BACILLOMYCIN F, A NEW ANTIBIOTIC OF ITURIN GROUP: ISOLATION AND CHARACTERIZATION

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Bacillomycin F, a new family of iturin group antibiotics, was isolated from *Bacillus subtilis*. It shows a potent antifungal activity and a narrow spectrum against bacteria. Acid hydrolysis gave a peptide moiety which contains 7 mole of amino acids: D-Asp_2, L-Glu_1, L-Thr_1, L-Pro_1, D-Tyr_1 and a lipid moiety which is a mixture of two main long chain β-amino acids: 3-amino-14-methylpentadecanoic acid (58%) and 3-amino-14-methylhexadecanoic acid (23%).

The antibiotics of iturin group are peptidolipidic compounds characterized by a high antifungal activity[1~4]. They all have a long chain β-amino acid linked to a peptidic moiety containing D and L α-amino acids[5~8]. In our work for the characterization of new antibiotics of this group, strong antifungal activity was detected in the culture broth of the strain I 164 of *Bacillus subtilis*.

The present paper describes the production, the purification, the antimicrobial properties and the composition of bacillomycin F.

Materials and Methods

Antibiotic Production

The antibiotic producing strain was *Bacillus subtilis* strain I 164, Institut Pasteur, Paris. It was grown in a medium containing 37 g/liter of a "brain-heart infusion" (Bio-Mérieux) at 32°C on a rotating shaker. A part (10%) of this culture was used to inoculate Erlenmeyer flasks containing the production medium.

The production medium was either the medium of LANDY *et al.*[2] or the medium of WALTON and WOODRUFF[5]. The culture was carried out for 6 days at 32°C. The production of bacillomycin F was followed with the bioassay procedure.

Isolation and Purification of Bacillomycin F

The broth was adjusted to pH 2.0 with 12 N HCl. The precipitate was collected by centrifugation and extracted twice by 95% ethanol with vigorous shaking. The extracts were evaporated to dryness (yield: 1.9 g/liter of broth). The crude powder was dissolved in methanol and purified by column chromatography on silicic acid BioSil HA 325 mesh (BioRad, U.S.A.). Elution was performed with a discontinuous gradient of methanol in the solvent hexane - chloroform - methanol (25: 45: 10, v/v/v). The antibiotic was eluted with hexane - chloroform - methanol (25: 45: 26, v/v/v). A further purification was made by preparative thin-layer chromatography on silica gel with chloroform - methanol - water (65: 25: 4, v/v/v), then the product was precipitated by methanol (yield 50 mg/liter of broth).

Bioassay Procedure

Antifungal activity of the ethanolic extract was determined by the cylinder method using *Penicillium chrysogenum* as the test organism and it was expressed by the diameters of growth inhibition zone.

The location of the antibiotic on thin-layer chromatograms was made by the detection of the antifungal activity on an agar culture of *Penicillium chrysogenum*.

Determination of Antibiotic Activity

The minimal inhibitory concentration (MIC) of bacillomycin F against Gram-positive, Gram-negative bacteria, yeasts and fungi was determined by liquid or agar dilution method. MIC's were measured after 24 hours of incubation at 37°C for the bacteria and after three to fourteen days at 28°C for the yeasts and the fungi.

Analytical Methods

The infrared spectrum was recorded in KBr pellet on an Infracord 137 Perkin Elmer apparatus and the ultraviolet spectrum on a M25 Beckman spectrophotometer. Electrophoresis was performed on Whatman No. 1 paper in a pH 8.0 veronal buffer for 1 hour at 45 volts/cm with a Pherograph apparatus.

Water-soluble amino acids were analyzed as *N*-heptafluorobutyryl isobutyl esters[9] by gas chromatography on a capillary column of silicone WCOT SE 30 with temperature programming from 90°C to 290°C (427 Packard apparatus).

Liposoluble amino acids were analyzed as *N*-acetylmethyl esters by gas chromatography on a capillary column of silicone WCOT SE 30 from 210°C to 220°C (IGC 120 FL Intersmat apparatus).

Spectrometric Methods

Mass spectra were obtained with a Finnigan spectrometer operating at 70 eV, using a source temperature of 240°C, coupled with the gas chromatograph.

Hydrolysis

Total hydrolysis of bacillomycin F was performed with 6 N HCl at 150°C for 8 hours and partial hydrolysis with 6 N HCl at 105°C for 15 hours.

Results

Culture Studies

The antibiotic production tested on *Penicillium chrysogenum* was not related to the nature of the culture media, LANDY *et al.*[2] or WALTON and WOODRUFF[3] medium. The time course of the culture of *B. subtilis* is shown in Fig. 1. The maximum concentration of bacillomycin F was reached after a four days culture.

Antibiotic Properties

Bacillomycin F showed a strong antifungal activity against various yeasts, fungi and phytopathogenic fungi but a weak antibacterial activity only against *Micrococcus luteus* (Table 1).

Physicochemical Properties

Bacillomycin F is a colorless powder, mp. 243°C, giving a positive reaction with PAULY reagent. It is soluble in diluted alkali and in 75% ethanol, slightly soluble in pyridine, dimethylsulfoxide and dimethylformamide and insoluble in water and in most organic solvents.

The ultraviolet spectrum in ethanol shows absorption at 203 nm (Eg/l=25.2) and at 275 nm (Eg/l=1.0). The infrared spectrum in KBr exhibits the bands of peptidic linkage at 3300 cm^{-1}, 1660 cm^{-1} and 1550 cm^{-1} (Fig. 2).

Fig. 1. Time course of bacillomycin F production.
Growth (●—●) was measured by the absorbance at 600 nm and the antifungal activity (▲—▲) was measured on *Penicillium chrysogenum* as described in Materials and Methods.

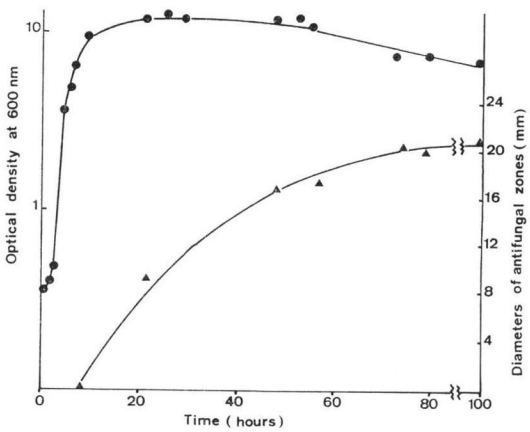
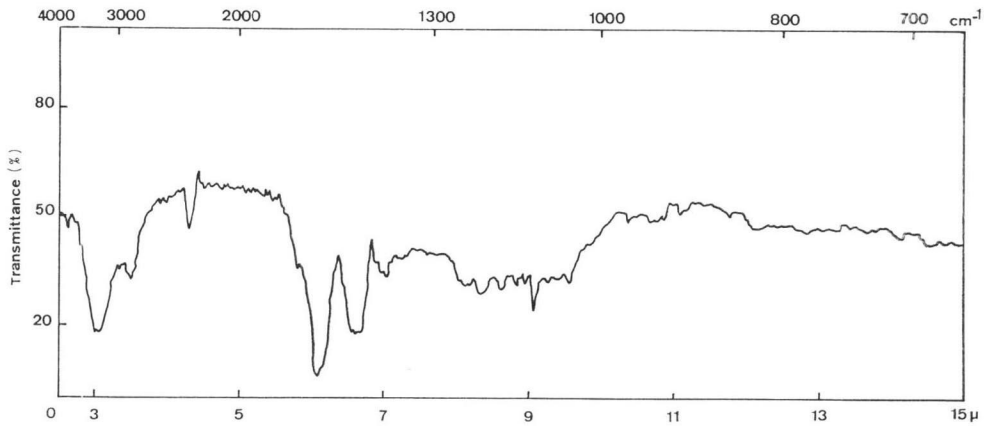


Table 1. Antimicrobial activity of bacillomycin F.

Test organisms	MIC (μg/ml)	Test organisms	MIC (μg/ml)
Aspergillus niger	40	*Candida albicans*	40
Botrytis cinerea (phytopathogenic)	20	*Candida tropicalis*	40
Fusarium oxysporum (phytopathogenic)	>320	*Saccharomyces cerevisiae*	10
Mycosphaerella pinodes (phytopathogenic)	10	*Azotobacter vinelandii*	>400
Neurospora crassa	80	*Brucella bronchiseptica*	>400
Penicillium chrysogenum	20	*Escherichia coli* K12	>400
Pleospora herbarum (phytopathogenic)	10	*Streptomyces albus* G	>400
Rhodotorula pilimanae	80	*Bacillus cereus*	>400
Sclerotinia fructigena (phytopathogenic)	40	*Micrococcus luteus*	200
Sclerotinia sclerotiorum (phytopathogenic)	50	*Sarcina lutea*	>400
Stemphylium radicinum (phytopathogenic)	320	*Staphylococcus aureus*	>400
Trichophyton mentagrophytes	20		

Bacteria were grown for 18 hours at 37°C whereas fungi were grown from 3 to 14 days at 28°C. Phytopathogenic fungi were assayed in a medium containing tomato juice 200 ml, $CaCO_3$ 3 g, agar 10 g per liter. Yeasts and other fungi were assayed in Sabouraud medium. *Streptomyces albus* G was grown on peptone agar medium (biotrypcase 10 g, K_2HPO_4 1 g, $MgSO_4 \cdot 7H_2O$ 1 g, $NaNO_3$ 2 g, KCl 0.5 g, 2% agar). The other bacteria were tested in a brain-heart infusion medium.

Fig. 2. Infrared spectrum of bacillomycin F in KBr.



The product was tested by thin-layer chromatography on silica gel 60, Rf 0.47 in chloroform - methanol - water (65: 25: 4, v/v/v), 0.63 in dimethylformamide - water - chloroform (44: 6: 50, v/v/v) and 0.45 in butanol - acetone - water (16: 24: 4, v/v/v). Bacillomycin F has no electric charge as shown by paper electrophoresis in pH 8.0 buffer, in comparison with neutral iturin A[7] and anionic bacillomycin L[6] as standards.

Analysis of Amino Acids

Bacillomycin F was hydrolyzed by 6 N HCl at 150°C for 8 hours. Hydrolysates were extracted with chloroform, a lipidic part and a water-soluble part were obtained.

The water-soluble amino acids were analyzed by thin-layer chromatography on cellulose powder in isopropanol - pyridine - acetic acid - water (40: 40: 5: 20, v/v/v/v) and pyridine - *tert*-amylalcohol -

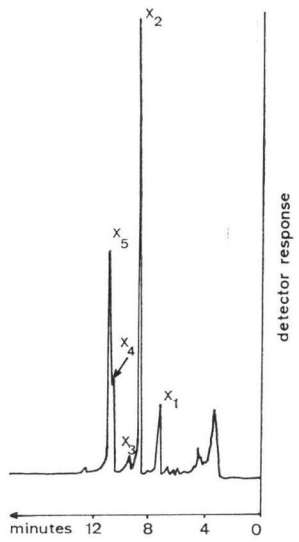
water (35: 35: 30, v/v/v) and identified as aspartic acid, glutamic acid, proline, threonine and tyrosine. A quantitative analysis of the *N*-heptafluorobutyryl isobutyl esters by gas chromatography gave the following molar ratios: $Asp_{2.9}$, Glu_1, $Pro_{0.9}$, $Thr_{0.7}$, $Tyr_{1.1}$.

The configuration of the amino acids was determined by enzymatic methods[5] with D-amino acid oxidase for proline, threonine and tyrosine, with L-glutamic decarboxylase for glutamic acid and L-glutamate oxalacetate transaminase for aspartic acid.

The amino acid composition is the following: D-Asp_2, L-Asp_1, L-Glu_1, L-Pro_1, L-Thr_1, D-Tyr_1.

Fig. 3. Gas chromatogram of the *N*-acetyl methylesters of the lipid part of bacillomycin F.

Temperature conditions: 210°C for 6 minutes, then for 3 minutes from 210°C to 220°C (rate: 6°C per minute) and then 220°C.



Analysis of the Lipid Moiety

The lipid moiety was extracted by chloroform and the yield was 28% of the initial product. Analysis by thin-layer chromatography on silica gel 60 with chloroform - methanol - water (65: 25: 4, v/v/v) and detection with a ninhydrin solution according to RUSSELL[10] gave a spot with a migration identical to that of the β-amino acids obtained from iturin A (Rf 0.63)[11].

The qualitative and quantitative analysis of the lipidic compounds was carried out by gas chromatography/mass spectrometry of the *N*-acetylmethylesters. The retention times were compared with those of the β-amino acid derivatives of mycosubtilin, iturin A and of synthetic 3-aminohexadecanoic and 3-aminoheptadecanoic acids. The gas chromatogram is shown in Fig. 3.

Two main peaks X_2 (58%) and X_5 (23%) and three minor peaks X_1 (6%), X_3 (3%) and X_4 (10%) were observed.

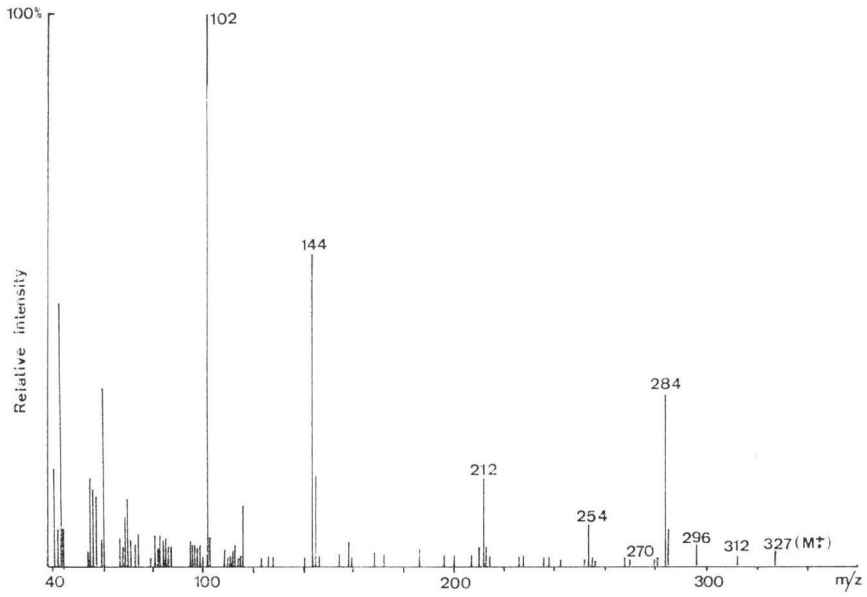
The mass spectra of X_2 and X_5 are shown in Fig. 4. Two homologous molecular ions at $m/z=327$ and $m/z=341$, correspond to the derivatives $C_{15}H_{30}$–(NH–$COCH_3$)–$COOCH_3$ (X_2) and $C_{16}H_{32}$–(NH–$COCH_3$)–$COOCH_3$ (X_5) respectively. Homologous peaks at $m/z=284$ for X_2 and $m/z=298$ for X_5 resulting of the loss of CH_3–CO from the molecular ions indicate the presence of an amide group in β of the carboxylic ester group[11].

Both spectra show the characteristic peaks of an acetamide group in β of the carboxylic ester group. The α fragmentations of amide group give an ion at $m/z=144$ $(CH_3–CO–NH=CH–CH_2–COOCH_3)^+$ and ions at $m/z=254$ $(C_{13}H_{27}CH=NH–COCH_3)^+$ for X_2 and $m/z=268$ $(C_{14}H_{29}CH=NH–COCH_3)^+$ for X_5. The peak at $m/z=102$ $(NH=CH–CH_2–COOCH_3)^+$ arises from the loss of the ketene group from the $m/z=144$ ion.

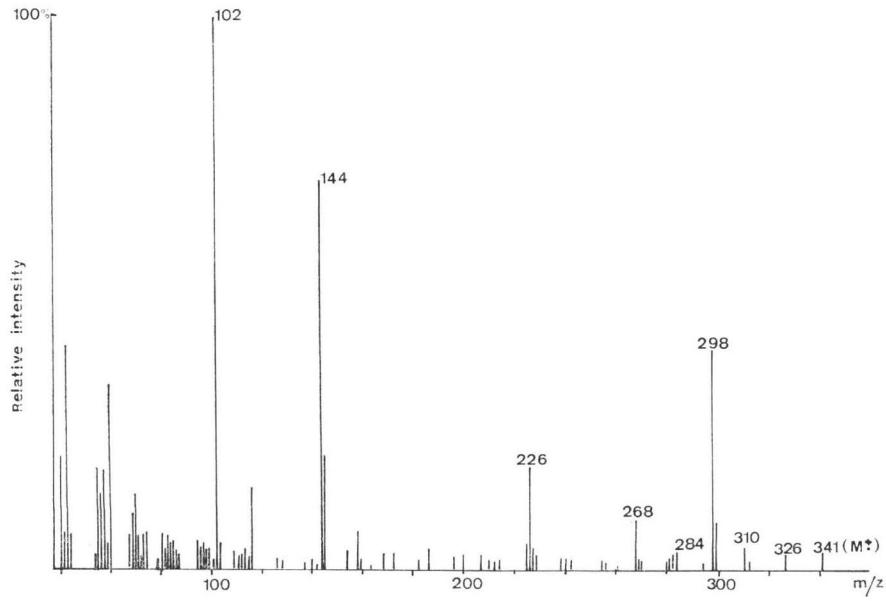
The spectra of the derivatives X_1, X_3 and X_4 exhibit the molecular ions at $m/z=313$, 326 and 341, respectively. Other peaks are consistent with the derivatives of C_{15}, C_{16} and C_{17} β-amino acids.

The nature of the hydrocarbon chain was determined by comparison of the retention times of β-amino acid derivatives of bacillomycin F and those of standard compounds. X_1 was identified to 3-amino-12-methyltetradecanoic acid, X_2 to 3-amino-14-methylpentadecanoic acid, X_3 to 3-amino-hexa-

Fig. 4. a) Mass spectrum of the *N*-acetyl methylester of the C_{16} β amino acid.



b) Mass spectrum of the *N*-acetyl methylester of the C_{17} β amino acid.



decanoic acid, X_4 to 3-amino-15-methylhexadecanoic acid, X_5 to 3-amino-14-methylhexadecanoic acid.

It should be noted that all β-amino acids have a chiral center at C_3 and moreover X_1 and X_5 have a second chiral center at the antepenultimate carbon atom. The configuration of these centers is not known. However, analogous β-amino acids which are constituents of iturin A, have been found to have the *R*-configuration at the C_3 center[12].

Determination of the Peptide-lipid Linkage

The chloroform extract of the 6 N HCl hydrolysate at 105°C for 16 hours contained, besides the β-amino acids, other liposoluble compounds. Their Rf (0.40 in chloroform - methanol - water, 65: 25: 4, v/v/v) was identical to the Rf of the compounds isolated in the same conditions from the hydrolysates of bacillomycin L and bacillomycin D. These compounds were recovered from thin-layer plates, dinitrophenylated with 2,4-dinitrofluorobenzene and hydrolyzed by 6 N HCl at 150°C for 8 hours. The hydrolysate was analyzed by thin-layer chromatography in chloroform - methanol - water (65: 25: 4, v/v/v). It contained dinitrophenylthreonine (Rf 0.37) and β-amino acids (Rf 0.63). Thus the compounds were threonyl-β-amino acids and the linkage between the peptide and lipid moieties is an amide bond between the carboxyl group of threonine and the amine group of a β-amino acid.

Conclusions

The present work describes the isolation of a new family of iturin group antibiotics. They were isolated from the culture broth of a strain of *Bacillus subtilis* by acidification and extraction with ethanol. They are potent antifungal agents and their bacterial activity are restricted to *M. luteus* as the other antibiotics of this group. The lipid moiety consists mainly of one *iso*-C_{16} or *anteiso*-C_{17} β-amino acid as it was found in mycosubtilin belonging to iturin group[5]. The amino acid composition of the peptide moiety was close to that of iturin A, with a L-threonyl residue in bacillomycin F instead of a L-seryl residue in iturin A. This threonyl residue links the peptide moiety to the β-amino acid in bacillomycin F as did the seryl residue in iturin A. The structure elucidation is being continued.

Acknowledgements

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