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PROTECTIVE EFFECT OF IRIDALS FROM SAPONIN INJURY IN *CANDIDA ALBICANS* CELLS

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Key Word Index—*Iris pallida*; Iridaceae; iridals; ergosterol; steroidal saponins; triterpenoids; membranes; *Candida albicans*.

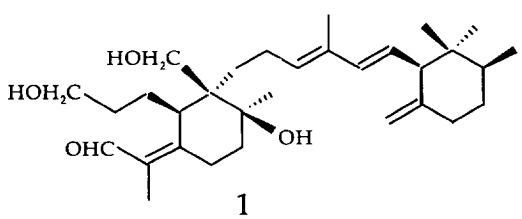
Abstract—Spirostanol saponin permeabilization of *Candida albicans* cells resulted in phosphate (Pi) leakage from these cells. Pre-incubation of *C. albicans* suspension cultures with ergosterol or cycloiridals inhibited Pi leakage when cells were subjected to saponins. Saponins were shown to precipitate ergosterol but not cycloiridals. Inhibition of Pi leakage in the presence of cycloiridals could thus be due to their incorporation into cell membranes. Cycloiridals, which were previously shown to modify fluidity parameters in artificial phospholipid bilayers, seem *in vivo* to have a protective effect on membranes against surfactant (saponin) injury.

INTRODUCTION

Iridals were first described in 1982 [1]. These triterpenoids, found in various *Iris* species, may be divided into three classes: monocycloiridals, bicycloiridals and spiroiridals [2]. Their structural diversity (about 30 different iridals are known to date) and high concentrations within rhizomes (around 3% of dry weight [3, 4]) led us to investigate their possible biological significance.

We previously hypothesized that iridals were involved in the maintenance of cell membrane integrity during water stress [5]. Recently, we highlighted the presence of iridals within microsomes of *I. germanica* rhizomes [6] and proposed a sterol-like structural role for these triterpenoids. This hypothesis was supported by ESR measurements of fluidity parameters of liposomal membranes containing phosphatidylcholine and cyloiridals [7]. These results indicated that cycloiridals (iriflorental + iripallidal) influence bilayer properties in the same manner, but at lower intensity, than cholesterol.

The membrane-damaging potential of some drugs may be evaluated following the release of ions [8–10], nucleotides [11] or proteins [12] contained within cells or liposomes. Among natural plant substances, saponins are able to induce cell membrane permeabilization [12, 13]. To determine iridal involvement in cell membranes, we studied the effect of these compounds on membrane leakage caused by saponins used as surfactants.



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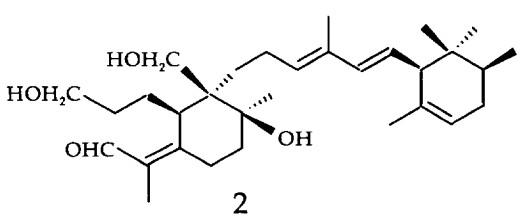

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RESULTS AND DISCUSSION

Candida albicans cell suspension cultures were used in the present study. These cells were incubated in water and the effects of various substances on inorganic phosphorus (Pi) leakage from cells were monitored. The investigated substances (cycloiridals, ergosterol, steroidal saponins) were dissolved in 95% ethanol and added to the cell culture [100 μl ethanol (95%) containing the tested substance into 10 ml cell suspension culture].

At first, we separately tested the effects of ethanol,

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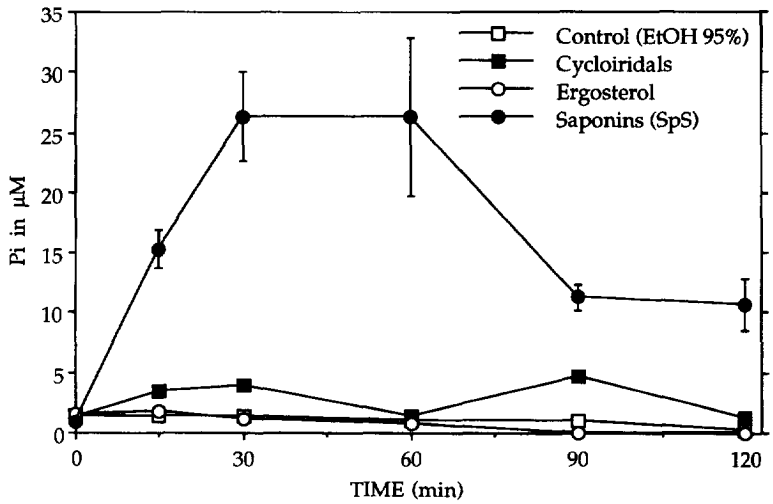

Fig. 1. Effects of 95% ethanol (10 μl ml^{-1}), cycloiridals (30 μM), ergosterol (30 μM) and spirostanol saponins (30 μM) on Pi leakage from *C. albicans* cells. Results are means of two replicates (except for spirostanol saponins with three replicates ±S.D., vertical bars).

cycloiridals, ergosterol and spirostanol saponins (SpS) on Pi leakage from *C. albicans* cell suspension cultures. The results are presented in Fig. 1. No significant release of Pi was induced by incubation of *C. albicans* cells with 1% ethanol (95%) or with ergosterol at 30 μM. The addition of cycloiridals (30 μM) to the cell culture induced very slight Pi leakage into the external medium, but it never reached 5 μM. Conversely, when SpS (30 μM) were incubated with *C. albicans* culture, we observed a substantial release of Pi into the external medium (Fig. 1). This leakage increased from the start until *ca* 60 min incubation, and reached about 25 μM Pi in the medium. During the second hour of incubation, the external Pi level dropped to *ca* 11 μM. The SpS concentration used here was not lethal for *C. albicans* culture, as indicated by a constant number of colony forming units (CFU) (see Experimental) in samples harvested during the experiments (data not shown, see ref. [14]). This seems to indicate that only membrane permeabilization was induced by SpS at this concentration, and that the drop of Pi observed between 60 and 120 min could have been due to cell re-absorption.

It is known that iridals show affinity for plant and animal microsomal membranes [6], and that they could be integrated into artificial phospholipid bilayers [7]. It was therefore of interest to investigate the behaviour of cell membranes containing iridals and submitted to surfactant injury. *Candida albicans* suspension cultures were pre-incubated for 10 min with a mixture of two cycloiridals (30 μM). Then SpS were added and Pi leakage was monitored in the external medium (Fig. 2). The same experiment was carried out with iridals substituted by ergosterol, a typical fungus sterol. In both experiments, the curves plotted from the results (Fig. 2) were comparable to control curves (Fig. 1) obtained in the absence of SpS. There was no obvious Pi leakage after pre-incubation of cells with cycloiridals. Only a slight Pi release was apparent (*ca* 5 μM) during the first 15 min, when *C. albicans* cells were pre-incubated with ergosterol. These results indicate that the presence of cycloiridals and ergosterol within the culture medium hinders saponin effects. We thus investigated the mechanisms underlying these effects.

Many saponins are known to precipitate sterols [15, 16], suggesting that SpS and ergosterol could precipitate within the culture medium. Such a reaction could reduce both the soluble SpS level and their membrane permeabilization effects. Since we previously noted that cycloiridals and sterols have some behavioural similarities, joint precipitation of cycloiridals and SpS should be considered. We investigated potential ergosterol–SpS and cycloiridal–SpS precipitate formation and tested another sterol (cholesterol) and saponin (digitonin) to check the experimental procedure (see Experimental). This latter substance could be used as a reference with respect to its effect on sterols [16].

Initial concentrations of ergosterol, cholesterol and cycloiridal were around 30 μM. Levels of these compounds were monitored after complex formation with SpS or digitonin (Table 1). The results indicated that the SpS mixture efficiently precipitated cholesterol and ergosterol, comparable to the results obtained with digitonin. Conversely, neither the SpS mixture nor digitonin were able to precipitate cyloiridals, whose concentration remained unchanged during the experiment.

Inhibition of Pi leakage from *C. albicans* cells due to the presence of both ergosterol and cycloiridals in the culture medium could involve two different mechanisms. Concerning ergosterol, its presence within the culture medium likely induced precipitate formation with SpS. This would reduce SpS levels and therefore their membrane permeabilization activity. Further investigations would be necessary to determine whether or not at least some ergosterol is incor-

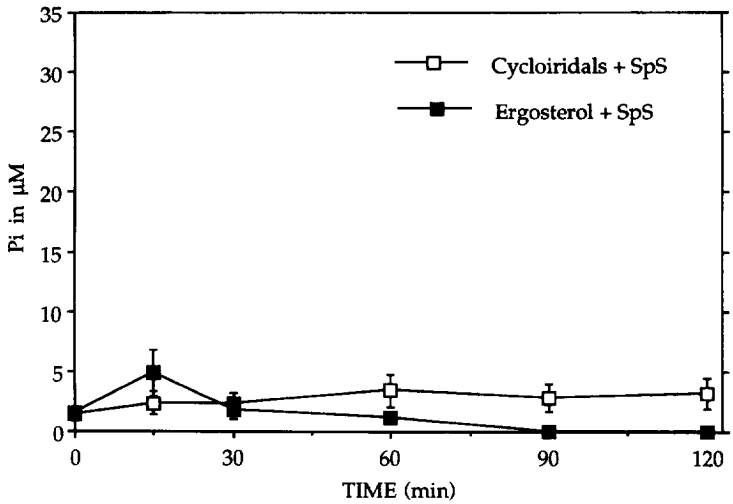

Fig. 2. Effects of spirostanol saponins (SpS) (30 μM) on Pi leakage from *C. albicans* cells pre-incubated for 10 min with cycloiridals (30 μM) or ergosterol (30 μM). Results are means of three replicates ±S.D.

Table 1. Sterol and cycloiridal levels in the supernatant of the complex formation medium, in the presence of SpS or digitonin

	Ergosterol (μM)	*n*	Cholesterol (μM)	*n*	Cycloiridals (μM)	*n*
SpS	0.90±0.04	3	0.30±0.01	3	32.11±1.52	3
Digitonin	0	1	0	1	33.40	2

SpS, spirostanol saponin mixture.
n, number of replications, when $n = 3$, results are means ±S.D.

porated into cell membranes during the preincubation period, and to define the molecular ratio in ergosterol/SpS precipitates.

On the other hand, no precipitate was obtained with cycloiridals and SpS. Cycloiridals were recently found to be easily integrated into phospholipid bilayers [7]. It could be hypothesized that at least some cycloiridals are incorporated into *C. albicans* cell membranes during preincubation. We monitored cycloiridal levels in cellular fractions over the course of the leakage experiments. The results are reported in Table 2. Clearly about half of the cycloiridals present in the culture medium were rapidly incorporated into slightly stirred cells.

Table 2. Percentages* of cycloiridals within *C. albicans* cellular fractions collected during the time-course of incubation

Time (min)	Cycloiridals (%)
0	46.6
15	48.0
30	52.0
60	39.0
90	27.3
120	26.6

* 1 ml fractions were collected at different times during incubation. At $T = 0$, the cycloiridal level within the medium was 30 μM (*ca* 15 μg ml^{-1}). Percentages represent iridal levels in the cellular fractions relative to theoretical iridal levels in the whole fraction (*ca* 15 μg ml^{-1}). Results are means of two replicates.

The present results seem to indicate that inhibition of Pi leakage from cells subjected to SpS injury could be mediated by the presence of cycloiridals. The fact that cycloiridals were shown to reduce membrane fluidity above the phase transition temperature [7], suggests that such physicochemical modifications may hinder surfactant injury of these membranes. Previous results of Grunwald [17] indicated that some sterols (cholesterol, ergosterol or campesterol) may modify membrane permeability and (depending on their concentration) reduce electrolyte leakage. Nevertheless, their presence can sensitize such membranes to sterol-binding lytic compounds such as saponins which leads to destabilization [18, 19]. Also, membranes lacking sterols (such as the fungi Pythanceae) are less disrupted compared with those containing sterols [20]. Cycloiridals appear to be able to stabilize membranes without also sensitising them to saponins, apparently because of their inability to bind with saponins. To our knowledge, this is the first report on the *in vivo* protective effect of cycloiridals in cell membranes.

EXPERIMENTAL

Materials. The *C. albicans* cloned strain VW 32 was provided by Dr F. Fruit, Department of Parasitology and Mycology, Universitary Hospital Center of Lille, France. *Iris pallida* Lam. rhizomes were obtained from

the Botanical Garden of Montpellier (France) and grown in an experimental field at the University. *Trigonella foenum graecum* L. (fenugreek) was also cultivated in the field at the University.

Iridal extraction. Iris pallida rhizomes were ground and extracted with EtOH (70%). The extract was filtered on PVPP Durapore 0.45 μm and concentrated *in vacuo* (35°). One volume of H_2O was added and the mix. was extracted 4× with Et_2O. Organic frs were pooled and the solvent evapd *in vacuo*. The residue was dissolved in $CHCl_3$–MeOH (2:1) before purification, as previously described [21]. Iriflorental and iripallidal were characterized by comparing their R_fs and UV spectra with those of authentic standards provided by Dr F.-J. Marner (University of Köln, Germany). The iriflorental:iripallidal ratio of the purified mixture was 2.7 ± 0.1.

Steroidal saponins. These substances were extracted from fenugreek seeds and obtained as indicated in ref. [22]. During the present study, we used SpS obtained after hydrolysis with β-glucosidase of a mixt. containing only steroidal saponins from *T. foenum-graecum*. The mean molecular weight of these SpS was regarded as representative of the mean molecular weights of saponins present in fenugreek, and was evaluated at 1000 g mol^{-1} according to refs [23] to [26].

Culture of Candida albicans. Cells were grown in 250 ml flasks (37°, 130 rpm) containing 50 ml of the following medium: yeast nitrogen base (YNB) supplemented with glucose and L-asparagine [27] and diluted 10-fold in a Pi buffer (NaH_2PO_4, $2H_2O$; 0.608 g l^{-1} and Na_2HPO_4, $12H_2O$; 2.183 g l^{-1}), adjusted to pH 7.0. During the early logarithmic growth phase, the culture was collected and centrifuged (15 min, 1500 *g*, 4°). The pellet was washed 2× in water by resuspending and repelleting. The washed pellet was resuspended in an adequate volume of water to obtain a suspension of *ca* 2×10^6 colony forming units per ml (CFU/ml^{-1}).

Incubation of C. albicans *with test compounds.* Incubations of cells with the different studied substances were carried out in 10 ml of the above suspension. Chemicals (SpS, iridals or ergosterol) were added to the suspensions dissolved in 30 μl EtOH (95%). The mixts were incubated at 37° and stirred (130 rpm) for various times.

Viability test. At 0 and 2 hr and at selected intervals during incubation in the presence of drugs, samples were removed for viability determinations. Standard dilutions (at least 1:100) and the plate count method were used to quantify CFU ml^{-1}.

Measurement of Pi leakage. At selected times during incubation, 1 ml of *C. albicans* cell suspension was collected and centrifuged (10 min, 5000 *g*, room temp.). The supernatant was used for Pi analysis according to ref. [28].

Precipitation assays. This procedure was carried out according to [16]. Ergosterol, cycloiridals, cholesterol, digitonin and a spirostanol saponin mixture were dissolved in 95% EtOH. Saponins (SpS or digitonin) and sterols (ergosterol or cholesterol) or cycloiridals were mixed in 95% EtOH and diluted to obtain equal concns of compounds (30 μM) and a final volume of 1 ml. Solns were vortexed, then left for 2 hr at room temp. Samples were then centrifuged for 20 min at 6000 *g* and 500 μl of supernatant was collected. Supernatants were analysed by GC (sterol analysis) or HPLC (cycloiridal analysis).

Sterol assay. Underivatized sterols were analysed by GC on a Delsi DI700 apparatus, equipped with a DB1 column (30 m × 25 μm). Injection, detection and column temperatures were, respectively, 270°, 280° and 280° isothermal. Sterols were quantified by the external standard method.

Iridals in yeast fractions. The pellets obtained during the preparation of samples of Pi determination contained the *C. albicans* cells. These frs were extracted twice with 1 ml of EtOAc. The solvent was evapd under a N_2 stream and the residue redissolved in a $CHCl_3$–MeOH (2:1) mixt. before analysis by RP-HPLC, as indicated previously [5].

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