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The *in vitro* antifungal activity of some dithiocarbamate organotin(IV) compounds on *Candida albicans* – a model for biological interaction of organotin complexes

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The *in vitro* antifungal activity of the dithiocarbamate organotin complexes $[Sn\{S_2CN(CH_2)_4\}_2Cl_2]$ (1), $[Sn\{S_2CN(CH_2)_4\}_2Ph_2]$ (2), $[Sn\{S_2CN(CH_2)_4\}Ph_3]$ (3), $[Sn\{S_2CN(CH_2)_4\}_2n\text{-}Bu_2]$ (4), $[Sn\{S_2CN(CH_2)_4\}Cy_3]$ {Cy = cyclohexyl} (5), $[Sn\{S_2CN(C_2H_5)_2\}_2Cl_2]$ (6), $[Sn\{S_2CN(C_2H_5)_2\}_2Ph_2]$ (7), $[Sn\{S_2CN(C_2H_5)_2\}Ph_3]$ (8), $[Sn\{S_2CN(C_2H_5)_2\}_3Ph]$ (9) and $[Sn\{S_2CN(C_2H_5)_2\}Cy_3]$ (10) has been screened against *Candida albicans* (ATCC 18804), *Candida tropicalis* (ATCC 750) and resistant *Candida albicans* collected from HIV-positive Brazilian patients with oral candidiasis. All compounds exhibited antifungal activities and complexes 3 and 8 displayed the best results. We have investigated the effect of compounds 1–10 on the cellular activity of the yeast cultures. Changes in mitochondrial function have not been detected. However, all drugs reduced ergosterol biosynthesis. Preliminary studies on DNA integrity indicated that the compounds do not cause gross damage to yeast DNA. The data suggest that these compounds share some mechanisms of action on cell membranes similar to that of polyene but not with azole drugs, normally used in *Candida* infections. Copyright © 2008 John Wiley & Sons, Ltd.

Keywords: antifungal activity; *Candida* species; tin(IV) dithiocarbamates complexes

Introduction

The opportunistic pathogen *Candida albicans* is responsible for superficial or systemic infections in immuno-compromised patients.[1] Patients on immuno-suppressive therapy, due to organ transplantation, chemotherapy of critical diseases or AIDS, are potential targets for infections.[1] The drugs of choice for treatment are polyene and azole compounds.[2,3] Amphotericin and nystatin, classical examples of polyene drugs, act in the membrane of the fungal cell by linking to ergosterol. These drugs interfere with the permeability of the cellular membrane, causing losses of macromolecules and ions essential for cell survival.[2,4] The antifungal activity of azoles, such as myconazole and fluconazole, arises from the interaction with the enzyme sterol-14α-demethyllase (CYP51), involved in the biosynthesis of ergosterol. Interaction with CYP51 results in a decreased mitochondrial function and availability of ergosterol and accumulation of 14-methylsterols. Changes in ergosterol levels and sterol structure influence the activity of several metabolic pathways and also membrane permeability.[3–5]

Genome studies have provided a better understanding of the closer distance between the fungal kingdom and human species.[6] It is possible that the chemical and genetic similarities explain why some fungal diseases of mammals are difficult to treat. Resistance of yeast to conventional treatment has been reported and it is related to (i) alterations in the target of the drug, (ii) interference with ergosterol biosynthesis and/or (iii) increase in the expression of drug efflux pumps.[7] Altogether these points make the search for alternative drugs mandatory and metal-based compounds might represent a novel group of antifungal agents, either alone or in combined formulation with drugs in current use to overcome resistance.

Biological activities of organotin compounds as biocide,[8] antitumor,[9] schizonticidal and antimalarial[10] have been reported. Also, derivatives with Schiff bases have potential use as amoebicidal agents.[11] Some 2-alkylindole derivatives have activity against *Bacillus subtilis, Bacillus pumilus, Staphylococcus aureus* and *Micrococcus luteus*.[12] Activity against leshmaniasis in mice and helminthes in cats has been found for dioctyltin maleate.[13]

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Dithiocarbamates comprises some important classes of compounds that have been used, for example, as catalysts, in the rubber industry and in pesticides.[14] Antitumor and antiviral activities have been reported for pyrrolidine dithiocarbamate salts.[15] Diethyldithiocarbamate salts have been investigated for possible application in chronic alcoholic therapy[16] and treatment of HIV-patients.[17]

Our recent efforts have focused on mechanistic aspects of biological activity of organotin compounds using fungus cultures as models.[18] Herein we report some results involving organotin dithiocarbamate derivatives. We have (i) determined the minimal inhibitory concentrations (MIC) for compounds **1–10**, (ii) examined the integrity of the genetic material and respiratory functions of drug-treated colonies of *C. albicans* and (iii) measured the levels of ergosterol in the cell membrane.

Materials and Methods

Chemicals

The synthesis of the organotin dithiocarbamate complexes has been reported previously, Fig. 1. Complexes **1–5** and **10** were prepared by us[18] and complexes **6**–**9** were obtained according to the literature procedure.[19] All chemicals were purchased from Aldrich, Merck or Synth and used as received.

Yeast strains and culture conditions

Standard *C. albicans* (ATCC 18804) and *C. tropicalis* (ATCC 750) and a drug-resistant *C. albicans* clinical isolated from a Brazilian HIV-positive patients with oral candidiasis (from the Reference Centre for Treatment for Sexually Transmitted Diseases of Belo Horizonte, Minas Gerais State, Brazil) were used in this work. Yeast cells were aerobically grown on Sabouraud dextrose broth, SDB (1% peptone, 0.5% yeast extract, 2% glucose), or agar, SDA (SDB + 1.5% agar), at 37 °C and stored at 4 °C. Dichloromethane was employed as solvent in all tests. For yeast cell viability, DNA integrity assays and ergosterol extraction experiments, *C. albicans* cultures were grown to stationary phase at 30 °C (1×10^9 CFU ml^{-1}) with continuous shaking in SDB. All experiments were carried out in triplicate.

Minimum inhibitory concentrations

MIC experiments were performed as recommend in the literature[20] with 10^5–10^6 CFU ml^{-1}. This experiment was performed with the following isolates: standard *C. albicans* (ATCC 18804) and *C. tropicalis* (ATCC 750) and a drug-resistant *C. albicans* clinical from a Brazilian HIV-positive patient.

MIC is defined as the minimum concentration of drug required to totally inhibit the visual growth of the fungal cells at 37 °C, expressed in micrograms of compounds in the study per 1 ml of broth. Owing to the limited solubility of the compound in aqueous solutions, aliquots of 10 µl dichloromethane containing the appropriate mass of the compounds were added to 1 ml SDB, instead of performing serial dilutions (1 : 2).

Evaluation of fungicidal and respiratory effects of tin compounds on yeast

A test was carried out to detect the basis of inhibitory effects and changes in the respiratory function induced by the drugs in the yeast.[21] Cultures of *C. albicans* (ATCC 18804) were grown for 24 h at 37 °C in the presence of the organotin compounds at concentrations of 10 and 40 µg ml^{-1}. Then, the mixture was centrifuged at 1.3×10^4 rpm for 5 min and the cells were suspended in phosphate buffered saline, PBS (NaCl, 137 mmol l^{-1}; KCl, 2.7 mmol l^{-1}; Na_2HPO_4, 10 mmol l^{-1}; KH_2PO_4, 2 mmol l^{-1}; pH = 7.4). Each cell suspension (100 µl) was spread over 15 ml of SDA on Petri dishes and incubated for 48 h at 30 °C. Then, samples were transferred to Petri dishes with molten-agar (0.5% glucose; 0.05% 2, 3, 5-triphenyltetrazolium chloride, TTC; 2% agar) and grown for 24 h at 30 °C. The number of colonies was determined

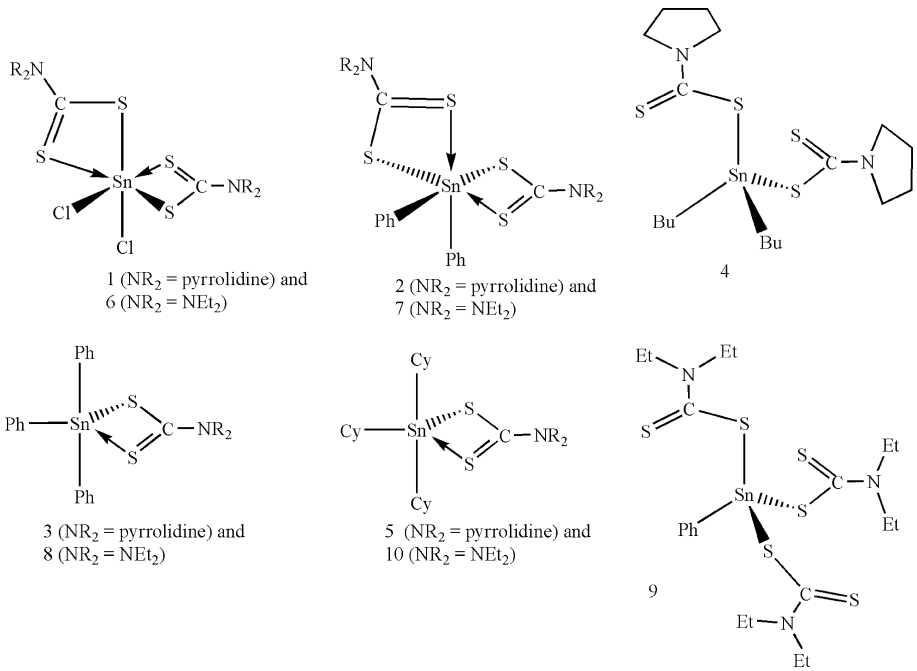

Figure 1. Structures of complexes **1–10**.

and expressed as a percentage of the control (100%). Colonies with active respiration reduce TTC to a dark red colour while those with reduced respiratory function remain white.

DNA fragmentation assay

This assay was performed in order to evaluate gross DNA damage caused by the complexes. We based our strategy on a previous study with yeast and mammalian cells.[22] *C. albicans* cells were grown in SDB at 30 °C with shaking for 18 h in three different conditions: (i) absence of dichloromethane and compounds; (ii) presence of 10 µl dichloromethane; and (iii) presence of each complex at concentrations of 10 and 100 µg ml^{-1}. The liquid cultures were centrifuged for 5 min in a microfuge. The liquid phase was eliminated and pellets were resuspended in 200 µl lysis buffer (2% Triton X-100; 1% sodium dodecylsulphate; 100 mmol l^{-1} NaCl; 10 mmol l^{-1} Tris–HCl pH = 8; 1 mmol l^{-1} EDTA pH = 8). The tubes were then immersed in liquid nitrogen for 2 min (twice) and transferred to a hot bath at 95 °C for 1 min. The tubes were vortexed for 30 s. Then, 200 µl of chloroform were added and the tube vigorously agitated for 2 min. The tubes were centrifuged again for 3 min and the aqueous phase was transferred to tubes containing 400 µl of cold ethanol. The tubes were incubated overnight at −20 °C, centrifuged and the pellets washed with 500 µl cold ethanol (70%) and dried at room temperature. Finally, the pellets were resuspended in 10 µl TE buffer (10 mmol l^{-1} Tris–HCl pH = 8 and 1 mmol l^{-1} EDTA pH = 8). Alterations in DNA integrity were assessed by comparing electrophoretic migration of the samples in a 0.8% agarose horizontal gel prepared in TAE buffer [40 mmol l^{-1} Tris(hydroxymethyl)aminomethane acetate and 1 mmol l^{-1} EDTA]. For each assay 5 µl of the DNA samples were mixed with 5 µl of loading buffer (0.025 mg bromophenol blue; 1 ml glycerol and 1 ml distilled water) and run at 70 mV for approximately 0.5 h; the gel was stained with ethidium bromide and DNA was visualized under UV light.

Sterol extraction

The lipids mentioned in this work are ergosterol and its intermediate the dehydroergosterol. The amount of ergosterol was measured in order to investigate the action of the organotin compounds on the cell membrane of *C. albicans*. Sterols were extracted employing a methodology described in the literature with minor modifications.[23] Cells were grown on 2 ml of SDB with continuous shaking at 30 °C for 24 h in the presence of compounds **1**–**10**, at a concentration of 10 µg ml^{-1}, or dichloromethane for the control. The liquid cultures were transferred to Eppendorf tubes and centrifuged for 5 min at 1.3 × 10^4 rpm. The pellets were washed with distilled water, dried at room temperature and weighted. The resulting cells were resuspended in 25% alcoholic potassium hydroxide solution (25 g of KOH in 35 ml of sterile distilled water and 65 ml of ethanol), giving a final cell density of 1 × 10^9 cells cm^{-3}. Then, the tubes were incubated at 90 °C for 1 h after which they were cooled to room temperature. For the extraction of ergosterol 0.5 ml sterile distilled water, 1 ml of *n*-heptane was added and the tube was vortexed for 3 min. The *n*-heptane layer was transferred to Eppendorf vessels and kept at −20 °C for 24 h. Aliquots of 125 µl were diluted into 3 ml ethanol and analyzed from 230 to 300 nm. The concentrations of ergosterol and dehydroergosterol were determined by the difference in the absorbances at 281.5 and 230 nm, according to the literature methodology.[23]

Statistical analysis

Finally, differences between means for respiratory effect and ergosterol extraction experiments were analyzed by non-paired Student's *t*-test with $p > 0.05$.

Results

Determination of the Minimal inhibitory concentrations

The MIC experiments were determined for compounds **1**–**10** towards *C. albicans* (ATCC 18804), a drug-resistant *C. albicans* clinical isolate and *C. tropicalis* (ATCC 750). The MIC values varied from 1.25 to 250 µg ml^{-1} according to the drug and microorganism tested, Table 1.

Compounds **3** and **8** had the lowest MIC values and they were active towards the three isolates. Complex **4** was effective against *C. tropicalis* and drug-resistant *C. albicans* isolate, **2** with respect to *C. tropicalis* and **1** and **6** to the drug-resistant *C. albicans* isolate only. The other derivatives, **5, 7, 9** and **10**, had no activity. The two ligands (ammonium pyrrolidine and sodium diethyldithiocarbamate) were previously tested in a disk diffusion assay and did not show any antifungal activity, and were thus not evaluated for MIC. The literature reports that the highest fungicidal activity is found for trialquil-tin(IV) based complex.[24] The activity observed for the triphenyl derivatives **3** and **8** agrees with that. Surprisingly the tricyclohexyl was not very effective in the presence of the fungal colonies.

Evaluation of fungicidal and respiratory effects of tin compounds

For this evaluation two concentrations (10 and 100 µg ml^{-1}) of all compounds were used. After 24 h of exposure to compounds **1**–**10**, *C. albicans* colonies were plated and counted. Numbers of surviving colonies were expressed as a percentage of the control without drug (Table 2). Significant differences in the number colonies for all compounds in relation to the control ($p > 0.05$) were observed at both concentrations (10 and 100 µg ml^{-1}). It was detected that the growth of the colonies was not recovered after removing the drug from the medium, therefore we concluded that compounds **1**–**10** are in fact fungicidal and not fungistactic compounds. The surviving colonies were overlaid with TTC, incubated and evaluated for respiratory dysfunction. All colonies

Table 1. The minimal inhibitory concentrations for the complexes **1**–**10**

	MIC (µg ml^{-1})		
Compound	*C. albicans*	Drug-resistant *C. albicans*	*C. tropicalis*
[Sn{S$_2$CN(CH$_2$)$_4$}$_2$Cl$_2$] (**1**)	50.0	2.50	75.0
[Sn{S$_2$CN(CH$_2$)$_4$}$_2$Ph$_2$] (**2**)	50.0	25.0	2.50
[Sn{S$_2$CN(CH$_2$)$_4$}Ph$_3$] (**3**)	5.00	1.25	1.25
[Sn{S$_2$CN(CH$_2$)$_4$}$_2$*n*-Bu$_2$] (**4**)	30.0	2.50	1.25
[Sn{S$_2$CN(CH$_2$)$_4$}Cy$_3$] (**5**)	>250	75.0	75.0
[Sn{S$_2$CN(C$_2$H$_5$)$_2$}$_2$Cl$_2$] (**6**)	30.0	1.25	40.0
[Sn{S$_2$CN(C$_2$H$_5$)$_2$}$_2$Ph$_2$] (**7**)	40.0	75.0	50.0
[Sn{S$_2$CN(C$_2$H$_5$)$_2$}Ph$_3$] (**8**)	2.50	5.00	2.50
[Sn{S$_2$CN(C$_2$H$_5$)$_2$}$_3$Ph] (**9**)	>250	75.0	75.0
[Sn{S$_2$CN(C$_2$H$_5$)$_2$}Cy$_3$] (**10**)	>250	75.0	75.0

Table 2. Yeast viability study for the complexes at 30 °C with *C. albicans* (ATCC 18804)

Concentration (μg ml^{-1})	10	100
[Sn{$S_2CN(CH_2)_4$}$_2Cl_2$] (**1**)	94.67 ± 3.51	2.33 ± 0.58
[Sn{$S_2CN(CH_2)_4$}$_2Ph_2$] (**2**)	93.33 ± 3.06	3.67 ± 0.58
[Sn{$S_2CN(CH_2)_4$}Ph_3] (**3**)	5.67 ± 0.33	133 ± 0.57
[Sn{$S_2CN(CH_2)_4$}$_2n$-Bu_2] (**4**)	82.33 ± 2.52	3.33 ± 0.56
[Sn{$S_2CN(CH_2)_4$}Cy_3] (**5**)	118.33 ± 1.53	62.67 ± 3.06
[Sn{$S_2CN(C_2H_5)_2$}$_2Cl_2$] (**6**)	78.00 ± 2.65	3.67 ± 0.58
[Sn{$S_2CN(C_2H_5)_2$}$_2Ph_2$] (**7**)	90.33 ± 1.53	5.33 ± 0.58
[Sn{$S_2CN(C_2H_5)_2$}Ph_3] (**8**)	1.67 ± 0.58	1.31 ± 0.57
[Sn{$S_2CN(C_2H_5)_2$}$_3$Ph] (**9**)	117.00 ± 2.00	54.67 ± 3.21
[Sn{$S_2CN(C_2H_5)_2$}Cy_3] (**10**)	119.67 ± 0.58	55.67 ± 3.21
Control	122.67 ± 2.52	122.67 ± 2.52
	Growth[a] /CFU	

[a] Numbers of surviving colonies expressed as a percentage of the control: (i) 10 μg ml^{-1} – compounds **1** (80%); **2** (76%); **3** (5%); **4** (67%); **5** (96%); **6** (64%); **7** (74%); **8** (1%); **9** (95%); **10** (98%). (ii) 100 μg ml^{-1} – compounds **1** (2%); **2** (3%); **3** (1%); **4** (3%); **5** (51%); **6** (3%); **7** (5%); **8** (1%); **9** (45%); **10** (45%).

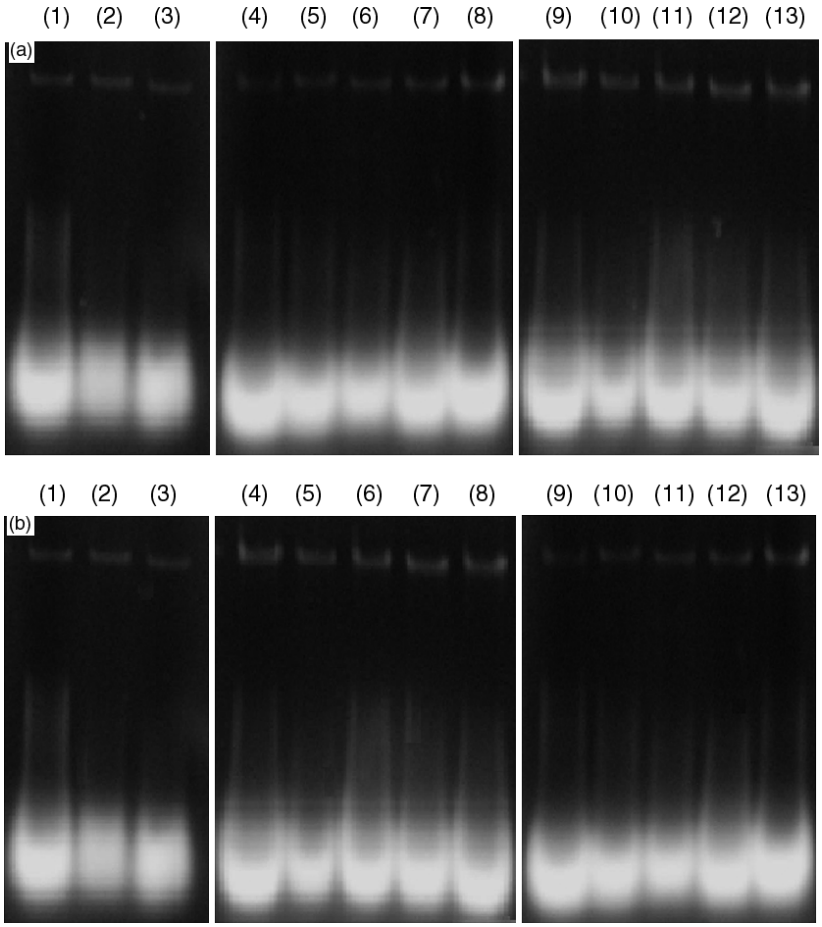

Figure 2. DNA binding assays for *C. albicans* (ATCC 18804) incubated with complexes **1–10** and dichloromethane solvent. Yeast cells were grown in the presence of (a) 10 and (b) 100 μg ml^{-1} of the complexes to late exponential phase.

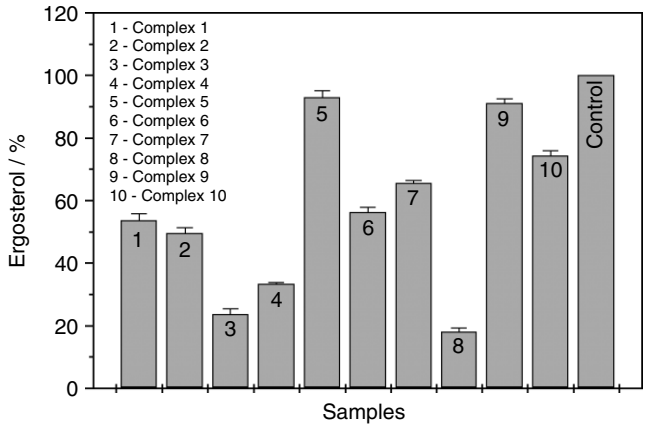

Figure 3. UV spectrophotometric sterol profiles: inhibition spectra of sterol in *C. albicans* (ATCC 18804) by (a) pyrrolidine and (b) diethyldithiocarbamate complexes series.

changed from white to red, suggesting that the tin complexes did not affect the respiratory ability of the cells.

DNA degradation assays

The method employed in the study was similar to that used in previous studies[22] where DNA degradation was evaluated. DNA was extracted from *C. albicans* control of yeast cells exposed to the complexes **1**–**10** at concentrations of 10 and 100 $\mu g\ ml^{-1}$. Degradation of high molecular weight DNA was not evidenced in the yeast cells exposed to the tin-based drugs, Fig. 2.

Sterol extraction

C. albicans was grown in the presence of compounds **1**–**10** and then, total lipids were extracted and analyzed by electronic spectroscopy in UV region. The position of the bands in the UV spectra of drug-treated cells was similar to that of the control (Fig. 3). However, a decrease in the concentration of sterols produced by the complexes was observed. The greatest reduction in ergosterol production occurred in the presence of compounds **3** and **8** (approximately 80%) and **4 (**approximately 70%), Fig. 4. Compounds **1, 2, 6** and **7** exhibited an intermediary effect on ergosterol production (30–50% reduction), in accordance to the MIC values. Compounds **5, 9** and **10** exhibited little activity, showing high ergosterol levels similar to the control.

Discussion

Most of the tin compounds analyzed in this study have been effective against the bacteria *S. Aureus*[18b] and filamentous fungi (data not published). In this study the interest was the mechanistic investigation of organotin complexes.

A number of mechanisms for the biological action of organotin derivatives have been proposed. However, a complete understanding has still to be found. Release of K^+ from cells, resulting from increased cytoplasmic membrane permeability, shows the cytoplasmic membrane to be a possible site of action.[25,26,27] The crossing of the cytoplasmatic membrane by organotin derivatives might be a consequence of lipid-solubility[28] effected by weak interactions with the amino acids, proteins,[29] nucleosides, carbohydrates and steroids[30] present in the cell membrane. Another proposition relates to the redox potential for the reaction $Sn^{4+} + 2e^- \rightarrow Sn^{2+}$ (0.154 V compared with standard hydrogen electrode); this lies within the physiological range found for several enzyme reactions, thus suggesting that enzymatic processes might be involved in the biological activity of organotin compounds.[31]

The type and the number of organic fragments attached in the Sn(IV) derivatives influences the biological activity of the organotin complexes towards the cultures screened in this work: $Ph_3 > Bu_2 > Cl_2 > Ph_2 > Ph \approx Cy_3$. As expected, the triphenyl–tin complexes, **3** and **8,** are the more active. The poor effect of the tricyclohexyl-tin derivatives was an unexpected result.[24] The Sn–Cl bearing compounds, **1** and **6,** are structurally different from the others. The presence of the halide might decrease the hydrophobic character of the complexes, rendering them biologically active.

Complexes **3** and **8** also displayed the highest inhibition of ergosterol biosynthesis. Compound **4** also have severely interfered with the levels of sterols. Although the MIC value for compound **4** was not as low as those for complexes **3** and **8** on *C. albicans*, they were very low for *C. tropicalis* and the *C. albicans* resistant isolate.

An important finding of our work was the indication that the organotin compounds studied here have a fungicidal rather than fungistatic effect on yeast cells. Compounds **1**–**10** had no effect on the respiratory ability of the drug-treated cells. It is known that $[Mn(phen)_2(mal).2H_2O]$, $[Ag_2(phen)_3(mal).2H_2O]$,

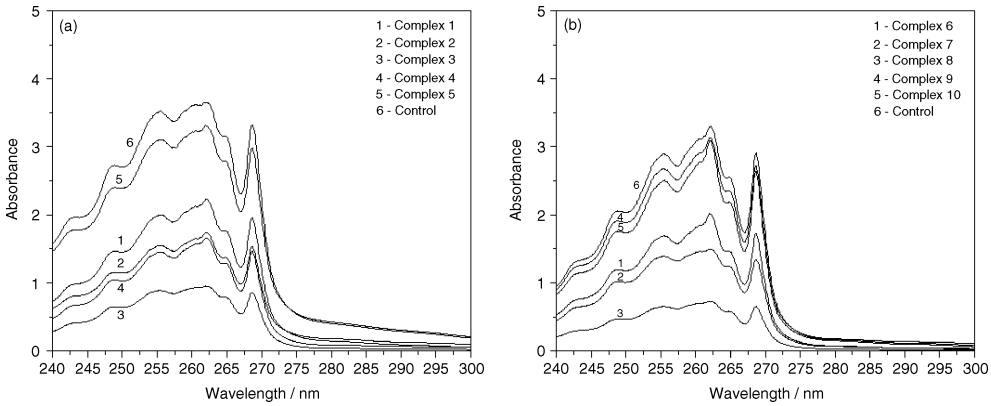

Figure 4. Ergosterol contents of yeast *C. albicans* (ATCC 18804) treated with pirrolidine and dietyl dithiocarbamate organotin derivatives and control cells.

$[Cu(phen)_2(mal)^{\bullet}2H_2O]$, cisplatin and palladium complexes cause a significant alteration in oxygen uptake in *C. albicans* cells.[32] A disruption of mitochondrial function normally results in loss of certain cytochromes responsible for lipid biosynthesis in the yeast cellular membrane.[21] No mitochondrial changes were detected in this work; therefore deactivation of cytochrome CYP51, sterol-14α-demethyllase was disregarded as possible mechanism.

Apoptotic DNA degradation was observed in mammalian and *C. albicans* cells exposed to Cu(II), Mn(II) and Ag(I) 1,10-phenanthroline complexes.[22] In the range of concentrations of 10 and 100 $\mu g\ ml^{-1}$, we have not detected patterns that could indicate DNA fragmentation of cells exposed to complexes **1**–**10**, Fig. 2. It is an indication that these organotin compounds seem to be less destructive to the DNA materials of *C. albicans* than other transition metal complexes reported previously.

The outcome of our results is that the fungicidal activity of the organotin dithicarbamate complexes arises from the decrease in the biosynthesis of ergosterol. Therefore they may share similarities with polyene drugs but not with the azoles, since no deactivation of cytochrome CYP51 was detected. In spite of the controversy around the toxicity of organotin towards higher species,[25,26,27] it is possible that some of their complexes are not so hazardous. Therefore, organotin complexes **3,8,1** and **6** might represent a new class of drugs to be employed alone or in combination with others in current use, either as new formulations for fungal diseases or to overcome resistance.

Acknowledgments

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