CHARACTERIZATION OF A NEW ANTIBIOTIC OF ITURIN GROUP: BACILLOMYCIN D

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(Received for publication July 18, 1980)

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 moiety 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-Asp_1, D-Asp_1, L-Glu_1, L-Pro_1, D-Ser_1, L-Thr_1 and D-Tyr_1. 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 subtilis*[1]. 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-aminoacids[2]. Other peptidolipids of the same group were studied and the structures of mycosubtilin[3], bacillomycin L[4], iturin C[5] 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 studied[6] and a new antibiotic, previously reported by RAUBITSCHEK and DOSTROVSKY[7], 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 described[2]. 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 fractions 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[8]. 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 α-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 α-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[2] 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 β-aminoacids with 14 and 15 carbon atoms in iturin A[2] and bacillomycin L[4], 16 and 17 carbon atoms in mycosubtilin[3].

Fig. 1. Infrared spectrum of bacillomycin D in KBr.

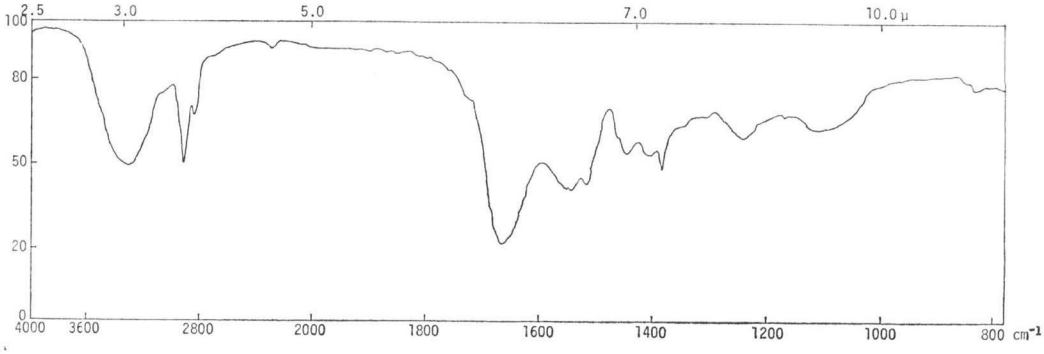
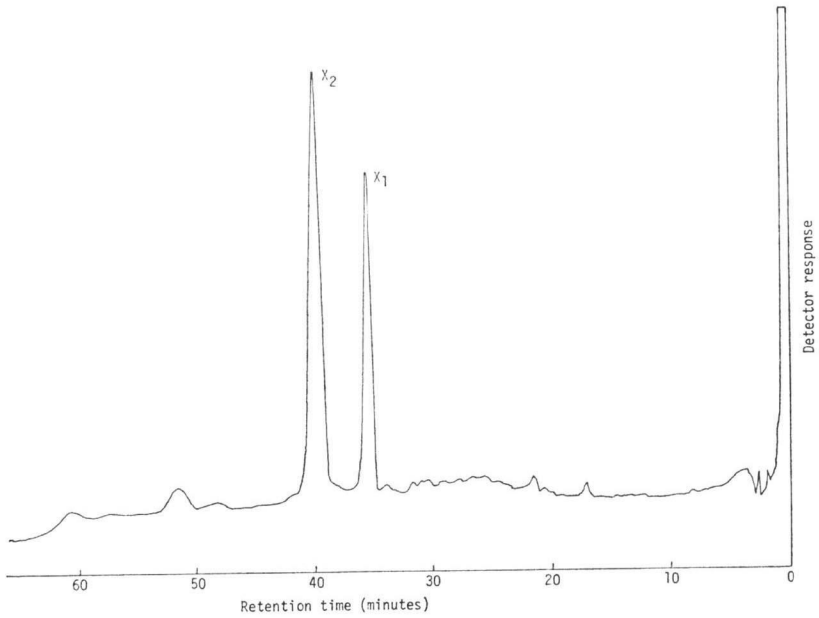


Fig. 2. Gas chromatogram of trifluoroacetyl-*n*-butylester derivatives of lipid moiety of bacillomycin D. X_1: 3-amino 12-methyl tridecanoic acid; X_2: 3-amino 12-methyl tetradecanoic acid.



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$\pm$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[2,3,4]. 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.

This work was supported by the Centre National de la Recherche Scientifique (E. R. A. n° 852).

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