

ISOLATION AND CHARACTERIZATION OF NEW ITURINS:
ITURIN D AND ITURIN E

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Two new antibiotics, iturin D and iturin E, were isolated from a strain of *Bacillus subtilis* producing iturin A. These compounds belong to the iturin group, the acid hydrolysates contained α -amino acids Asp₃, Glu₁, Pro₁, Ser₁, Tyr₁, and a mixture of *n*-C₁₄, *iso*-C₁₅, *anteiso*-C₁₅, *iso*-C₁₆ and *n*-C₁₆ β -amino acids.

They differ from iturin A by the presence of a free carboxyl group in iturin D and a carboxymethyl group in iturin E.

Iturin A is an antifungal antibiotic produced by several strains of *Bacillus subtilis*¹⁻³. Its structure has been determined; a cyclolipopeptide containing seven residues of D and L- α -amino acids and one residue of a β -amino acid (Fig. 1)³⁻⁵.

Another lipopeptide, iturin C, has been isolated from the culture medium of a strain producing iturin A³. In contrast to iturin A, iturin C has no antifungal activity and differs by the presence of an aspartyl residue, instead of an asparaginyl residue in iturin A (Fig. 1)⁷. During studies on the influence of the culture medium on the production of antibiotics, two new antifungal compounds were isolated, they were related to iturin A and the names iturin D and iturin E are proposed.

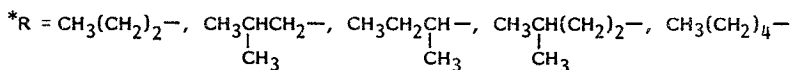
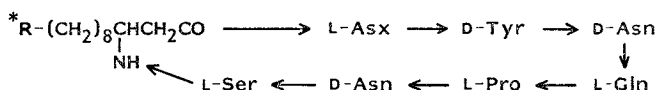
This paper describes the identification and the characterization of these antibiotics.

Culture Conditions

Iturin-producing *Bacillus subtilis* was kindly supplied by Dr. L. DELCAMBE, C.N.P.E.M., Liège, Belgium. *B. subtilis* was grown in a medium containing 37 g/liter of brain-heart infusion (Bio-Mérieux, France) on a rotating shaker. A part (10%) of this culture was used to inoculate Erlenmeyer flasks containing the production medium; either a Biotrypcase (10 g/liter) medium or a brain-heart infusion (37 g/liter) medium or the medium of LANDY *et al.*³. The culture was carried out for 60 hours at 35°C.

Fig. 1. Structure of iturins.

Iturin A: L-Asx=L-Asn. Iturin C: L-Asx=L-Asp.



Isolation and Purification of Iturins

The culture supernatant was adjusted to pH 2.0 with 12 N HCl, the precipitate was collected by centrifugation and lyophilized. 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 with chloroform-methanol-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 a Sabouraud agar culture of *Saccharomyces cerevisiae* and incubated for 2 days at 28°C, bioautograms revealed the presence of iturin A in all the crude antibiotic preparations. In addition, two new antifungal compounds iturin D and iturin E were detected; they were about 20-fold less abundant than iturin A.

A further purification of iturin D and iturin E was made by preparative thin-layer chromatography on silica gel in the same solvent.

Biological Properties

The antifungal activities of iturins D and E were determined in Sabouraud medium containing increasing amounts of antibiotic. MIC values are given in Table 1.

Iturin D and iturin E showed a strong antifungal activity against yeasts and fungi; they did not exhibit any antibacterial activity.

Chromatographic and Spectroscopic Characterization

Iturins D and E are colorless powders; they gave a negative reaction with ninhydrin and a positive reaction with Pauly reagent.

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

Both products were tested by TLC on Silica gel 60 in various solvent systems. Their R_f are summarized in Table 2. Iturins D and E are distinguishable from other antibiotics of the iturin group; bacillomycins D, F, L and mycosubtilin.

The presence of a free carboxyl group in iturin D and the absence of such a group in iturin E were suggested by paper electrophoresis; at pH 8.0 iturin D moved toward anodic compartment while

Table 1. Antifungal activity of iturins D and E.

Microorganisms	MIC ($\mu\text{g/ml}$)	
	Iturin D	Iturin E
<i>Saccharomyces cerevisiae</i>	15	30
<i>Candida albicans</i>	45	30
<i>C. tropicalis</i>	>100	>100
<i>Botrytis cinerea</i>	30	10
<i>Stemphylium radicinum</i>	45	40
<i>Mycosphaerella pinodes</i>	>100	>100
<i>Fusarium oxysporum</i>	30	40

The minimal inhibitory concentrations (MICs) were determined by the agar dilution method. Yeasts and fungi were assayed in Sabouraud medium. MICs were measured after 48 hours of incubation at 28°C 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	R _f		
	Solvent A	Solvent B	Solvent C
Iturin A	0.35	0.53	0.45
Iturin D	0.18	0.35	0.34
Iturin E	0.60	0.77	0.83
Bacillomycin D	0.21	0.40	0.42
Bacillomycin F	0.47	0.45	0.63
Bacillomycin L	0.16	0.38	0.38
Mycosubtilin	0.26	0.48	0.45

Solvent A: Chloroform-methanol-water (65:25:4).

Solvent B: Butanol-acetone-water (4:6:1).

Solvent C: Chloroform-dimethylformamide-water (25:22:3).

iturin E did not migrate.

Analysis of α -Amino Acids

Iturins D and E were hydrolyzed by 6 N HCl at 150°C for 8 hours. The hydrolysates were extracted with chloroform, a lipidic part and a water-soluble part were obtained.

The water-soluble amino acids were analyzed by TLC on cellulose powder in 2-propanol - pyridine - acetic acid - water (40:40:5:20) and identified in both antibiotics as aspartic acid, glutamic acid, proline, serine and tyrosine. Quantitative analysis of the dinitrophenyl derivatives⁹⁾ gave the following molar ratios; Asp_{3.2}, Glu₁, Pro_{0.8}, Ser_{0.9}, Tyr_{0.8}, for iturin D and Asp_{3.1}, Glu₁, Pro_{0.9}, Ser_{0.7}, Tyr_{0.7}, for iturin E.

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 RUSSELL¹⁰⁾, the chromatograms showed a spot with a Rf identical to that of β -amino acids obtained from iturin A (Rf 0.63).

The structure of these β -amino acids was determined by gas chromatography of the *N*-trifluoroacetylmethyl esters in comparison with the derivatives of β -amino acids from iturin A¹¹⁾.

Quantitative analysis showed that the β -amino acids of iturins D and E are identical to those of iturin A produced in the same medium (Table 3). Three major components; 3-amino tetradecanoic acid, 3-amino 13-methyl tetradecanoic acid and 3-amino 12-methyl tetradecanoic acid were identified. The amounts of the different β -amino acids varied according to the culture medium.

Determination of Lipid-peptide Linkage

Iturins D and E 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

Table 3. Analysis of β -amino acids of iturins.
(The values are given in % of total β -amino acids.)

Medium ^a	Antibiotic	β -Amino acids ^b				
		<i>n</i> -C ₁₄	<i>iso</i> -C ₁₅	<i>anteiso</i> -C ₁₅	<i>iso</i> -C ₁₆	<i>n</i> -C ₁₆
I	Iturin A	52	26	12	7	2
	Iturin D	55	25	11	7	1
	Iturin E	53	22	13	9	2
II	Iturin A	24	40	20	9	6
	Iturin D	26	42	19	7	5
	Iturin E	24	42	20	8	5
III	Iturin A	15	60	18	6	1
	Iturin D	14	58	20	7	<1
	Iturin E	16	61	17	5	<1

^a Medium I: Brain-heart infusion (37 g/liter), medium II: of LANDY *et al.*⁹⁾, medium III: Biotrypcase medium (10 g/liter).

^b *n*-C₁₄: 3-Amino tetradecanoic acid, *iso*-C₁₅: 3-amino 13-methyl tetradecanoic acid, *anteiso*-C₁₅: 3-amino 12-methyl tetradecanoic acid, *iso*-C₁₆: 3-amino 14-methyl pentadecanoic acid, *n*-C₁₆: 3-amino hexadecanoic acid.

ninhydrin according to RUSSELL¹⁰). They showed, beside the β -amino acids, a further ninhydrin positive spot (Rf 0.4) which co-migrated with the peptides Ser \rightarrow β -amino acids, isolated in the same conditions from the hydrolysates of iturin A.

The peptides obtained from iturins D and E were hydrolyzed with 6 N HCl at 150°C for 8 hours, they contained serine and β -amino acids. Moreover these peptides were dinitrophenylated with 2,4-dinitrofluorobenzene and hydrolyzed with 6 N HCl at 150°C for 8 hours: Dinitrophenyl (DNP)-serine and β -amino acids were identified. Thus iturins D and E contain the same sequence Ser \rightarrow β -amino acid as iturin A.

Fast Atom Bombardment Mass Spectrometry (FAB-MS)

The FAB-MS of iturin D displayed three major (M+H)⁺ peaks at m/z 1,044, 1,058 and 1,072, the differences of 14 mass units being due to the presence of the homologous C₁₄, C₁₅ and C₁₆ β -amino acids. The corresponding (M+Na)⁺ peaks were observed at m/z 1,066, 1,080, 1,094 (Fig. 2) when the spectra were run in the presence of NaCl.

In the case of iturin E, the FAB-MS showed three (M+H)⁺ peaks at m/z 1,058, 1,072 and

Fig. 2. Na-Cationized FAB-MS of iturin D showing the (M+Na)⁺ peak region.

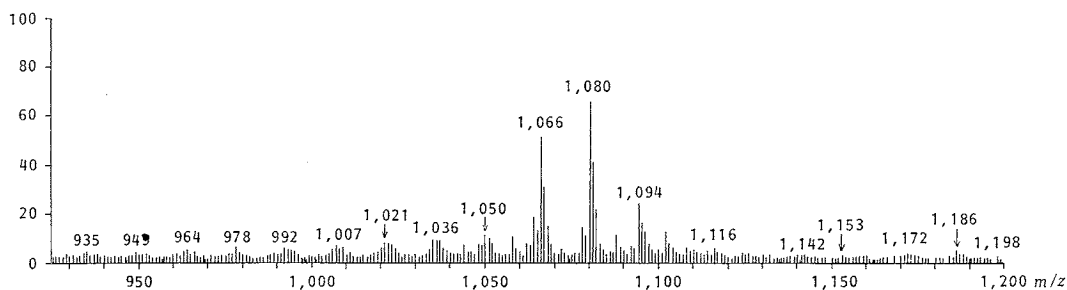
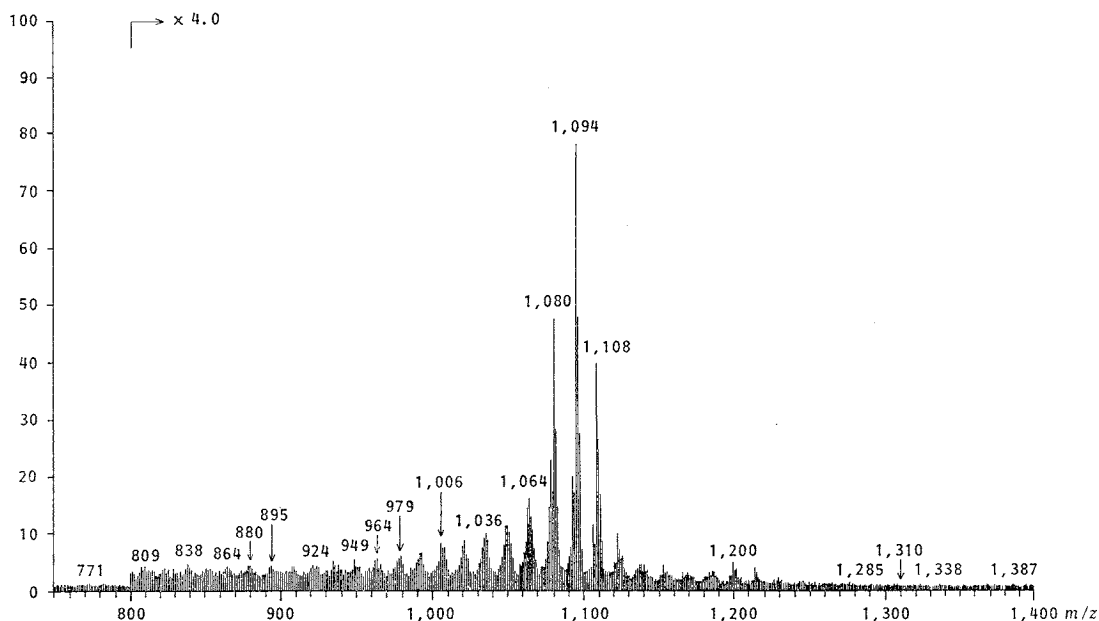


Fig. 3. Na-Cationized FAB-MS of iturin E showing the (M+Na)⁺ peak region.



1,086 and, in the presence of NaCl, the homologous peaks $(M+Na)^+$ at m/z 1,080, 1,094 and 1,108 (Fig. 3) corresponding to $M_r=1,057, 1,071$ and $1,085$, *i.e.* 14 mass units higher than the values found for iturin D.

Structure of Iturins D and E

The formula $C_{48}H_{74}N_{12}O_{14}$, $M_r=1,042$, and $C_{49}H_{76}N_{12}O_{14}$, $M_r=1,056$ for iturin A homologues corresponded to the following composition $\beta AA_{14,15}^*$, Asn₃, Gln₁, Pro₁, Ser₁, Tyr₁⁴⁾. The molecular weights 1,043, 1,057, found for iturin D are one mass unit higher than the values obtained for iturin A and correspond to the formula $C_{48}H_{73}N_{11}O_{15}$ and $C_{49}H_{75}N_{11}O_{15}$ as in the case of iturin C⁷⁾, *i.e.* one carboxamide group of asparagine or glutamine residue must be replaced by a carboxyl group. In iturin E the molecular weights given by the mass spectra correspond to the formula $C_{48}H_{75}N_{11}O_{15}$ and $C_{50}H_{77}N_{11}O_{15}$, *i.e.* a methyl ester must substitute the carboxyl group of iturin D.

In accordance with these results the electrophoretic behavior of iturin D in an alkaline buffer was that of an anionic compound while iturin E was neutral (see above).

The total sequence of these antibiotics has not been determined on account of the very small available amounts but their sequences are likely to be identical to those of iturins A and C. In fact, it does not seem likely that one strain of *Bacillus subtilis* would produce peptidolipidic antibiotics having the same composition but different sequences. One Asn or Gln residue of iturin A (Fig. 1) is replaced by an Asp or Glu residue in the case of iturin D and by an Asp-OCH₃ or Glu-OCH₃ residue in the case of iturin E.

It is not possible to claim that iturins D and E are, or are 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 asparagine or glutamine residue of iturin A was hydrolyzed giving rise to the aspartyl or glutamyl residue of iturin D. Similarly, the methyl ester found in iturin E might have been formed during the methanol extraction of the antibiotic.

However, an interesting result is the antifungal activity of iturins D and E. Iturin C, another companion of iturin A, has been previously described; it is devoided of antifungal activity. As iturin D, it differs from iturin A by the presence of a free carboxyl group⁷⁾. The position of this group must be different in both antibiotics as iturins C and D have not similar migrations in TLC. Thus the position of the free carboxyl group must be essential for the biological activity of iturin antibiotics.

Acknowledgments

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* $\beta AA_{14,15}$ is a C₁₄ or C₁₅ β -amino acid.

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