STRUCTURES OF BACILLOMYCIN D AND BACILLOMYCIN L PEPTIDOLIPID ANTIBIOTICS FROM *BACILLUS SUBTILIS*

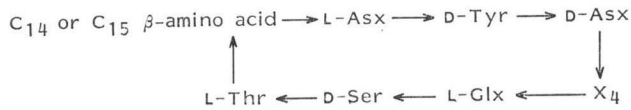
FRANÇOISE PEYPOUX, MARIE-THÉRÈSE POMMIER, BHUPESH C. DAS†, FRANÇOISE BESSON, LUCIEN DELCAMBE†† and GEORGES MICHEL

Laboratoire de Biochimie Microbienne, Université de Lyon I,
43, Boulevard du 11 Novembre 1918, 69622 Villeurbanne Cedex, France
†Institut de Chimie des Substances Naturelles,
Gif sur Yvette, France
††C.P.E.M., Boulevard de la Constitution,
32, Liège, Belgique

(Received for publication July 7, 1984)

The complete structures of bacillomycin D and bacillomycin L were revised by FAB mass spectrometry and by Edman degradation of the derivatives resulting from the *N*-bromosuccinimide reaction. The homologous components of both series of antibiotics were separated by HPLC and the β-amino acids were identified by capillary gas chromatography.

Bacillomycin D and bacillomycin L are peptidolipid antibiotics isolated from strains of *Bacillus subtilis*[1,2]. Their structural determination by chemical methods indicated that they consist of a heptapeptide chain linked to a liposoluble β-amino acid[3,4]. Among the amino acids of the peptidic moieties, aspartyl, glutamyl, asparaginyl and glutaminyl residues were found and the following structures had been proposed:



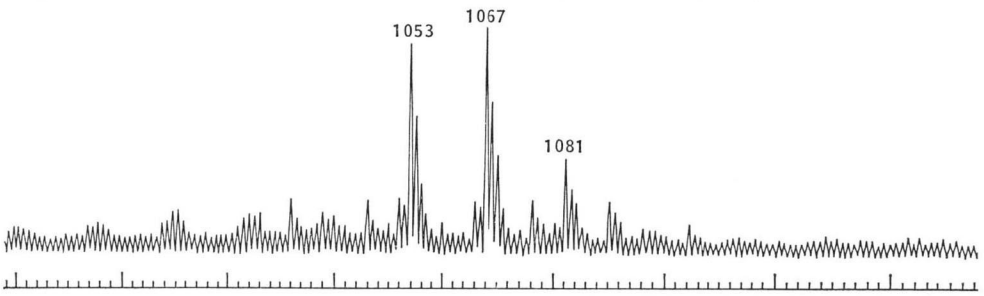
Bacillomycin D: L-Asx=L-Asp, D-Asx=D-Asn, X_4=L-Pro, L-Glx=L-Glu
Bacillomycin L: L-Asx=L-Asp, D-Asx=D-Asp, X_4=L-Ser, L-Glx=L-Gln

The presence of amide groups on the dicarboxylic amino acids was suggested from the formation of α,ω-diamino acids by the reaction of RESSLER and KASHELIKAR[5] and the free carboxyl groups were estimated by titration with the hydroxymate method applied to methyl esters[6].

Recently, these antibiotics were studied by fast atom bombardment (FAB) mass spectrometry and the molecular weights were found one mass unit less than the expected values. This difference could be due to the presence of an Asn or a Gln residue instead of an Asp or a Glu residue and such a discrepancy is not surprising in view of the imprecision of quantitative methods used in the previous work.

On the other hand, reinvestigation of homologous β-amino acids from iturin A using HPLC and NMR spectrometry by ISOGAI *et al.* showed that they consist of a mixture of *n*-C_{13}, *n*-C_{14}, *anteiso*-C_{15}, *iso*-C_{15}, *n*-C_{15} and *n*-C_{16} β-amino acids with *n*-C_{14} and *iso*-C_{15} as major components[7]. More recently, WINKELMANN *et al.* isolated from a strain of *B. subtilis* a peptidolipid complex of the iturin group containing six β-amino acids with a high proportion of *iso*-C_{16}[8].

Fig. 1. Na-Cationized FAB mass spectrum of bacillomycin D showing the $(M+Na)^+$ peak region.



These new results prompted us to reinvestigate the structure of both the peptidic and the lipophilic moieties of bacillomycin D and of bacillomycin L.

Fast Atom Bombardment Mass Spectrometry

Mass spectra were obtained with a Kratos MS80 mass spectrometer. The FAB ion source was of the standard Kratos design and was equipped with an Ion Tech atom gun. The bombardment was with 6~7 kV xenon atoms. The peptides (*ca.* 5 μg) was placed on the copper target end of a direct insertion probe using glycerol as matrix. For obtaining Na-cationized spectra 1 μl of a 10% aqueous solution of NaCl was added to the glycerol matrix.

The FAB mass spectrum of bacillomycin D displayed two major $(M+H)^+$ peaks at *m/z* 1,031 and 1,045, the difference of 14 mass units being due to the presence of the homologous C_{14} and C_{15} β-amino acids. The corresponding $(M+Na)^+$ peaks were observed at *m/z* 1,053 and 1,067 (Fig. 1) when the spectra were run with NaCl. The previously reported formula $C_{48}H_{73}N_9O_{16}$ and $C_{49}H_{75}N_9O_{16}$ for bacillomycin D with M=1,031 and 1,045, *i.e.*, one mass unit higher than the values obtained by FAB mass spectrometry, should therefore be changed to $C_{48}H_{74}N_{10}O_{15}$ and $C_{49}H_{76}N_{10}O_{15}$. Similarly, the FAB mass spectrum of bacillomycin L showed two major $(M+H)^+$ peaks at *m/z* 1,021 and 1,035 as also the $(M+Na)^+$ peaks at *m/z* 1,043 and 1,057, *i.e.*, corresponding to M=1,020 and 1,034. The molecular weights calculated from the previously reported formula $C_{46}H_{71}N_9O_{17}$ and $C_{47}H_{73}N_9O_{17}$ are M=1,021 and 1,035. Accordingly, the correct formula of bacillomycin L should be revised to $C_{46}H_{72}N_{10}O_{16}$ and $C_{47}H_{74}N_{10}O_{16}$.

Notably no fragmentation peaks were observed in the FAB mass spectra of bacillomycin D and bacillomycin L, which was possibly due to their cyclic structure.

Edman Degradation of the Peptide Chain

The cyclic structure of the peptide chain was opened up as described previously[3] by *N*-bromosuccinimide treatment which cleaves the *C*-peptidyl bond of the tyrosyl residue. The peptide sequence was then determined by Edman degradation according to TARR[9]. The degradation discontinued at the β-amino acid and, as a consequence, the next α-amino acid (Asp or Asn) could not be identified by this method. After each step of degradation the *N*-terminal amino acid was identified as its PTH derivative by thin-layer chromatography on Silica gel 60 in chloroform-methanol (85: 15) or (95: 5). This method had

Table 1. *N*-Terminal amino acid after each step of Edman degradation of bacillomycin D and bacillomycin L treated with *N*-bromosuccinimide.

Antibiotic	*N*-Terminal amino acid				
	Step of degradation				
	0	1	2	3	4
Bacillomycin D	Asn	Pro	Glu	Ser	Thr
Bacillomycin L	Asn	Ser	Gln	Ser	Thr

Fig. 2. Gas chromatogram of *N*-trifluoroacetyl β-amino acyl methyl esters on a 50 m SP 2100 fused-silica capillary column.

Temp: 180°C for 15 minutes then programmation from 180°C to 240°C at 1°C/minute.
A: iturin A, B: bacillomycin D, C: bacillomycin L.

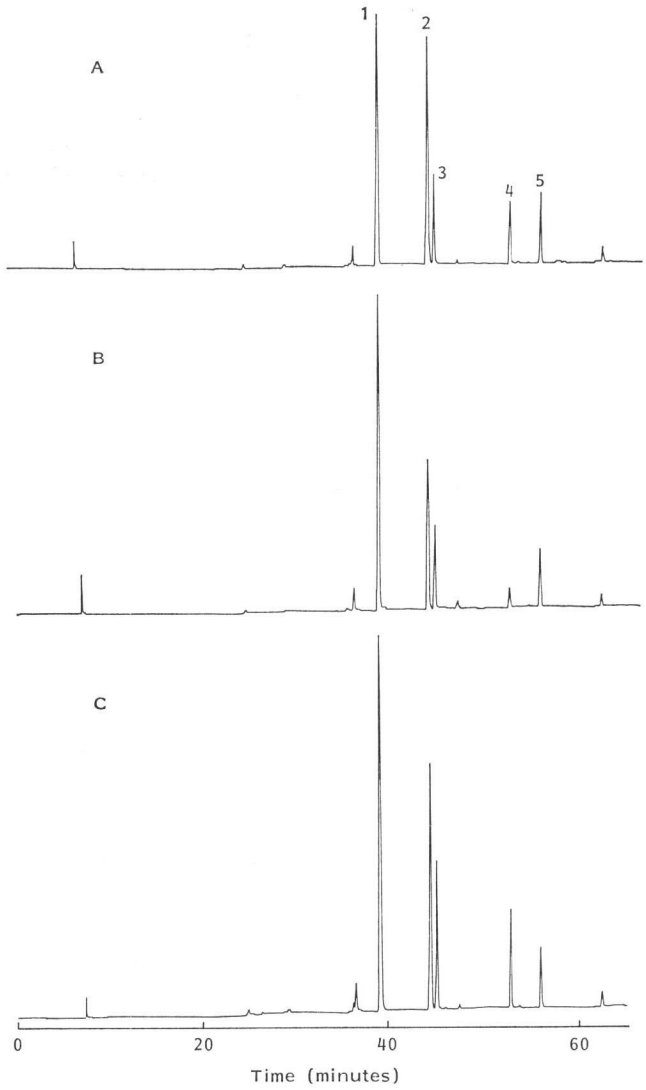
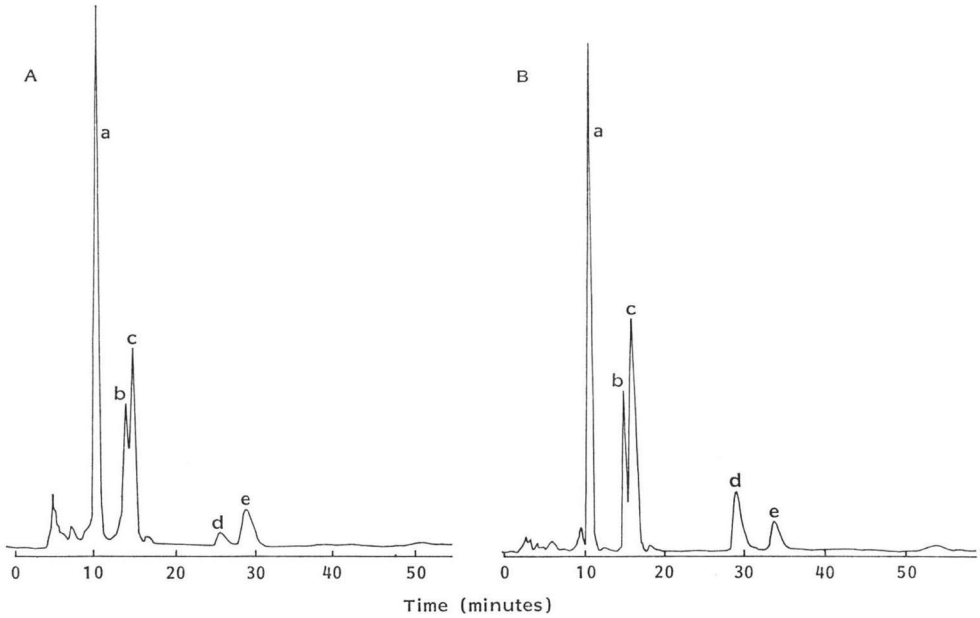


Table 2. Percentage of β-amino acids in antibiotics.

Peak	Nature of carbon chain	Iturin A	Bacillomycin D	Bacillomycin L
1	*n*-C_{14}	34	47.6	38.9
2	*iso*-C_{15}	30.7	22.7	25.2
3	*anteiso*-C_{15}	12.1	12.5	15.4
4	*iso*-C_{16}	8.5	3.3	10.1
5	*n*-C_{16}	9.8	8.8	6.1

Fig. 3. HPLC separation of homologous components of bacillomycin D (A) and bacillomycin L (B).

Elution solvent: acetonitrile - 10 mM ammonium acetate (1.6: 3).



been used previously with bacillomycin D[8] and the results are presented with those of bacillomycin L in Table 1.

Thus, in bacillomycin D, the free carboxyl group belongs to the L-glutamyl residue thereby indicating that the L-Asx residue linked to the COOH group of the β-amino acid must be an L-asparaginyl residue. In bacillomycin L both L-Asx and L-Glx residues of the sequenced peptide chain are amidated and the free carboxyl group must be that of L-Asp residue linked to the COOH group of the β-amino acid.

Identification of β-Amino Acid and Separation of Homologous Antibiotics

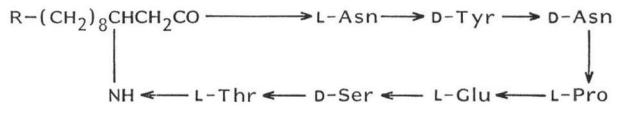
The structure of the β-amino acid components of bacillomycin D and bacillomycin L were specified by gas chromatography on SP 2100 capillary column of the *N*-trifluoroacetyl methyl esters in comparison with those of iturin A which have been extensively studied previously[7].

The elution profiles are drawn in Fig. 2 and the percentages of each component are indicated in Table 2.

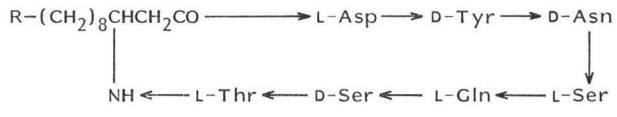
In both antibiotics, C_{14} and C_{15} β-amino acids were the major components with a lower percentage of C_{16} components.

The homologous bacillomycins D and L were separated by HPLC on a Lichrosorb RP18 column with acetonitrile - 10 mM ammonium acetate (1.6: 3) as elution solvent; Fig. 3 gives the elution profiles. Each peak may be associated with the nature of the β-amino acid component: peak a: *n*-C_{14} β-amino acid, b: *anteiso*-C_{15}, c: *iso*-C_{15}, d: *iso*-C_{16} and e: *n*-C_{16}.

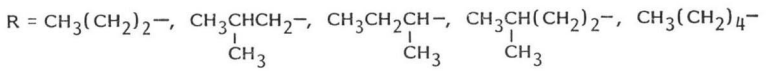
In conclusion, the following revised structures have been established for bacillomycin D and bacillomycin L.



Bacillomycin D



Bacillomycin L



Acknowledgments

This work was supported by the Centre National de la Recherche Scientifique (U.A. 528) and by the Ministère de l'Industrie et de la Recherche (Contrat n° 83.C.1012).

References

1) Raubitschek, F. & A. Dostrovsky: An antibiotic active against dermatophytes derived from *Bacillus subtilis*. Dermatologica 100: 45~49, 1950
2) Landy, M.; G. H. Warren, S. B. Rosenman & L. G. Colio: Bacillomycin: An antibiotic from *Bacillus subtilis* active against pathogenic fungi. Proc. Soc. Exp. Biol. Med. 67: 539~541, 1948
3) Peypoux, F.; F. Besson, G. Michel & L. Delcambe: Structure of bacillomycin D, a new antibiotic of iturin group. Eur. J. Biochem. 118: 323~327, 1981
4) Besson, F.; F. Peypoux, G. Michel & L. Delcambe: Structure de la bacillomycine L, antibiotique de *Bacillus subtilis*. Eur. J. Biochem. 77: 61~67, 1977
5) Ressler, C. & D. V. Kashelikar: Identification of asparaginyl and glutamyl residues *in endo* position in peptides by dehydration-reduction. J. Am. Chem. Soc. 88: 2025~2035, 1966
6) Augustyn, J. M. & W. B. Elliot: A modified hydroxymate assay of phospholipidase A activity. Anal. Biochem. 31: 246~250, 1969
7) Isogai, A.; S. Takayama, S. Murakoshi & A. Suzuki: Structures of β-amino acids in antibiotics iturin A. Tetrahedron Lett. 23: 3065~3068, 1982
8) Winkelmann, G.; H. Allgaier, R. Lupp & G. Jung: Iturin Al — A new long chain iturin A possessing an unusual high content of C_{16}-β-amino acids. J. Antibiotics 36: 1451~1457, 1983
9) Tarr, G. E.: A general procedure for the manual sequencing of small quantities of peptides. Anal. Biochem. 63: 361~370, 1975