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Interactions of the lipopeptide antifungal iturin A with lipids in mixed monolayers

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The miscibility and the interactions of the antifungal lipopeptide iturin A with lipids, DMPC and cholesterol, are studied in monolayers at the air/water interface and a comparison of the respective behaviour of iturin A and the biologically inactive methylated derivative MeTyr-iturin A is made. Each lipopeptide is miscible with anyone of the lipids. This behaviour is revealed by the dependence of the transition pressure upon composition and by deviations from the additivity rule of the mean molecular area. The thermodynamic properties of the mixed systems are studied by the method of Goodrich. The mixed monolayers are always more stable than the two separate components, subsequently there are interactions between the components. However, the excess free energy of mixing ΔG_m^{ex} is positive for the iturin A/DMPC system which is an indication that the interactions between lipopeptide and lipid molecules are weaker than the interactions between the pure components themselves. This is compatible with the presence of self-associated lipopeptide molecules. However, ΔG_m^{ex} is highly negative for the iturin A/cholesterol system giving evidence of the formation of a specific complex between iturin A and cholesterol which is not the case with the methylated derivative. These data are analysed in connection with previous results concerning the pore-forming properties of these lipopeptides and the lack of biological activity of MeTyr-iturin A.

Introduction

Iturins are cyclic lipopeptide antibiotics produced by *Bacillus subtilis* [1] which exhibit an antifungal activity depending on the primary structure (Fig. 1) of the peptide cycle [2–4]. The D-Tyr residue which is present in position 2 in all iturins seems to play a crucial role since its methylation dramatically decreases the antibiotic properties [2,3]. The biological activity of iturins is related to their interactions with cytoplasmic membranes [5] the increased permeability of which leads to a leakage of intracellular K^+ ions [6]. We have demonstrated that these lipopeptides induce ion conducting pores in planar membranes and their characteristics also depend on both the primary structure of the peptide cycle and the lipid composition of the membrane [7–9]. In addition, we have shown that the methylation of the D-Tyr residue modified the interaction of iturin A with lipid vesicles [10]. In the present work we compare the behaviour of iturin A and MeTyr-iturin A in mixed monolayers with phospholipid and cholesterol.

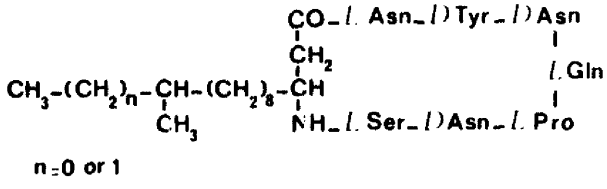

Fig. 1. Primary structure of iturin A.

Materials and Methods

Iturin A was extracted and purified as described previously [11] and the *O*-methyl derivative was prepared according to Ref. 2. DMPC was from Calbiochem. Cholesterol from Prolabo was recrystallized three times from methanol. Pyridin, hexafluoroisopropanol (1,1,1, 3,3,3-hexafluoropropanol [2]), hexane and absolute ethanol were from Merck. Cyclohexane was from Fluka. Pure water was obtained from a Millipore (Milli-Q) apparatus.

Abbreviations: DMPC, dimyristoylphosphatidylcholine; MeTyr-iturin A, *O*-methyltyrosine iturin A.

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Monolayer experiments were done using a Langmuir film balance system previously described [12]. The lipopeptide/lipid mixtures were dissolved in the following solvent system: pyridine/cyclohexane/ethanol/hexafluoroisopropanol/hexane (56:13:13:10:8, by vol.) and spread at the air/water interface of pure distilled water with a 50 µl Hamilton microsyringe. After the film stood to equilibrate at a large molecular area for at least 5 min, it was compressed at a rate less than 0.05 $nm^2 \cdot mol \cdot^{-1} min^{-1}$ in order to record the isotherm (surface pressure–area) curves. The temperature in the trough was kept constant at 20 °C by circulating water.

Results

Lipopeptide / DMPC mixed monolayer systems

Typical isotherm (π–A) curves of the pure components and their mixtures are given at different molar fractions, $x_i = n_i/(n_i + n_l)$ (n: number of molecules at the interface; the subscripts i and l refer, respectively, to iturin and lipid), of iturin A or MeTyr-iturin A (Fig. 2). The monolayer characteristics of the pure lipopeptides have already been described [12]. The isotherm curve of iturin A monolayer presents a transition region the significance of which is not yet really understood. The surface pressure π_t at the beginning of this transition increases with the DMPC amount in the monolayer up to $x_i = 0.4$. When the molar fraction of iturin A in the mixed monolayer is lower than 0.4 the (π_t–ln x_i) plot presents a discontinuity. The linear plot (coefficient of correlation $r = 0.997$) has then a slope of 6.24 $mN \cdot m^{-1}$. For ideal miscibility behaviour, the slope should be $kT/A_t = 6.32$ $mN \cdot m^{-1}$ (k, Boltzmann constant; T, 293° K; $A_t = 66 \cdot 10^{-20} m^2$, molecular area of pure iturin A at π_t) (Fig. 2a, inset). The isotherm curve of MeTyr-iturin A presents an inflection at 33 $mN \cdot m^{-1}$ that is taken as the π_t value. For the mixed MeTyr-iturin A-DMPC monolayers, the variation of π_t versus x_i are more complex than in the case of iturin A. A small amount of DMPC in the monolayer decreases extensively the value of π_t that is only 22 $mN \cdot m^{-1}$ at $x_i = 0.75$. Then π_t increases up to $x_i = 0.33$ to decrease again when the molar fraction of the lipopeptide is less than 0.33 (Fig. 2b, inset). The values of the mean molecular area, A, of the mixed films (data taken from the isotherms) are plotted against the molar fraction x_i of the lipopeptide, at several surface pressures, in order to examine eventual deviations from the additivity rule [13] as an indication of the miscibility of the components. The mean molecular areas in mixed films of iturin A with DMPC show positive deviations from the additivity rule at all surface pressures (Fig. 3a). Conversely, the deviations are negative in mixed monolayers of MeTyr-iturin A with DMPC (Fig. 3b).

To study the spontaneity of the miscibility process and the interactions of the molecules in the mixed films, the thermodynamic properties of the systems have been analysed by calculating the excess free energy of mixing

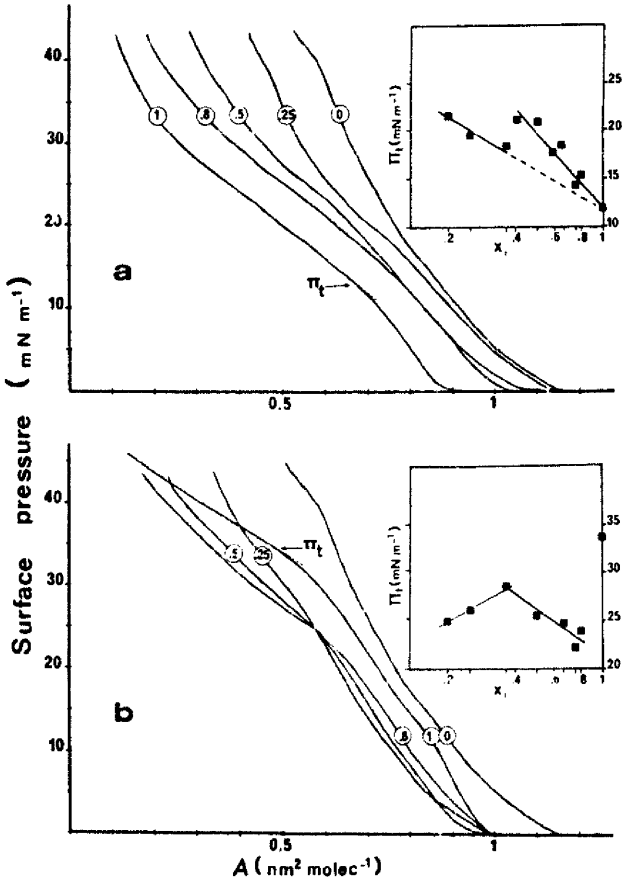

Fig. 2. Isotherm curves of mixed films of iturins and DMPC. The molar fraction x_i is indicated on the curves: (0) pure DMPC, (1) pure iturin. Insets: variation of the transition pressure π_t as a function of composition. (a) Iturin A, (b) MeTyr-iturin A.

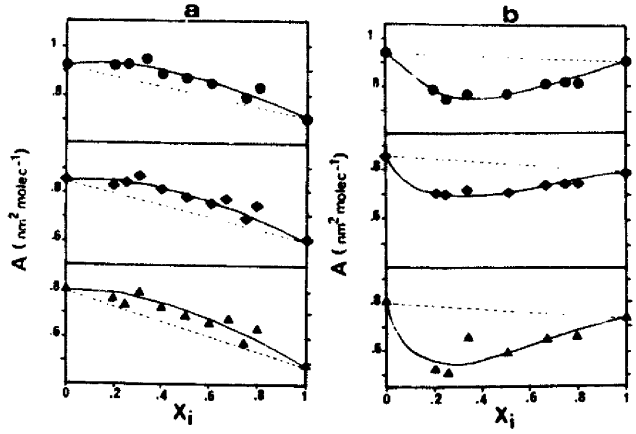

Fig. 3. Mean molecular areas of mixed films of iturins and DMPC as a function of composition at various surface pressures. ●, 10 $mN \cdot m^{-1}$; ◆, 15 $mN \cdot m^{-1}$; ▲, 20 $mN \cdot m^{-1}$. — — — represent the additivity rule values. (a) Iturin A, (b) MeTyr-iturin A.

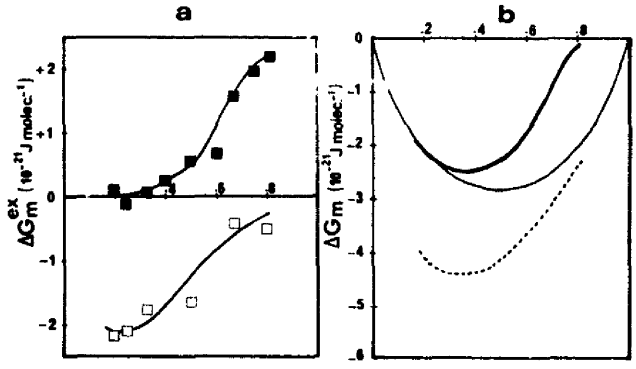

Fig. 4. Free energy of mixing as a function of composition for iturin/DMPC mixed films. (a) Excess free energy of mixing: ■, iturin A; □, MeTyr-iturin A. (b) Total free energy of mixing: ———, iturin A; — — —, MeTyr-iturin A. The fine line represents the free energy of mixing of an ideal mixture.

ΔG_m^{ex} by the relationship of Goodrich [13,14]:

$$\Delta G_m^{ex} = \int_0^{\pi} A_{12}\,d\pi - x_1\int_0^{\pi} A_1\,d\pi - x_2\int_0^{\pi} A_2\,d\pi$$

where A is the mean molecular area, x is the molar fraction, subscripts 1, 2, and 12 refer, respectively, to pure components 1 and 2 and to their mixtures.

The values of ΔG_m^{ex} were obtained by integrating under π–A curves of the mixture and of each the pure component (upper limit of integration: 30 mN · m^{-1}). The results are shown in Fig. 4a. For iturin A/DMPC systems, ΔG_m^{ex} is positive on the whole molar fraction range. Conversely, for MeTyr-iturin A/DMPC systems, ΔG_m^{ex} is always negative. For the two mixed systems ΔG_m^{ex} increases with the amount of lipopeptide in the monolayer. When the amount of iturin A is low (x_i < 0.4), ΔG_m^{ex} is near zero.

However if we examine the values of the total free energy change $\Delta G_m = \Delta G_m^{ex} + \Delta G_m^{id}$ where $\Delta G_m^{id} = kT(x_1 \ln x_1 + x_2 \ln x_2)$ [13,14] (ΔG_m^{id}, free energy change for an ideal mixture; k, Boltzmann constant; T, absolute temperature) we can see that the ΔG_m values are always negative for the two lipopeptide/DMPC systems studied (Fig. 4b). We observe a minimum in the ΔG_m values at $x_i = 0.4$ for iturin A and at $x_i = 0.33$ for MeTyr-iturin A.

Lipopeptide / sterol mixed monolayers systems

Fig. 5 shows typical isotherm curves of iturin A/cholesterol and MeTyriturin A/cholesterol mixed monolayers. The transition pressure π_t does not vary extensively with x_i when the amount of iturin A in the monolayer is high ($x_i > 0.66$). But when the molar fraction of iturin A is lower, π_t increases abruptly and then we note the absence of any transition on the isotherm curves when x_i is less than 0.4 (Fig. 5a, inset). For the MeTyr-iturin A/cholesterol systems the value of π_t at $x_i = 0.8$ is very low (19.5 mN · m^{-1}) but subsequent

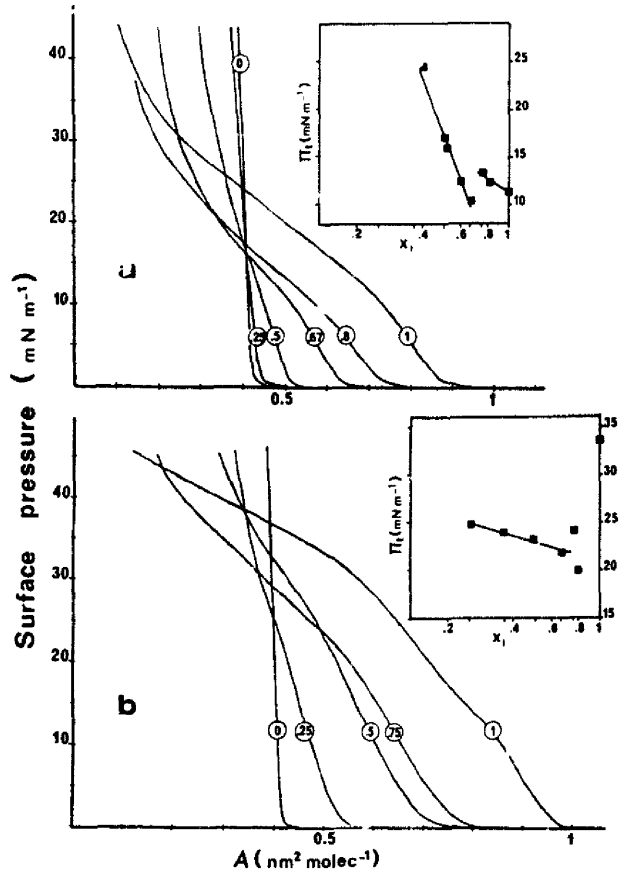

Fig. 5. Isotherm curves of mixed films of iturins and cholesterol. The molar fraction x_i is indicated on the curves: (0) pure cholesterol, (1) pure iturin. Insets, variation of the transition pressure π_t as a function of composition. (a) Iturin A, (b) MeTyr-iturin A.

increase of the amount of cholesterol leads to an increase of π_t (Fig. 5b, inset).

Fig. 6 shows the (A–x_i) plots at several surface pressures. For the two lipopeptide/cholesterol mixed

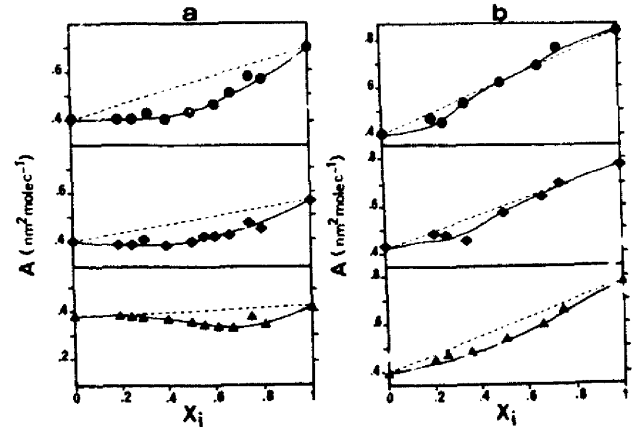

Fig. 6. Mean molecular areas of mixed films of iturins and cholesterol as a function of composition at various surface pressures. ●, 10 mN·m^{-1}; ◆, 15 mN·m^{-1}; ▲, 20 mN·m^{-1}; — — — represent the additivity rule values. (a) Iturin A, (b) MeTyr-iturin A.

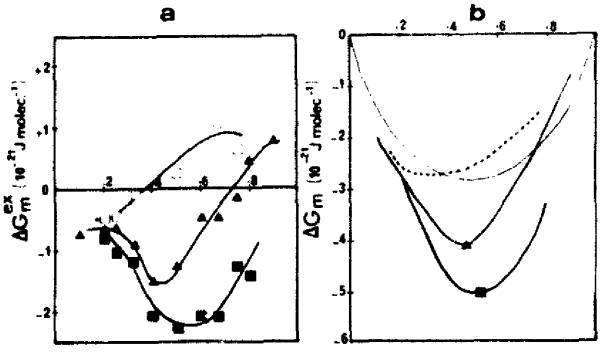

Fig. 7. Free energy of mixing as a function of composition for iturin/sterol mixed films. (a) Excess free energy mixing: ■, iturin A/cholesterol mixtures; ▲, iturin A/ergosterol mixtures; □, MeTyr-iturin A/cholesterol mixtures. (b) Total free energy of mixing: -■-, iturin A/cholesterol mixtures; -▲-, iturin A/ergosterol mixtures; — — —, MeTyr-iturin A/cholesterol mixtures. The fine line represents the free energy of mixing of an ideal mixture.

monolayers we observe negative deviations from the additivity rule at all surface pressures.

Fig. 7 shows the variations of the free energy of mixing versus the composition of the mixed sterol/lipopeptide monolayers. In the case of the methyl derivative, the values of the excess free energy of mixing ΔG_m^{ex} increase with the amount of lipopeptide in the monolayer (Fig. 7a). We observe negative values only below $x_i = 0.4$. However, the total free energy change ΔG_m is negative on the whole range of composition with a minimum near $x_i = 0.33$ (Fig. 7b). The behaviour of the iturin A/cholesterol mixed monolayers is very different. Indeed ΔG_m^{ex} is always negative. At the equimolecular ratio iturin A/cholesterol (1 : 1), the total free energy change ΔG_m is minimum. Its value is nearly twice that of an ideal mixed monolayer. The replacement of cholesterol by ergosterol in the mixed iturin A/sterol monolayer leads to an increase of the ΔG_m values although they are always negative with a minimum near the equimolecular ratio. However, the ΔG_m^{ex} values are positive above $x_i = 0.75$ (i.e., three molecules of iturin A for one of ergosterol).

Discussion

One important question that must be considered concerns the miscibility of lipopeptide and lipid molecules in the monolayer.

The dependence of the transition pressure π_t on the composition of the mixed lipopeptide/lipid monolayer is an indication that the compounds are at least partially miscible. The discontinuity in the (π_t–ln x_i) plots observed in mixed iturin A/DMPC monolayers at $x_i = 0.4$ could correspond to a sudden change in the mutual array of the two compounds when the amount of iturin A increases. Up to $x_i = 0.4$, the mixture behaves ideally.

The dramatical decrease of the transition pressure of MeTyr-iturin A as soon as lipid (DMPC or cholesterol) is put in the monolayer could be the sign of a change of either the orientation or the conformation of the lipopeptide at the air/water interface. For MeTyr-iturin A/DMPC systems, π_t is maximum at $x_i = 0.33$.

The absence of any detectable transition in the mixed iturin A/cholesterol monolayers rich in cholesterol ($x_i < 0.4$) could indicate that the two compounds are not only miscible but form an intimate complex [15]. The discontinuity observed in the (π_t–ln x_i) plots at high iturin A level can be interpreted too as a change in the bidimensional packing of the molecules. The disappearance of the transition pressure takes place only below $x_i = 0.25$ for mixed MeTyr-iturin A/cholesterol systems.

The miscibility in monolayer of iturin lipopeptides with lipids is confirmed by the deviations of the (A–x_i) plots from the additivity rule. In addition, this result is consistent with non ideality of the mixture and gives the evidence of steric perturbations due to interactions between the component molecules of the monolayers. In the case of DMPC/lipopeptides systems the sign of the deviation is not the same when iturin A is methylated. This in an indication that the mixed structures are of different natures.

But among all the properties of the mixed monolayers studied as a function of its composition in order to test ideality, the most generally accepted one is that which is based on the excess free energy of mixing ΔG_m^{ex} [16]. Using this criterion we can conclude that, in all cases, the lipopeptide/lipid system is non-ideal, i.e. there are privileged interactions between the monolayer components.

However, let us examine more carefully the different results obtained. ΔG_m^{ex} of the iturin A/DMPC system is near zero at low amount of lipopeptide ($x_i < 0.4$). Therefore the corresponding mixed monolayers (that contain less than two molecules of iturin A for three molecules of phospholipid) behave almost as ideal mixtures as deduced too from surface pressure values. The positive values of ΔG_m^{ex} above $x_i = 0.4$ reveal interactions between the pure component molecules themselves which are more important than mutual interactions between two components [17]. Then, the molecules of a component have a tendency to self associate. This is compatible with the formation of lipopeptide patches (bidimensional aggregates) in the monolayer as and when the amount of lipopeptide increases. This state is nevertheless more stable than the unmixed state and cannot be assimilated to a phase separation since the total free energy change ΔG_m is negative on the whole range of composition with a minimum at the iturin A/DMPC molar ratio 2 : 3 ($x_i = 0.4$). This suggests that the lipopeptide aggregates are in interaction with DMPC molecules to form mixed structures.

ΔG_m^{ex} of the MeTyr-iturin A/DMPC systems always is negative. This implies interactions between the methylated lipopeptide and the phospholipid molecules. The interaction is maximum at $x_i = 0.33$ according to the $(\Delta G_m - x_i)$ plot. We can then suppose the existence of a MeTyr-iturin A/DMPC 1:2 complex. We have also to point out that the monolayer composition $x_i = 0.33$ corresponds to the maximum observed in the $(\pi_i - \ln x_i)$ plots.

For iturin A/cholesterol systems ΔG_m^{ex} values are widely negative. This supposes strong interactions between the lipopeptide and the sterol molecules. In the case of the ergosterol, the interactions with iturin A are weaker, and above the lipopeptide/sterol molar ratio 3:1 the lipopeptide molecules reassemble to form bidimensional patches. For the two sterols the interactions are optimum near the stochiometric ratio lipopeptide/sterol 1:1. These results are consistent with the formation of an iturin A-sterol 1:1 complex more stable with cholesterol than with ergosterol. From the ΔG_m^{ex} values we can see, besides, that the interaction of the methylated lipopeptide with cholesterol is weak, and above the lipopeptide/cholesterol molecular ratio 2:3, the lipopeptide molecules tend to form 'patches'.

General conclusion

The main result of this work is that iturin lipopeptides interact with lipids in monolayers. The methylated iturin A interacts strongly with DMPC and forms a lipopeptide-DMPC 1:2 complex. At the opposite, the interaction of iturin A with DMPC is weak and the lipopeptide molecules tend to form bidimensional patches in the phospholipid layer. The presence of lipopeptide aggregates interacting with DMPC molecules, is consistent with previous findings [18]. Indeed, it has been shown using pulse fluorimetry that when iturin A micelles interact with DMPC vesicles, iturin A is fully incorporated in the lipid bilayer but self associated inside the membrane. In the same conditions, the MeTyr-iturin A aggregates are less stable. The presence of such lipopeptide aggregates in the lipid membranes have already been suggested to explain in part the biological activity of these compounds and specially their faculty of increasing membrane permeability. Such aggregate structures would be involved in the formation of ion conducting pores [7–9].

Another important result is the existence of a strong interaction between iturin A and cholesterol, leading to the formation of an equimolecular complex. Surprisingly, the interaction of iturin A with ergosterol is weaker than with cholesterol. As a matter of fact, iturin A has an antifungal power specially against yeasts whose membranes contain mainly ergosterol. However, it has been shown on a mutant strain of *Saccharomyces cerevisiae* whose membrane contains only cholesterol that the leakage of K^+ ions from the cell, induced by iturin A, is then more important than in the wild strain containing ergosterol [19]. Therefore the increased permeability of the membrane, induced by iturins is related partly to the nature of the sterol and probably to interactions between the lipopeptide and the sterol molecules. To support this view, we notice that the biologically inactive MeTyr-iturin A does not interact greatly with cholesterol. Can we see there one of the key of the lack of biological activity of MeTyr-iturin A? As a matter of fact, the formation of a complex with cholesterol has often been proposed to explain the antifungal properties of compounds as amphotericin B [20] or mycobacillin [21]. A model of association of iturin A with cholesterol can bring in the hydroxyl group of tyrosine and the hydroxyl group of the sterol as proposed for mycobacillin [21]. If actual, the methylation prevents this binding. It is also possible that the sterols interact with a peculiar conformation of iturin A, absent in MeTyr-iturin A structures. Indeed, we have shown by pulse fluorimetry of D-Tyr that iturin A has three conformers, the proportions of which are modified in aggregated molecules [18]. Then the active conformation of iturin A may be only in a low proportion in the methylated derivative.

In conclusion, the monolayer experiments have provided a useful technique to specify molecular associations between antifungal lipopeptides and lipids. They confirm unambiguously the existence of oligomeric structures of iturin A in mixture with phospholipids. They give evidence of the formation of a specific iturin A-cholesterol complex and, at the opposite, they point out the weaker interaction of the biologically inactive methylated iturin A with cholesterol. All these results allow us to suggest that the biological activity of iturins is the consequence of a whole set of phenomena including among them, but not exclusively, the presence of lipopeptide aggregates in the lipid layer and the formation of lipopeptide-sterol complexes. Furthermore we have to consider that a biological membrane is not made of only phospholipid or cholesterol but of a lipid mixture (and proteins). Therefore, we have now to study the iturin lipopeptides behaviour in a mixed phospholipid/sterol monolayer and the influence of other important parameters as monovalent or divalent ions [9].

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