4: 4: 75)
3) 2-butanol-acetic acid-water (8: 3: 4)
Thin-layer chromatography system:
4) 10% trisodium citrate, pH 8.2, as a developing solvent for Brinkman Polygram® CEL-300 $UV_{254}$ cellulose plates.

[b] LL-BM123$\gamma$ designates a mixture of $\gamma_1$ and $\gamma_2$.

## References

1) Ellestad, G.; D. Cosulich, R. Broschard, J. Martin, M. Kunstmann, G. Morton, J. Lancaster, W. Fulmor & F. M. Lovell: Glycocinnamoylspermidines, a new class of antibiotics. III. Structures of LL-BM123$\beta$, $\gamma_1$, and $\gamma_2$. J. Am. Chem. Soc. 100: 2515~2524, 1978

2) MARTIN, J. H.; M. P. KUNSTMANN, F. BARBATSCHI, M. HERTZ, G. ELLESTAD, M. DANN, G. S. REDIN, A. C. DORNBUSH & N. A. KUCK: Glycocinnamoylspermidines, a new class of antibiotics. II. Isolation, characterization and biological properties of LL-BM123$\beta$, $\gamma_1$ and $\gamma_2$. J. Antibiotics 31: 398~404, 1978
3) HLAVKA, J. J.; P. BITHA, J. BOOTHE & T. FIELDS: Glycocinnamoylspermidines, a new class of antibiotics. IV. Chemical modification of LL-BM123$\gamma$. J. Antibiotics 31: 477~479, 1978
4) KUCK, N. A. & G. S. REDIN: Glycocinnamoylspermidines, a new class of antibiotics. V. Antibacterial evaluation of the isopropyl derivative of LL-BM123$\gamma$. J. Antibiotics 31: 405~409, 1978
5) ELLESTAD, G. A.; J. H. MARTIN, G. O. MORTON, M. L. SASSIVER & J. E. LANCASTER: Structure of LL-BM123$\alpha$, a new *myo*-inosamine-2 containing antibiotic. J. Antibiotics 30: 678~680, 1977
6) SHIRLING, E. B. & D. GOTTLIEB: Methods for characterization of *Streptomyces* species. Internat. J. System. Bacteriol. 16: 313~340, 1966
7) BECKER, B.; M. P. LECHEVALIER, R. E. GORDON & H. A. LECHEVALIER: Rapid differentiation between *Nocardia* and *Streptomyces* by paper chromatography of whole-cell hydrolysates. Appl. Microbiol. 12: 421~423, 1964
8) LECHEVALIER, M. P.: Identification of aerobic actinomycetes of clinical importance. J. Lab. Clin. Med. 71: 934~944, 1968
9) LECHEVALIER, M. P.; A. C. HORAN & H. LECHEVALIER: Lipid composition in the classification of *Nocardia* and *Mycobacteria*. J. Bacteriol. 105: 313~318, 1971
10) PRIDHAM, T. G. & D. GOTTLIEB: The utilization of carbon compounds by some *Actinomycetales* as an aid for species determination. J. Bacteriol. 56: 107~114, 1948
11) LECHEVALIER, M. P. & H. LECHEVALIER: Chemical composition as a criterion in the classification of aerobic actinomycetes. Internat. J. System. Bacteriol. 20: 435~443, 1970