ANTIFUNGAL ACTIVITY UPON *SACCHAROMYCES CEREVISIAE* OF ITURIN A, MYCOSUBTILIN, BACILLOMYCIN L AND OF THEIR DERIVATIVES; INHIBITION OF THIS ANTIFUNGAL ACTIVITY BY LIPID ANTAGONISTS

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The antifungal activity of three antibiotics of the iturin group: iturin A, mycosubtilin, bacillomycin L and of eleven methylated and acetylated derivatives of these antibiotics was tested upon *Saccharomyces cerevisiae*. The lowest MIC values were found for natural antibiotics. The substitution of polar groups diminished the antifungal activity.

Various lipids, sterols, fatty acids, fatty acid methyl esters and phospholipids were tested as inhibitors of the antifungal activity of iturin A, mycosubtilin and bacillomycin L. Cholesterol was the strongest inhibitor upon the three antibiotics; ergosterol, oleic acid and *cis*-vaccenic acid were less potent inhibitors. Among phospholipids, phosphatidyl choline inhibited bacillomycin L and iturin A while diphosphatidyl glycerol inhibited bacillomycin L and mycosubtilin. The inhibitory effect appeared to be dependent on the nature of both the hydrophilic group and the fatty acid part of phospholipids.

Three antibiotics of the iturin group were isolated from various strains of *Bacillus subtilis*[1]. Their structures were determined[2,3,4]: A lipid-soluble β-aminoacid[5] is linked to a peptide which contains D and L α-aminoacids to form a cyclic peptidolipid (Fig. 1). These antibiotics exhibit a restricted antibacterial activity against some *Micrococcus* and *Sarcina* strains and a strong antifungal activity against a wide variety of fungi and yeasts[6]. Recently some methyl and acetyl derivatives of these antibiotics were prepared and their antibacterial activity upon *Micrococcus luteus* was reported in comparison with the activity of natural antibiotics[7,8,9]. Recent studies on the mode of action of these antibacterial compounds show variable lytic activities upon protoplasts from *M. luteus*[7,8,10]. These results could suggest an interaction between these antibiotics and some lipid components of the bacterial cytoplasmic membrane. If such an interaction occurs the antibiotic activity should be modified by addition of lipids to the antibiotic. The present work reports the antifungal activities of the antibiotics of iturin group and of their derivatives upon *Saccharomyces cerevisiae* and the effect of various lipids upon the antifungal activity of the three natural antibiotics.

Materials and Methods

Lipids, antibiotics and derivatives

Cholesterol and ergosterol were purchased from Prolabo (France); dipalmitoyl phosphatidyl ethanolamine from Fluka (Switzerland); cholesterol methyl ether, cholesterol acetate, cholesterol stea-

rate, fatty acids, fatty acid methyl esters, dipalmitoyl phosphatidic acid from Sigma (U.S.A.); phosphatidyl glycerol from egg yolk, phosphatidyl inositol from soybean, phosphatidyl serine from bovine brain, diphosphatidyl glycerol from bovine heart were purchased from Sigma (U.S.A.); phosphatidyl choline was prepared from egg yolk according to SINGLETON *et al.*[11]

Iturin A was prepared as described previously[5]. Mycosubtilin was a gift of Dr. H. B. WOODRUFF, Merck and Company, Rahway, New Jersey, U.S.A., bacillomycin L was a gift of Dr. G. H. WARREN, Wyeth Institute of Applied Biochemistry, West Chester, Pennsylvania, U.S.A. Methyl and acetyl derivatives of iturin A and of mycosubtilin, methyl derivatives of bacillomycin L were prepared as described previously[9].

Determination of MIC values

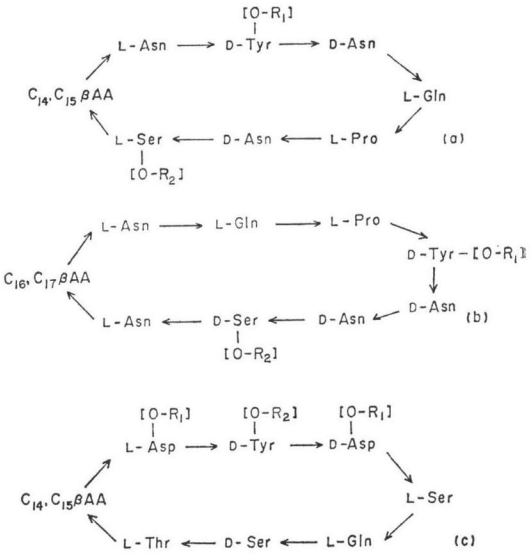
The minimum inhibitory concentration (MIC) was determined by the dilution method in liquid medium. Various quantities of antibiotics were added to a culture of *Saccharomyces cerevisiae* (200 μl). Absorbance: 0.55; culture medium composition: glucose 40 g, peptone (Biotrypcase, Bio Mérieux) 10 g, yeast extract (Bio Mérieux) 2 g in 1 liter, pH 7.2. The growth was observed after 24 hours at 28°C. When growth occurred a sediment was observed in the bottom of the tube, a total inhibition of the growth suppressed the sediment. All experiments were made in duplicate.

Fig. 1. Structure of antibiotics.

(a) Iturin A: $R_1=R_2=H$; compound I: $R_1=CH_3$, $R_2=H$; compound II: $R_1=COCH_3$, $R_2=H$; compound III: $R_1=H$, $R_2=COCH_3$; compound IV: $R_1=R_2=COCH_3$.

(b) Mycosubtilin: $R_1=R_2=H$; compound V: $R_1=CH_3$, $R_2=H$; compound VI: $R_1=COCH_3$, $R_2=H$; compound VII: $R_1=H$, $R_2=COCH_3$; compound VIII: $R_1=R_2=COCH_3$.

(c) Bacillomycin L: $R_1=R_2=H$; compound IX: $R_1=CH_3$, $R_2=H$; compound X: $R_1=R_2=CH_3$; compound XI: $R_1=H$, $R_2=CH_3$.



Determination of antagonists

Each test tube contained the antibiotic at the minimum inhibitory concentration and various concentrations of lipids. To this mixture was added a culture of *Saccharomyces cerevisiae* (200 μl), absorbance: 0.55 and the growth was observed after 24 hours at 28°C.

Results

MIC Determination

The MIC values of antibiotics and of their derivatives are given in Table 1. The most active antibiotic is mycosubtilin, MIC=10 μg/ml; iturin A and bacillomycin L have a good antifungal activity, MIC=30 μg/ml, while all the derivatives have much smaller activities. The substitution of tyrosyl residue by a methyl or by an acetyl group has a strong inhibitory effect upon the antifungal activity of iturin A, mycosubtilin and bacillomycin L. MIC values have not been determined for these derivatives but they are more than 6 fold the MIC values of natural antibiotics. The esterification of carboxyl groups of bacillomycin L and the acetylation of seryl group of mycosubtilin and of iturin A increases to 4 fold the MIC values of natural antibiotics.

Role of Lipids upon Antifungal Activities

Various lipids, sterols, fatty acids and phospholipids were tested for their antagonistic action

Table 1. Activity of iturin A, mycosubtilin, bacillomycin L and their derivatives on *Saccaromyces cerevisiae*.

	Concentration (μg/ml)							
	1 μg	5 μg	10 μg	20 μg	30 μg	60 μg	120 μg	180 μg
Iturin A	+	+	+	+	−	−	−	−
O-Methyltyrosine-iturin A (compound I)	+	+	+	+	+	+	+	+
Acetyltyrosine-iturin A (compound II)	+	+	+	+	+	+	+	+
Acetylserine-iturin A (compound III)	+	+	+	+	+	±	−	−
Diacetyl iturin A (compound IV)	+	+	+	+	+	+	+	+
Mycosubtilin	+	+	−	−	−	−	−	−
O-Methyltyrosine-mycosubtilin (compound V)	+	+	+	+	+	+	+	+
Acetyltyrosine-mycosubtilin (compound VI)	+	+	+	+	+	+	+	+
Acetylserine-mycosubtilin (compound VII)	+	+	+	±	−	−	−	−
Diacetyl mycosubtilin (compound VIII)	+	+	+	+	+	+	+	+
Bacillomycin L	+	+	+	+	−	−	−	−
Bacillomycin L dimethylester (compound IX)	+	+	+	+	+	±	−	−
O-Methyltyrosine-bacillomycin L dimethylester (compound X)	+	+	+	+	+	+	+	+
O-Methyltyrosine-bacillomycin L (compound XI)	+	+	+	+	+	+	+	+

Symbols: + growth, − no growth, ± restricted growth.

upon antifungal activity of iturin A, mycosubtilin and bacillomycin L. The results are summarized in Table 2.

Cholesterol is a strong inhibitor for all antibiotics while methyl ether, acetyl and stearyl esters of cholesterol have no inhibitory activity. Ergosterol shows a lesser action than cholesterol upon the three antibiotics.

In the group of fatty acids, oleic acid and *cis*-vaccenic acid have an inhibitory activity which is greatly lost by esterification. The results obtained with phospholipids are quite dependent on the nature of these phospholipids. Phosphatidyl choline has but a little activity upon iturin A and a stronger activity upon bacillomycin L; phosphatidyl serine and diphosphatidyl glycerol have an intermediate activity upon bacillomycin L and mycosubtilin.

Discussion

The antifungal activities of iturin A, mycosubtilin and bacillomycin L upon *Saccharomyces cerevisiae* are much stronger than their antibacterial activities upon *Micrococcus luteus*. In previous work the inhibition of growth of *M. luteus* was reported as 70~80% at 60 μg/ml of antibiotics[9]; the inhibition of growth of *S. cerevisiae* was total at 10 μg/ml for mycosubtilin, 30 μg/ml for iturin A and bacillomycin L. The antifungal activity was reduced when antibiotics were substituted by acetyl or methyl groups; thus the methylation of the carboxyl groups of bacillomycin L, the acetylation of seryl group of iturin A and of mycosubtilin greatly diminished the antifungal activity. However the most drastic effect is observed when the tyrosine residue is substituted: no activity was observed in the concentration range which have been tested. As it was reported for the antibacterial activity[9], it seems that phenolic group of the tyrosine residue is essential for the antifungal activity.

Table 2. Antagonisation of antifungal activity of iturin A, mycosubtilin and bacillomycin L by lipids.

Lipid	Antibiotic Ratio antibiotic / lipid (in weight)											
	Iturin A				Mycosubtilin				Bacillomycin L			
	1/1	1/2	1/4	1/6	1/1	1/2	1/4	1/6	1/1	1/2	1/4	1/6
Cholesterol	+	+	+	+	+	+	+	+	+	+	+	+
Cholesterol methyl ether	−	−	−	−	−	−	−	−	−	−	−	±
Cholesterol acetate	−	−	−	−	−	−	−	−	−	−	−	−
Cholesterol stearate	−	−	−	−	−	−	−	−	−	−	−	−
Ergosterol	−	−	+	+	−	−	±	±	±	±	+	+
Elaidic acid	−	−	−	−	−	−	−	−	−	−	−	−
Elaidic methyl ester	−	−	−	−	−	−	±	+	−	−	−	−
Petroselinic acid	−	−	−	+	−	−	−	−	−	±	+	+
Petroselinic methyl ester	−	−	−	−	−	−	−	+	−	−	−	−
Oleic acid	−	−	+	+	−	±	+	+	−	±	+	+
Oleic methyl ester	−	−	−	−	−	±	±	±	−	−	±	±
cis-Vaccenic acid	−	−	+	+	±	+	+	+	+	+	+	+
cis-Vaccenic methyl ester	−	−	−	−	−	−	−	−	−	−	−	−
trans-Vaccenic acid	−	−	−	−	−	−	−	−	−	−	−	+
trans-Vaccenic methyl ester	−	−	−	−	−	−	+	+	−	−	−	−
Phosphatidyl choline	−	−	±	±	−	−	−	−	±	+	+	+
Phosphatidyl ethanolamine	−	−	−	−	−	−	−	−	−	−	−	−
Phosphatidyl glycerol	−	−	−	−	−	−	−	±	−	−	−	−
Phosphatidyl serine	−	−	−	−	−	−	−	+	−	−	+	+
Phosphatidyl inositol	−	−	−	−	−	−	−	−	−	−	±	±
Diphosphatidyl glycerol	−	−	−	−	±	±	±	+	−	±	+	+
Phosphatidic acid	−	−	−	±	−	−	−	−	−	−	−	−

− No growth of *Saccharomyces cerevisiae*, no sediment in the test tube
+ Normal growth giving a sediment in the test tube
± Restricted growth giving a small sediment in the test tube

Previous works on a polypeptidic antibiotic, mycobacillin, had already shown a reduction of the antifungal activity when the natural antibiotic was acetylated or methylated[11,12]. The importance of the tyrosine residue in the antimicrobial activity of iturin A, mycosubtilin and bacillomycin L could suggest an interaction of the antibiotic with polar lipids of the microbial cell membrane. In this case, an inhibition of the antimicrobial activity by some lipids could be expected.

The reversal of antifungal activity by sterols, fatty acids and phospholipids was reported for several polyene antibiotics[13,14] and for the cyclic polypeptide antibiotic mycobacillin[15,16,17]; cholesterol and lecithin were found to be antagonists of mycobacillin upon *Aspergillus niger*[17]. An antagonistic effect was observed with oleic acid while petroselinic acid, elaidic acid and *trans*-vaccenic acid did not reverse growth inhibition by mycobacillin[17].

We have tested a large variety of lipids and our results lead to the following conclusions: cholesterol is the more potent antagonist of the three antibiotics, ergosterol is also an antagonist but at higher concentrations. This property must be tightly related to the hydroxyl group of sterols as methyl ether and esters of cholesterol do not have any inhibitory effect. A polar interaction between the alcohol group of sterols and the phenolic group of antibiotics agrees with previous results showing a direct participation of tyrosine residue in the antibacterial activity[9].

Fatty acids have variable effects. The most active are *cis*-unsaturated acids, oleic acid and *cis*-vaccenic acid which have an inhibitory effect upon the three antibiotics. However another *cis*-unsaturat-

ed fatty acid, petroselinic acid, has a more selective effect on bacillomycin L, a smaller effect on iturin A and no effect on mycosubtilin, thus the position of the insaturation in the hydrocarbon chain plays a role in the inhibitory activity of fatty acids. The methyl esters of *cis*-unsaturated fatty acids have a smaller activity or no activity. *Trans*-unsaturated fatty acids do not show any inhibitory action except a slight one upon bacillomycin L. Thus the spatial structure of acids is an important factor for an inhibition of antibiotic activity; one can suppose an interaction between the carboxyl group of the acid and the polar groups of antibiotics, this hypothesis is supported by the fact that bacillomycin L, the most polar of the three antibiotics, displays the strongest inhibition by unsaturated acids. A *cis*-configuration corresponds to a more compact molecule and the hydrocarbon chain of fatty acids could wrap up the antibiotic and inhibit the antifungal activity.

The effect of phospholipids is more complex as both the nature of fatty acids and the nature of the polar molecule substituting the phosphate group can be involved in the interaction with antibiotics. Phosphatidyl choline is the best inhibitor of bacillomycin L but does not interact with mycosubtilin. A lesser inhibitory effect is observed with phosphatidyl serine, phosphatidyl inositol and diphosphatidyl glycerol but these phospholipids display no activity upon iturin A.

In conclusion peptidolipidic antibiotics react strongly with cholesterol and to a lesser degree with other lipidic components of the cytoplasmic membrane: *cis*-fatty acids and phospholipids. The lipid-antibiotic interaction must certainly be one of the factors involved in the activity of antibiotics on cytoplasmic membranes.

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