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Interaction of odorous lactones with phospholipids: implications in toxicity towards producing yeast cells

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Abstract

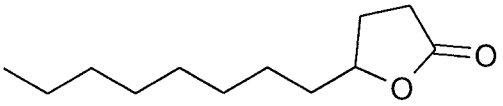
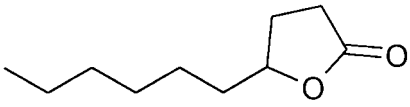
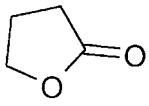
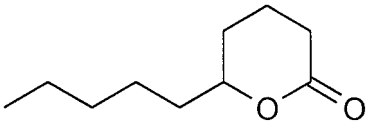
Some lactonic aroma compounds, that can be produced industrially by microorganisms, become toxic towards the producing cells as these compounds reach high concentrations in the culture medium. To determine the manner by which these metabolites may influence yeast physiology, the effects of four lactones (concentration range of 100 to 300 mg l^{-1}) on the growth of *Yarrowia lipolytica* and on the phase behaviour of deuterated dimyristoylphosphatidylcholine (DMPC-d27) were studied. The results showed that the hydrophobic lactones decrease the phase transition temperature (Tm) of DMPC-d27 bilayers and that Tm decrease (ΔTm) was related to the inhibitory action of the lactones on yeast growth (evaluated by the lag time). These results suggest that whatever the lactone, a ΔTm of at least 2.5 °C resulted in a total growth inhibition: this implicates the lactone-phospholipids interaction in the mechanism of yeast growth inhibition. The test used in the present study may be a predicting method to assess the *in vivo* action of potential membrane active compounds.

Introduction

Lactones, which are formed by intramolecular esterification within a hydroxy-acid, are widely distributed in fruits and fermented products. Some of these compounds have fruity odors (Table 1) and they have thus a commercial interest. Biotechnological processes have been developed in order to produce natural lactones, mainly by biotransformation of a long-chain fatty acid (ricinoleic acid) (Aguedo *et al.* 2000). For example, γ-decalactone, which has a peach-like odor, can be produced by yeast species such as *Sporidiobolus salmonicolor* or *Yarrowia lipolytica*. During the process, concentration of lactones in the biotransformation medium can reach several grams per litre, which makes the metabolites toxic towards the producing microorganisms. Antimicrobial properties of lactones have been described and a concentration-dependent inhibitory action on yeast growth has been reported (Endrizzi-Joran 1994, Feron *et al.* 1996). Odorous lactones, which are mainly 4- or 5-alkanolides, possess a hydrophobic character, allowing to suppose that their antimicrobial effect is based on the interaction of the molecule with cell membranes. In a previous study γ-decalactone was demonstrated to have a fluidising action *in vivo* on the membrane of *Y. lipolytica* (Aguedo *et al.* 2002).

In the present study, different lactones were chosen for their molecular structures (Table 1) that are related to that of γ-decalactone but differing in ring size or lateral chain length. Thus γ-dodecalactone, γ-decalactone and γ-butyrolactone possess the same lactone ring and differ for the lateral acyl chain. δ-Decalactone, which has the same number of carbon as γ-decalactone but with a 5-carbon lactone ring, was also tested. The influence of these compounds on the growth of the yeast, *Y. lipolytica*, has now been determined. Then, with the aim of characterising the way lactones may interact with cell membranes, the influence of the four lactones on the phase behaviour of

Table 1. Structure, sensorial properties (Dufossé *et al.* 1993) and octanol/water partition coefficients (Log P) (determined here according to the method of Rekker & Kort (1979), of the lactones used in this study.

Structure	Name	Odorant notes	Log P
	γ-Dodecalactone	Peach, butter, fatty	4.409
	γ-Decalactone	Peach, fatty, fruity	3.351
	γ-Butyrolactone	Butter, fetid acrid	0.247
	δ-Decalactone	Peach, oily, creamy	3.441

pure phospholipid bilayers were studied by means of Fourrier transform infrared spectroscopy (FTIR).

Materials and methods

Materials

γ-Butyrolactone, γ-decalactone, γ-dodecalactone and δ-decalactone were from Sigma. Dimyristoylphosphatidylcholine deuterated on the *sn*-2 acyl chain (DMPC-d27) (Avanti Polar Lipids, Alabaster, USA), was used without any purification.

Yeast cells growth

Yarrowia lipolytica W29 (ATCC20460; CLIB89) was grown at 27 °C, 140 rev/min in 500 ml baffled Erlenmeyer flasks containing 200 ml liquid medium containing per litre: 15 g glucose, 2.5 g NH_4Cl, 0.1 g yeast extract and salts as previously described (Pagot *et al.* 1998) and 150 or 300 mg lactone l^{-1}. Inoculation consisted of 6.5×10^6 cells ml^{-1} [= turbidity at 600 nm (OD_{600}) of 0.25]. Lag time corresponds to the time interval between inoculation and exponential cell growth.

Partition coefficients of the lactones

Octanol/water partition coefficients (Log P) of the lactones were determined theoretically according to the method of Rekker & Kort (1979), using the Molecular Modeling Pro software.

Preparation of samples

Dimyristoylphosphatidylcholine deuterated on the *sn*-2 acyl chain (DMPC-d27) was dissolved in chloroform at 20 mg ml^{-1} and used as the spreading solution in the typical amount of 150 μl. The films were prepared *in situ* over an IR-transparent ZnSe window (7 cm $\times$ 1 cm) after total evaporation of the solvent. The films were then covered with a special water-tight cell (Bruker, Karlsruhe, Germany) that allowed the introduction of γ-decalactone solutions with a glass syringe directly onto the phospholipid films. Hydration of the films was achieved at least 10 °C above the gel to liquid-crystalline transition temperature (20.3 °C). Phospholipids were maintained in these conditions for at least 1 h and then cooled to 12 °C by circulation of a cold fluid around the water-tight cell.

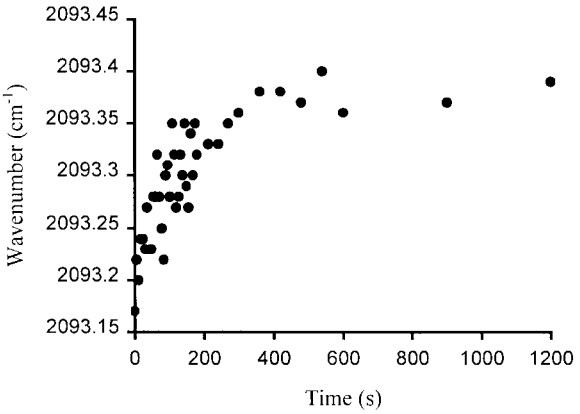


Fig. 1. Time dependence of CD_2 symmetric stretching vibration wavenumber after introduction of γ-decalactone onto DMPC-d27 film at 27 °C. Introduction of γ-decalactone (300 mg l^{-1}) was achieved at time zero second.

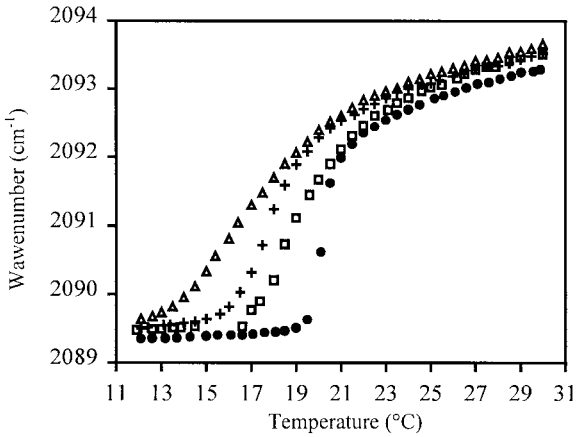


Fig. 2. Temperature dependence of the CD_2 symmetric stretching vibration wavenumber for pure DMPC-d27 (•) and DMPC-d27 with γ-decalactone at 175 (□), 250 (+) and 300 mg l^{-1} (Δ).

FTIR measurements

Infrared spectra were recorded by a vector 22 FTIR spectrometer (Bruker). Ten scans were co-added with a resolution of 4 cm^{-1}. Data processing was done using the OPUS software package (Bruker, Karlsruhe) with a wavenumber accuracy of 0.1 cm^{-1}. The phase transition temperature of DMPC-d27 was monitored in the presence of the different lactones by following the frequency shift of the 2090 cm^{-1} band which is assigned to the CD_2 symmetric stretching mode (Bouchard *et al.* 1996). It was followed for each lactone concentration during heating of the phospholipid films from 12 to 30 °C. Temperature was regulated by water circulation around the water-tight cell. Temperature of the lipids was measured via a K-thermocouple directly inserted at the surface of the ZnSe crystal and measured with a 0.1 °C precision. Heating rate in the 12–30 °C range was 0.6 ± 0.2 °C min^{-1}. One example of the effect of lactone introduction into DMPC-d27 films is given in Figure 1 and shows that the interaction (at 27 °C) is achieved within 400 s. So, phase transition measurements between 12 and 30 °C in the presence of lactones were systematically performed after an insertion period of 1 h.

Results and discussion

Influence of the lactones on the growth of the yeast Yarrowia lipolytica

Growth of *Y. lipolytica* was monitored turbidimetrically at 600 nm in a liquid culture medium containing one of the four lactones at a concentration of 150 or 300 mg l^{-1}. The lag phases (latency phase) that were determined from each growth curve, are reported in Table 2. γ-Butyrolactone had no significant influence on the cell growth, δ-decalactone slowed down the growth in a concentration-dependent manner. Growth was also diminished with 150 mg γ-decalactone per litre and totally inhibited for 300 mg l^{-1}. No growth was detected with both the tested concentrations of γ-dodecalactone.

From the lag phase determined in the presence of the two tested concentrations of lactones, an order in the inhibitory action of the four lactones was established: γ-dodecalactone was the most effective, then γ-decalactone, δ-decalactone and γ-butyrolactone that exhibited no effect. The comparison between this order and the Log P values determined for each lactone (Table 1), showed that the most hydrophobic molecule, i.e. γ-dodecalactone, was the most inhibitory one and that the most hydrophilic one did not inhibit cell growth. γ-Decalactone and δ-decalactone gave inhibitory effects that were included between the effects of γ-dodecalactone and γ-butyrolactone, however γ-decalactone was more inhibitory than δ-decalactone despite its lower Log P. Thus, the physiological action of the lactones on this yeast seems to be related to their hydrophobic character. However, the effect observed with δ-decalactone may indicate that the structure of lactone rings is also implicated in growth inhibition. Within the following experiments, the interactions of these four lactones with model phospholipids were investigated.

Table 2. Lag phase of the yeast *Y. lipolytica* inoculated in media containing 150 or 300 mg lactones l^{-1}.

Compounds	Lag phase (h), in the presence of the lactones at two concentrations	
	150 mg l^{-1}	300 mg l^{-1}
γ-Butyrolactone	6.3 ± 0.1	6.2 ± 0.1
γ-Decalactone	9.4 ± 0.1	No growth after 30 h
δ-Decalactone	6.8 ± 0.1	9.2 ± 0.1
γ-Dodecalactone	No growth after 30 h	No growth after 30 h
Reference (without lactone)	6.1 ± 0.1	

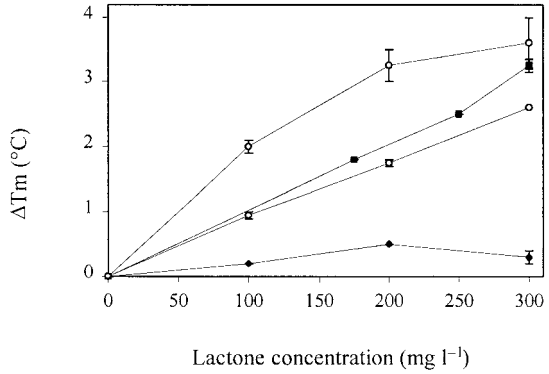


Fig. 3. Variation in Tm value (ΔTm) of DMPC-d27 induced by four lactones with different concentrations: γ-dodecalactone (□), δ-decalactone (○), γ-decalactone (■) and γ-butyrolactone (♦). ΔTm was determined by subtracting Tm in the presence of lactone to Tm of pure DMPC-d27, i.e. 20.3 °C.

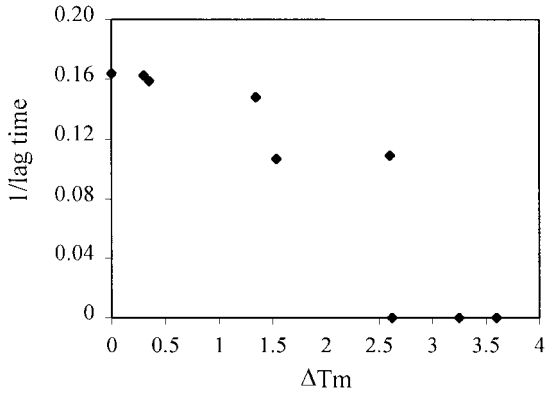


Fig. 4. Representation of 1/lag time for *Y. lipolytica* inoculated in lactone enriched media (determined from the values in Table 2), versus ΔTm, corresponding to the difference between Tm of pure DMPC-d27 and Tm of DMPC-d27 in the presence of lactones.

Interactions of the lactones with DMPC-d27

We have used Fourrier transform infrared spectroscopy to investigate the effects of the lactones on the gel-to-liquid-crystalline phase transition temperature (Tm) of the acyl chains of DMPC-d27. The use of deuterated phospholipids allowed the investigation of the acyl chain thermotropic behaviour without contribution of the CH_2 groups of the lactones. The typical phase transition curves that were obtained are illustrated in Figure 2, which was obtained with different concentrations of γ-decalactone. Tm was assigned to the inflection point of the curves in the region of the maximal slope, i.e. 20.3 °C for DMPC-d27 alone. Lactone introduction into DMPC-d27 multibilayers decreased the Tm: probably because of the introduction of gauche conformers in the acyl chains resulting from the lactone-lipids interaction (Casal & Mantsch 1984). This decrease in Tm caused by lactone corresponds to a fluidising effect. For each concentration of the tested compounds, the shift in Tm (ΔTm) was determined and these values were then used to compare the effect of the different compounds.

Influence of the length of the lateral carbon chain of the lactones on the interactions with DMPC-d27

ΔTm determined with γ-lactones (4-alkanolides) differing in the length of the acyl chain, i.e. γ-butyrolactone, γ-decalactone and γ-dodecalactone which have a lateral carbon chain with respectively 0, 6 and 8 carbons (Table 1), are given in Figure 3. γ-Butyrolactone had a weak effect on ΔTm. γ-Dodecalactone and in a lesser extent γ-decalactone strongly depressed the Tm of DMPC-d27, in a concentration-dependent manner. The most important ΔTm (decrease of 3.6 °C) was obtained with 300 mg l^{-1} of γ-dodecalactone. According to these results, ΔTm in DMPC-d27 bilayers, is linked to the length of the lateral carbon chain of the tested lactones, which indicates that it is also linked to the global hydrophobic character of the compounds (Table 1).

Influence of the lactone ring size of the lactones on the interactions with DMPC-d27

γ-Decalactone and δ-decalactone (5-decanolide) have the same molecular weight and the same number of carbons (10), but they differ in the size of the lactone ring (respectively 4 and 5 carbons on the lactone ring). Their influence on Tm is also exposed in Figure 3. For the tested concentrations range, a slightly more pronounced effect on ΔTm was systematically observed with γ-decalactone, which has a smaller lactone ring than δ-decalactone. According to Log P values of the two tested compounds, it can be observed that in this case Log P is not the only parameter that governs the interaction of the compounds with DMPC-d27. Considering the carbon repartition between the acyl chains and the ring of the two molecules, we can suppose that the steric hindrance, mainly related to the ring size, also influences the interaction.

Hypothetical relation between the cell adaptation and the structural effects of lactones on lipid bilayers

Figure 4 was obtained by plotting 1/lag time, which corresponds to the time period of the yeast adaptation to the lactone-enriched medium, versus ΔTm which represents the influence of lactones on the bilayer physical state. This curve was drawn with the results obtained for the two tested concentrations of the lactones, in order to test the hypothesis that the time of adaptation of *Y. lipolytica* to an increase in lactone concentration is related to the degree of structural change of phospholipid bilayers induced by lactones. The general profile of the graph indicates that lag time increased in an exponential manner with increasing values of ΔTm and that no growth was observed with ΔTm values higher than 2.5 °C. Thus, lactone concentrations inducing at least a ΔTm of 2.5 °C, also inhibited the restart of cell growth. As a fluidising effect of a lactone on the membrane of *Y. lipolytica* has already been reported *in vivo* (Aguedo *et al.* 2002), we speculate that the adaptation time of this yeast to lactones may be predicted from their fluidising action (ΔTm) on a model bilayer.

This study shows that the hydrophobic lactones produced by yeast cells, rapidly interact with membrane lipids (Figure 1), increasing the fluidity of the phospholipid bilayers and thereby possibly leading to toxic and antimicrobial effects of the molecules previously reported (Endrizzi-Joran 1994, Feron *et al.* 1996).

From these results, the study justifies the relevance of a continuous extraction of the lactones during bioproduction processes (Dufossé *et al.* 1997). It may be possible to elaborate strategies to improve yeast adaptation to high lactone concentrations by modifying the physical properties of the cell membranes in view to prevent an increase in their fluidity: for example by decreasing the temperature or by adding in the medium membrane stabilising agents (Beney & Gervais 2001).

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