BACILLOMYCINS F_b AND F_c: ISOLATION AND CHARACTERIZATION

FRANÇOISE BESSON and GEORGES MICHEL

Laboratoire de Biochimie Microbienne, UA (C.N.R.S.) 1176,
Université Claude Bernard Lyon I,
43, Boulevard du 11 Novembre 1918,
69622, Villeurbanne Cédex, France

(Received for publication October 12, 1987)

Bacillomycins F_b and F_c, new antifungal antibiotics, were isolated from a strain of *Bacillus subtilis* producing bacillomycin F. Because of the presence of β-amino acids, these compounds belong to the iturin group. The acid hydrolysates contained α-amino acids Asp_3, Glu_1, Pro_1, Thr_1, Tyr_1 and a mixture of *iso*-C_{15}, *anteiso*-C_{15}, *iso*-C_{16}, *iso*-C_{17} and *anteiso*-C_{17} β-amino acids. The ratios of the different β-amino acids depend on the nature of the culture medium.

Bacillomycins F_b and F_c differ from bacillomycin F by the presence of free carboxyl groups.

Bacillomycin F is an antifungal antibiotic produced by *Bacillus subtilis*[1]. Its structure has been determined; it is a cyclolipopeptide containing seven residues of D and L α-amino acids and one residue of a β-amino acid (Fig. 1)[2].

During studies on the influence of the composition of the culture medium on the production of antibiotics, new antifungal compounds were isolated, they were related to bacillomycin F and we proposed the name bacillomycin F_a for the bacillomycin F previously identified[1,2] and the names bacillomycins F_b and F_c for the new antibiotics.

This paper describes the identification and the characterization of these antibiotics.

Culture Conditions

Bacillus subtilis I-164 was grown in a medium containing 37 g/liter of brain-heart infusion (Bio-Mérieux, France) on a rotary shaker. A part (2%) of this culture was used to inoculate Erlenmeyer flasks containing the production medium: the medium of LANDY *et al.*[3] where D,L-valine, D,L-leucine or D,L-isoleucine were added at a 4 g/liter-concentration. The submerged cultures were carried out at 32°C for 5 days.

Isolation and Purification of Bacillomycins F

The culture supernatant was adjusted to pH 2.0 with 12 N HCl, the precipitate was collected by centrifugation and dissolved in 0.5 N NaOH until the pH was about 7. After lyophilization, the crude antibiotic preparation was extracted with chloroform - methanol (2:1) and purified by column chromatography on silicic acid Bio Sil HA 325 mesh (Bio Rad, U.S.A.). Elution was performed first with hexane - chloroform - methanol (25:45:10): Only impurities were eluted. Second elution solvent was chloroform - methanol -

Fig. 1. Structure of bacillomycin F_a.

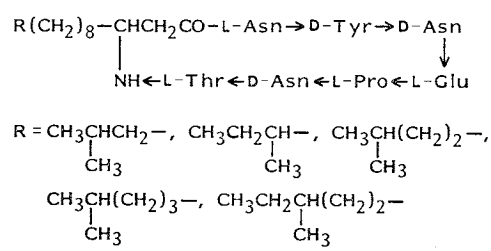


Table 1. Antifungal activity of bacillomycins F_b and F_c.

Microorganisms	MIC (μg/ml)	
	Bacillomycin F_b	Bacillomycin F_c
Candida albicans	>200	>200
C. tropicalis	>200	>200
Kluyveromyces bulgaricus	75	40
Saccharomyces cerevisiae	25	30
Aspergillus niger	25	30
Cladosporium cladosporioides	25	40
Fusarium oxysporum	>200	>200
Mycosphaerella pinodes	100	30

The MICs were determined by the agar dilution method, on Sabouraud medium, after 48 hours of incubation for yeasts and after a week for fungi.

Table 2. TLC of antibiotics of iturin group on Silica gel 60 in various solvent systems.

Antibiotics	Rf		
	Solvent A	Solvent B	Solvent C
Bacillomycin F_a	0.47	0.45	0.63
Bacillomycin F_b	0.23	0.39	0.42
Bacillomycin F_c	0.15	0.30	0.36
Bacillomycin D	0.21	0.40	0.42
Bacillomycin L	0.16	0.38	0.38
Mycosubtilin	0.26	0.48	0.45
Iturin A	0.35	0.53	0.45
Iturin D	0.18	0.35	0.34
Iturin E	0.60	0.77	0.83

Solvent A: $CHCl_3$ - MeOH - H_2O (65 : 25 : 4).
Solvent B: BuOH - Me_2CO - H_2O (4 : 6 : 1).
Solvent C: $CHCl_3$ - DMF - H_2O (25 : 22 : 3).

water (65 : 25 : 4).

The fractions were analyzed by TLC on Silica gel 60 with chloroform - methanol - water (65 : 25 : 4). The chromatograms were included in Sabouraud agar using *Saccharomyces cerevisiae* and incubated for 2 days at 28°C; bioautograms revealed the presence of bacillomycin F_a as the major antibiotic in all the crude preparations. In addition, two new antifungal compounds: Bacillomycins F_b and F_c were detected; they were 10~20 times less than bacillomycin F_a.

Further purification of bacillomycins F_b and F_c was made by TLC on Silica gel 60 in chloroform - methanol - water (65 : 25 : 4).

Biological Properties

The antifungal activities of bacillomycins F_b and F_c were determined in Sabouraud medium containing increasing amounts of antibiotic. MIC values are given in Table 1.

Bacillomycins F_b and F_c showed a strong antifungal activity against yeasts and fungi; they did not exhibit any antibacterial activity.

Chromatographic and Spectroscopic Characterization

Bacillomycins F_b and F_c are colorless powders; they gave a negative reaction with ninhydrin and a positive reaction with Pauly reagent.

The UV spectra of both antibiotics in ethanol revealed a maximum at 277 nm which is typical for all tyrosyl containing antibiotics of iturin group.

Both products were tested by TLC on Silica gel 60 in various solvent systems. Their Rf were compared with others iturinic antibiotics (Table 2). Bacillomycins F_b and F_c are distinguishable from bacillomycins D, L, iturins A, D, E and mycosubtilin.

The presence of free carboxyl group in bacillomycins F_b and F_c is suggested by paper electrophoresis; at pH 8.0, they moved toward anodic compartment while bacillomycin F_a did not migrate.

Analysis of α-Amino Acids

Bacillomycins F_b and F_c were hydrolyzed with 6 N HCl at 150°C for 8 hours. Hydrolysates were extracted with chloroform, a lipidic and a water-soluble part were obtained.

Table 3. Analysis of β-amino acids of bacillomycins F.
The values are given in % of total β-amino acids.

Medium[a]	Bacillomycin	β-Amino acids[b]				
		iso-C_{15}	*anteiso*-C_{15}	*iso*-C_{16}	*iso*-C_{17}	*anteiso*-C_{17}
I	F_a			90		10
	F_b			88		12
II	F_a	21		25	26	28
	F_b	19		26	29	26
III	F_a		6	6		88
	F_b		5	4		91

[a] The different media were the LANDY medium where valine (medium I), leucine (medium II) or isoleucine (medium III) were added at a 4 g/liter concentration.

[b] *iso*-C_{15}: 3-Amino-13-methyltetradecanoic acid, *anteiso*-C_{15}: 3-amino-12-methyltetradecanoic acid, *iso*-C_{16}: 3-amino-14-methylpentadecanoic acid, *iso*-C_{17}: 3-amino-15-methylhexadecanoic acid, *anteiso*-C_{17}: 3-amino-14-methylhexadecanoic acid.

The water-soluble amino acids were analyzed by TLC on cellulose powder in 2-propanol - pyridine - water - acetic acid (40 : 40 : 20 : 5) and identified in both antibiotics as aspartic acid, glutamic acid, proline, threonine and tyrosine. Quantitative analysis of the dinitrophenyl derivatives[4] gave the following molar ratios: $Asp_{3.1}$, Glu_1, $Pro_{0.7}$, $Thr_{0.6}$, $Tyr_{0.6}$ for bacillomycin F_b and $Asp_{2.9}$, Glu_1, $Pro_{0.8}$, $Thr_{0.7}$, $Tyr_{0.5}$ for bacillomycin F_c.

Since amino acid identification was carried out on acid hydrolysates, it was impossible to distinguish between aspartyl and asparaginyl or glutamyl and glutaminyl residues in the native antibiotics.

Analysis of β-Amino Acids

The lipidic fractions of acid hydrolysates were analyzed by TLC on Silica gel 60 with chloroform - methanol - water (65 : 25 : 4). After spraying with the ninhydrin reagent according to RUSSELL[5], the chromatograms showed a spot with a Rf identical to that of β-amino acids obtained from bacillomycin F_a (Rf 0.63).

It had been demonstrated that the nature of the β-amino acid components of bacillomycin F_a varied according to the composition of the culture medium[6]. Thus it was interesting to analyze the β-amino acids of bacillomycins F produced in the various media.

The structure of these β-amino acids was determined by gas chromatography of the *N*-trifluoroacetyl methyl esters in comparison with the derivatives of β-amino acids from bacillomycin F_a[7].

Quantitative analysis showed that the β-amino acids of bacillomycin F_b are identical to those of bacillomycin F_a obtained in the same medium (Table 3).

Determination of the Lipid-peptide Linkage

Bacillomycins F_b and F_c were hydrolyzed with 6 N HCl at 105°C for 16 hours. The chloroform extracts were analyzed by TLC on Silica gel 60 in chloroform - methanol - water (65 : 25 : 4) and revealed with ninhydrin according to RUSSELL[5]. The chromatograms showed two ninhydrin positive spots: One which comigrated with β-amino acids, the other one with the peptide Thr → β-amino acid isolated in the same conditions from hydrolysate of bacillomycin F_a[1].

The peptides obtained from bacillomycins F_b and F_c were hydrolyzed with 6 N HCl at 150°C for 8 hours; they contained threonine and β-amino acids. Moreover, these peptides were dinitro-

Table 4. Characterization of methyl derivatives of bacillomycins F_b and F_c.

Native antibiotic	Methylated compound	Rf value[a]	Pauly reaction	Antifungal activity[b]	Nature of the methyl derivative
Bacillomycin F_b	I	0.82	+	+	Methyl ester
	II	0.75	−	−	Methyl ester *O*-methyl tyrosine
Bacillomycin F_c	III	0.77	+	+	Dimethyl ester
	IV	0.82	−	−	Dimethyl ester *O*-methyl tyrosine

[a] Rf value after TLC on Silica gel 60 in $CHCl_3$ - MeOH - H_2O (65 : 25 : 4) and revelation with water.

[b] The 50-μg of each derivative were chromatographed on Silica gel 60 in the same solvent and the chromatograms were included in Sabouraud agar culture of *Saccharomyces cerevisiae*.

phenylated with 2,4-dinitrofluorobenzene and hydrolyzed with 6 N HCl for 8 hours at 150°C; the hydrolysates were analyzed by TLC in chloroform - methanol - water (65 : 25 : 4): DNP-Threonine (Rf 0.37) and β-amino acids (Rf 0.63) were identified. Thus, bacillomycins F_b and F_c contain the same sequence Thr → β-amino acids as bacillomycin F_a.

Preparation and Characterization of Methyl Derivatives of Bacillomycins F_b and F_c

The methyl derivatives of bacillomycins F_b and F_c were prepared as follows: The antibiotics were dissolved in anhydrous methanol and gaseous diazomethane was bubbled in the solution for 15 minutes at 20°C. The excess of diazomethane was blown out with a stream of nitrogen. Methyl derivatives of both antibiotics were tested by TLC on Silica gel 60 in chloroform - methanol - water (65 : 25 : 4). The revelation of the compounds were performed by spraying the plates with water and slow drying which gave white spots with peptidolipids[8].

Two methyl derivatives were obtained with bacillomycins F_b and F_c. Their Rf are given in Table 4. When the phenolic group of tyrosine was substituted, the methyl derivatives gave a negative reaction to Pauly reagent. Bacillomycins F_b and F_c gave methyl ester derivatives (compounds I and III, respectively) and methyl ester *O*-methyl tyrosine derivatives (compounds II and IV, respectively) (Table 4).

The antifungal activity of the different methyl derivatives on *S. cerevisiae* was determined by bioautography (Table 4): Only methyl ester derivatives (compounds I and III) gave an inhibition of the yeast growth.

Fast Atom Bombardment Mass Spectrometry (FAB-MS)

In the medium of LANDY containing isoleucine, *anteiso*-C_{17} β-amino acid represents about 90% of total β-amino acids (Table 3). In order to have antibiotics containing this β-amino acid, bacillomycins F_b and F_c were prepared from this medium for obtaining methyl derivatives as described above. All these compounds were analyzed by FAB-MS.

The FAB-MS of bacillomycin F_b displayed a major (M+H) peak at *m/z* 1,100. When the spectrum was run in presence of NaCl, the corresponding $(M+Na)^+$ peak was observed at *m/z* 1,122 (Fig. 2). With the methyl derivatives of bacillomycin F_b, the major $(M+H)^+$ peaks were observed at *m/z* 1,114 and *m/z* 1,128. In presence of NaCl, the corresponding $(M+Na)^+$ peaks were at *m/z* 1,136 and *m/z* 1,150.

In the case of bacillomycin F_c, the FAB-MS showed a major $(M+H)^+$ peak at *m/z* 1,101 and in presence of NaCl, a $(M+Na)^+$ peak at *m/z* 1,123 (Fig. 3). When bacillomycin F_c was methylated, the major $(M+H)^+$ peaks were observed at *m/z* 1,129 and *m/z* 1,143 and in presence of NaCl, $(M+Na)^+$

Fig. 2. Na-Cationized FAB-MS of bacillomycin F_b showing the $(M+Na)^+$ peak region.

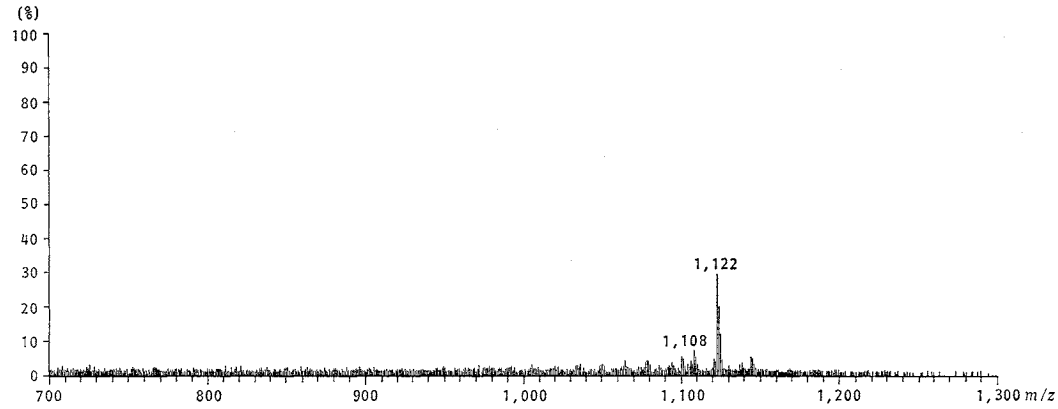
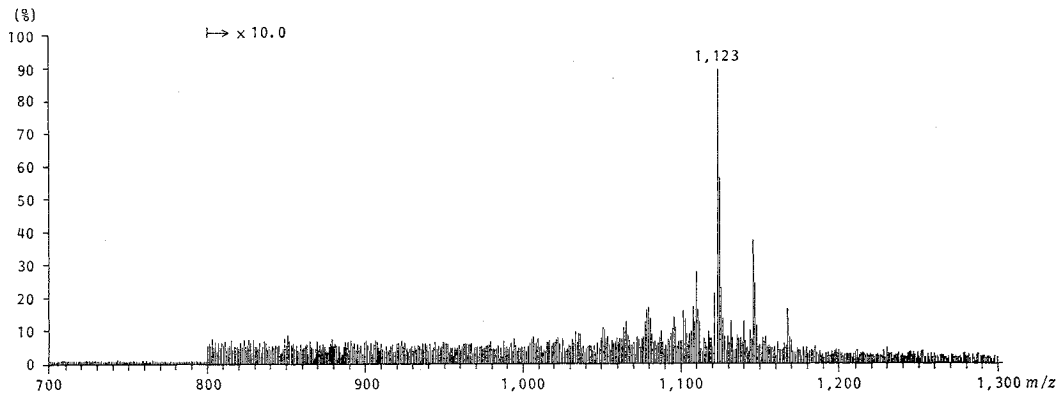


Fig. 3. Na-Cationized FAB-MS of bacillomycin F_c showing the $(M+Na)^+$ peak region.



peaks were at *m/z* 1,151 and *m/z* 1,165.

The $(M+H)^+$ peaks obtained in the case of bacillomycins F_b and F_c are one and two units higher than those obtained in the case of bacillomycin F_a where the major $(M+H)^+$ peak was observed at *m/z* 1,099 and the major $(M+Na)^+$ peak at *m/z* 1,121.

Structure of Bacillomycin F_b

The formula $C_{52}H_{82}N_{12}O_{14}$, M_r 1,098, found for bacillomycin F_a corresponds to the following amino acid composition: C_{17} β-amino acid, Asn_3, Gln_1, Pro_1, Thr_1, Tyr_1[2].

The molecular weight 1,099, found for bacillomycin F_b, is one mass unit higher than the value obtained for bacillomycin F_a and corresponds to the formula $C_{52}H_{81}N_{11}O_{15}$. It can be proposed that, as in the case of iturins C and D[9,10], one carboxamide group of asparagine or glutamine residue is replaced by a carboxyl group.

The presence of such a carboxyl group in bacillomycin F_b was confirmed by the mass spectra of the methyl derivatives. Their molecular weights, 1,113 and 1,127, are 14 and 28 mass units higher than the value obtained for bacillomycin F_b and correspond to the formula $C_{53}H_{83}N_{11}O_{15}$ and $C_{54}H_{85}N_{11}O_{15}$. These formula can be attributed to the methyl ester derivative and methyl ester *O*-methyl tyrosine derivative of bacillomycin F_b respectively (compounds I and II in Table 4).

Structure of Bacillomycin F_c

The molecular weight, 1,100, found for bacillomycin F_c, is two mass units higher than the values obtained for bacillomycin F_a and corresponds to the formula $C_{52}H_{80}N_{10}O_{16}$. This can be explained if two carboxamide groups of asparagine or glutamine are replaced by two carboxyl groups.

This hypothesis was confirmed by the mass spectra of the methyl derivatives. Their molecular weights, 1,128 and 1,142, are 28 mass units and 42 mass units higher than the value obtained for bacillomycin F_c and correspond to the formula $C_{54}H_{84}N_{10}O_{16}$ and $C_{55}H_{86}N_{10}O_{16}$. These formula can be attributed to the dimethyl ester derivative and dimethyl ester *O*-methyl tyrosine derivative of bacillomycin F_c respectively (compounds III and IV in Table 4).

Conclusion

Bacillomycins F_b and F_c differ from bacillomycin F_a by the presence of one and two carboxyl group respectively instead of carboxamide groups. The total sequence of these antibiotics has not been determined on account of the very small available amounts. The amino acid sequence of another carboxyl containing compound, iturin C, had been determined[9]: Iturin C differs from iturin A only by the presence of an Asp residue instead of an Asn residue but the remaining amino acid sequence of iturin C was identical to that of iturin A. Thus, it does not seem likely that one strain of *B. subtilis* would produce peptidolipidic antibiotics having the same amino acid composition but different sequences. One Asn or Gln of bacillomycin F_a is replaced by an Asp or Glu residue in the case of bacillomycin F_b and two Asn and/or Gln residues by Asp and/or Glu residues in the case of bacillomycin F_c.

It is not possible to claim that bacillomycins F_b and F_c are, or not, genuine components of the antibiotic mixture. Because of the acidic conditions prevailing in the initial operations of the isolation procedure, it is quite possible that the side chain of an Asn and/or Gln residue of bacillomycin F_a was hydrolyzed giving rise to the Asp and/or Glu residues of bacillomycins F_b and F_c.

However, interesting results are the antifungal activities of bacillomycins F_b and F_c, especially that of bacillomycin F_c: It is the only iturinic antibiotic possessing two carboxyl residues. All the others possess one carboxyl group (bacillomycins D, L and iturin D) or do not possess any carboxyl group (iturins A, E, bacillomycin F_a and mycosubtilin)[2,7,10,11].

Acknowledgments

We are grateful to Dr. M. T. POMMIER for providing us with the strains of yeasts and fungi. This work was supported by the Centre National de la Recherche Scientifique (UA 1176).

References

1) MHAMMEDI, A.; F. PEYPOUX, F. BESSON & G. MICHEL: Bacillomycin F, a new antibiotic of iturin group: Isolation and characterization. J. Antibiotics 35: 306~311, 1982
2) PEYPOUX, F.; D. MARION, R. MAGET-DANA, M. PTAK, B. C. DAS & G. MICHEL: Structure of bacillomycin F, a new peptidic antibiotic of the iturin group. Eur. J. Biochem. 153: 335~340, 1985
3) 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
4) GHUYSEN, J. M.; D. J. TIPPER & J. L. STROMINGER: Enzymes that degrade bacterial cell walls. *In* Methods in Enzymology. Vol. 8. *Eds.*, E. F. NEUFELD & Y. GINSBURG, pp. 685~699, Academic Press, New York, 1966
5) RUSSELL, D. W.: Ninhydrin as a reagent for *N*-methyl amino acids. J. Chromatogr. 4: 251~252, 1960
6) BESSON, F. & M. L. HOURDOU: Effect of amino acids on the biosynthesis of β-amino acids, constituents of bacillomycins F. J. Antibiotics 40: 221~223, 1987
7) PEYPOUX, F.; M.-T. POMMIER, B. C. DAS, F. BESSON, L. DELCAMBE & G. MICHEL: Structures of bacillomycin D and bacillomycin L peptidolipid antibiotics from *Bacillus subtilis*. J. Antibiotics 37: 1600~1604, 1984
8) IRMSCHER, G.; G. BOVERMANN, G. BOHEIM & G. JUNG: Trichotoxin A 40, a new membrane-exciting

peptide. Part A. Isolation, characterization and conformation. Biochim. Biophys. Acta 507: 470~484, 1978

9) PEYPOUX, F.; F. BESSON, G. MICHEL, L. DELCAMBE & B. C. DAS: Structure de l'iturine C de *Bacillus subtilis*. Tetrahedron 34: 1147~1152, 1978

10) BESSON, F. & G. MICHEL: Isolation and characterization of new iturins: Iturin D and iturin E. J. Antibiotics 40: 437~442, 1987

11) PEYPOUX, F.; M. T. POMMIER, D. MARION, M. PTAK, B. C. DAS & G. MICHEL: Revised structure of mycosubtilin, a peptidolipid antibiotic from *Bacillus subtilis*. J. Antibiotics 39: 636~641, 1986