CHARACTERIZATION OF A NEW ANTIBIOTIC OF ITURIN GROUP:
BACILLOMYCIN D

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The characterization of an antibiotic isolated from a strain of Bacillus subtilis revealed
that this compound is a new antifungal antibiotic of the iturin group. It contains a lipid moi-
ety which is a mixture of 3-amino 12-methyl tridecanoic acid (40%) and 3-amino 12-methyl
tetradecanoic acid (60%) and a peptide moiety: L-Asp₁, D-Asp₁, L-Glu₁, L-Pro₁, D-Ser₁, L-Thr₁
and D-Tyr₁. These two moieties are joined by a threonyl-β-aminoacid linkage.

Iturin is an antifungal antibiotic which was isolated by DELCAMBE from a strain of Bacillus subtilis1). The structure of the major component of crude iturin, iturin A, was determined by PEYPoux et al., as a
cyclic peptidolipid containing a lipophilic β-aminoacid and a peptide moiety with L and D α-amino-
acids2). Other peptidolipids of the same group were studied and the structures of mycosubtilin3), bacil-
lozymycin L4), iturin C5) were determined. In the course of a screening program for new antibiotics of the
iturin group, the distribution of the known antibiotics in various strains of B. subtilis was studied6) and a
new antibiotic, previously reported by RAUBITSCHEK and DOSTROVSKY7), was found to belong to the iturin
group. This paper describes the purification and the composition of this new antibiotic, bacillomycin D.

Material and Methods

Production and Purification of Bacillomycin D

The culture of B. subtilis and the extraction of bacillomycin D from culture medium were carried
out as previously described8). The crude extracts were purified by column chromatography on Sephadex
LH 20, the elution was performed with hexane - chloroform - methanol (25: 45: 10, v/v/v). The frac-
tions which have an antibiotic activity were pooled and concentrated in vacuo. The antibiotic was
crystallized at room temperature in a chloroform - methanol (2: 1, v/v) solution and the crystals were
washed twice with methanol and dried (yield 10% of crude extract.)

Hydrolysis

The complete hydrolysis of bacillomycin D was performed with 6 N HCl at 150°C for 8 hours and a
partial hydrolysis with 6 N HCl at 105°C for 15 hours.

Chromatographic methods

Thin-layer chromatography on silica gel C-60 with chloroform - methanol - water (65: 25: 4, v/v/v)
was used for the purification and the identification of the lipidic compounds obtained by hydrolysis
of bacillomycin D. The spots were visualized by the ninhydrin reagent or by the PAULY reagent.
Water-soluble aminoacids were characterized by thin-layer chromatography on cellulose with isopro-
panol - pyridine - acetic acid - water (8: 8: 4: 1, v/v/v/v).

The lipid moiety was studied as N-trifluoroacetyl-n-butylesters which were prepared by the method of ROACH and GEHRKE\(^b\). These derivatives were analyzed by gas-chromatography in a Fractovap GT 200 apparatus with 0.65% ethyleneglycol adipate on Chromosorb W, with temperature programming from 80°C to 215°C.

**Analytical methods**

The infrared spectrum was recorded in KBr pellet on an Infrascan, Hilger and Watts apparatus and the ultraviolet spectrum on a M 25 Beckman spectrophotometer. Electrophoresis was performed on Whatman No. 1 paper with a Pherograph apparatus in a pH 8.6 veronal buffer for 1 hour at 50 volts/cm.

The quantitative analysis of \(\alpha\)-aminoacids was carried out with a Technicon autoanalyser.

The molecular weight was determined by a thermoosmotic method according to BRADY et al.\(^9\) using a Mechrolab apparatus 301 A.

**Detection of active substances**

In order to detect the presence of a substance with an antifungal activity on thin-layer chromatograms the chromatographic sheets were covered with a thin agar film with Penicillium chrysogenum in a Petri dish; active substances appeared as spots clear of fungal growth.

**Results**

**Physico-chemical Properties**

Bacillomycin D is a colorless powder, m.p. 287°C, giving a negative reaction with ninhydrin and with EHRlich reagent but a positive reaction with PAULY reagent. It gave a single spot on thin-layer chromatography: Rf 0.21 in chloroform - methanol - water (65: 25: 4, v/v/v), Rf 0.41 in butanol - acetic acid - water (65: 10: 25, v/v/v), Rf 0.40 in butanol - acetone - water (16: 24: 4, v/v/v). Its acidic nature was shown by paper electrophoresis in pH 8.0 buffer, bacillomycin D had a migration toward anodic compartment while neutral iturin A used as control had no migration.

Bacillomycin D is insoluble in water and in most organic solvents, sparingly soluble in pyridine and aqueous ethanol and soluble in alkalies and in the mixture pyridine - ethanol - water (4: 3: 1, v/v/v).

The infrared spectrum in KBr pellet showed strong bands at 3300 cm\(^{-1}\) (OH, NH), 1660 cm\(^{-1}\) and 1560 cm\(^{-1}\) (CO-NH) (Fig. 1). The ultraviolet spectrum exhibited absorption at 200 nm (E g/l: 31.7) and at 275 nm (E g/l: 1.6) in ethanol.

**Composition**

Acid hydrolysis with 6 N HCl at 150°C for 8 hours yielded a lipidic part and a water-soluble part.

The water-soluble components were identified as \(\alpha\)-aminoacids by thin-layer chromatography on cellulose with isopropanol - pyridine - acetic acid - water (8: 8: 4: 1, v/v/v/v): aspartic acid, glutamic acid, proline, serine, threonine and tyrosine were identified. A quantitative analysis with a Technicon autoanalyser gave the following molar ratios: 2, 1, 1.2, 0.9, 0.7, 0.9.

The optical configuration of aminoacids was determined by enzymatic methods\(^5\) and the molecular formula could be: L-Asp\(_1\), D-Asp\(_1\), L-Glu\(_1\), L-Pro\(_1\), D-Ser\(_1\), L-Thr\(_1\), and D-Tyr\(_1\).

The lipid part was studied by thin-layer chromatography on silica gel and detected by a ninhydrin solution according to RUSSELL\(^10\). A comparative chromatography showed the same migration (Rf 0.63) for the lipid moiety of bacillomycin D and for the lipid moieties of bacillomycin L, iturin A and mycosubtilin. The structures of these last lipidic fractions were demonstrated previously, they are \(\beta\)-aminoacids with 14 and 15 carbon atoms in iturin A\(^2\) and bacillomycin L\(^4\), 16 and 17 carbon atoms in mycosubtilin\(^3\).
The lipid moiety of bacillomycin D was studied by gas chromatography of the N-trifluoroacetyl-n-
butylesters in comparison with the derivatives of the known antibiotics. The chromatogram is similar
to that of the derivatives of iturin A and of bacillomycin L. Quantitative analysis showed the presence
of two major components: 3-Amino 12-methyl tridecanoic acid (40%) and 3-amino 12-methyl tetradecanoic acid (60%) (Fig. 2). The mean molecular weight M.W. = 1,039 calculated for one lipidic β-
aminoacid and a heptapeptidic moiety was in good agreement with the experimental value: M.W. = 1,060 ± 32.

**Determination of Lipid-peptide Linkage**

The hydrolysis of bacillomycin D with 6 N HCl at 105°C for 15 hours gave a mixture of water soluble
aminoacids and a lipid soluble part which was different from the β-aminoacids. This fraction was studied by thin-layer chromatography in comparison with lipidic compounds obtained by hydrolysis of iturin A, mycosubtilin and bacillomycin L; these compounds were respectively seryl-β-aminoacid,
aspartyl-β-aminoacid and threonyl-β-aminoacid\(^2,5,14\). The Rf for the unknown compound from bacillomycin D was identical to the Rf of the compound from bacillomycin L, Rf 0.40 in chloroform - methanol - water (65: 25: 4, v/v/v).

The unknown compound was isolated from thin-layer plates, dinitrophenylated by 2,4-dinitrofluorobenzene and hydrolysed by 6 N HCl at 150°C for 8 hours: dinitrophenyl threonine and β-aminoacid were identified by thin-layer chromatography, Rf 0.37 and 0.63, respectively, in chloroform - methanol - water (65: 25: 4, v/v/v). After Edman degradation, the threonine was eliminated and the β-aminoacid was identified. Thus a threonyl-β-aminoacid linkage is present in bacillomycin D.

Conclusions

Bacillomycin D was previously reported by RAUBITSCHEK and DOSTROVSKY\(^7\) as an antifungal crude preparation from a strain of B. subtilis but the nature of the active compound had not been determined. The present work establishes the peptidolipidic nature of bacillomycin D and permits its classification in the iturin group. The lipidic moiety consists of one of C\(_{14}\) or C\(_{15}\) β-aminoacid and the peptide moiety is linked to this aminoacid by a threonyl residue. The electrophoretic migration agrees with the occurrence of two free carboxylic groups from aspartic or glutamic acid residues. The determination of the complete structure of bacillomycin D is under investigation.

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References

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