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Original Research Article

^{31}P-NMR study for determination of coumarin antifungal mechanism of action

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ABSTRACT

Coumarin derivatives as antifungal bioactive agents have been considered for many years. However, their mechanism of action is not very clear. In the current paper, antifungal activity of natural and synthetic coumarins are studied against *Cryptococcus neoformans* and *Saccharomyces cerevisiae.* Mechanism of pH-dependent activity of coumarin compounds was investigated using ^{31}P-NMR spectra and even intracellular estimate of the pH values. The results showed that coumarin derivatives have inhibitory effects without any noticeable side effects through a selective ATPase inhibitory mechanism.

Keywords: Coumarin, antifungal activity, ^{31}P-NMR, *Cryptococcus neoformans*, *Saccharomyces cerevisiae*

INTRODUCTION

Curiosity and achieving scientific recognition might be the primary motivations in studying the mechanism of action of new bioactive agents. However, this is not the answer to the high cost and the time consumed by such studies. In fact,

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there is more than curiosity in this type of research [1]. Although mode of action studies are more on the basic side of pharmaceutical research, the results can easily find their way into applied research, and drug development [2]. Research on the mechanism of action of antifungal agents can be useful in elucidation of the biological and biochemical processes. For example, sterol biosynthetic inhibitors were useful in the clarification and study of sterol biosynthetic pathways [3]. Mode of action studies can also play a role in finding new targets involved or improving the knowledge of existing targets. In addition to the benefits of this type of study in drug development, it is possible to predict the human toxicity of these agents and refine them [4]. As an example, knowing the action site of azole fungicides, cytochrome P450, any side effect involving human P450 could be possible for this group of antifungals [5]. It is known that ketoconazole is a potent inhibitor of testosterone synthesis and even may be a good candidate for androgen-dependent prostate carcinoma [6-11]. In this study, we intend to report mechanism of antifungal action for couamrins, through NMR experiments.

MATERIALS AND METHODS

Interaction studies

A modified method of the broth dilution technique was utilized. Fungal strains: *Candida albicans* ATCC 14053, *Saccharomyce cerevisiae* PLM 454, and *Cryptococcus neoformans* KF-33 were used.

Medium: RPMI 1640 sigma culture medium with no extra buffer at pH 7. Unless otherwise specified. One of the buffers used is MOPS (3-[N-Morpholino] propane-sulfonic acid), 34.5 g/L plus $NaHC0_3$, 2 g/L.

Inoculum: 5×10^3 (CFU/ml) or 5×10^2 (CFU/well) unless otherwise specified. Temperature 30°C, unless otherwise specified.

Staining method

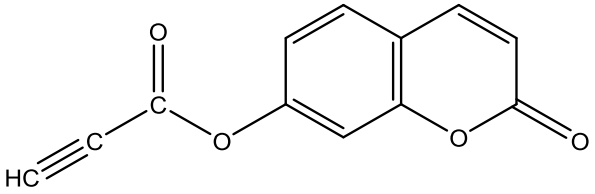
Cells were stained with a 0.5% solution of methylene blue before each examination with light microscope.

Compounds

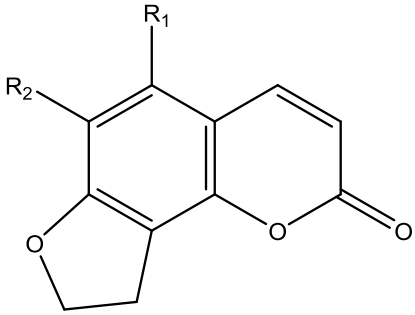
Coumarin compounds: Chemicals used in this study as antifungal (except the drugs) were of synthesized coumarin derivatives [7]. The following structures were used most often.

Reference compounds: A group of other drugs and chemicals were used as cAMP analogs, db-cAMP (ICN®), or phosphodiesterase inhibitors, including theophylline, IBMX, pentoxyfylline (all from Sigma®). In addition, the effect of morpholine compounds such as MOPS, NMO, and MES on the antifungal activity of coumarins was assessed. The test compounds were dissolved in the following solvents: AmB, itraconazole, ketoconazole, sodium orthovanadate (Na3V04) in DMSO;

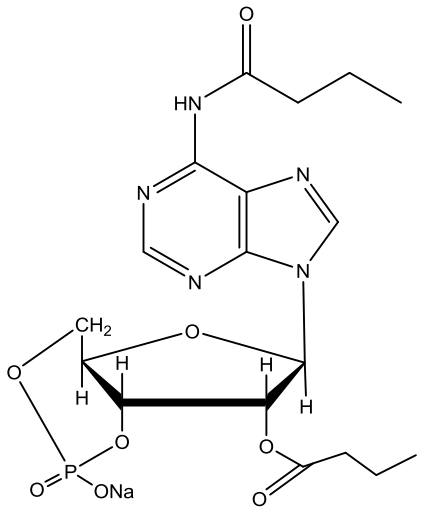
coumarins, pentoxifylline, IBMX and MM in methanol (depending on the concentration, angelicin might need acetone as co-solvent); fluconazole, db-cAMP, theophylline, hygromycin B, ouabain, MOPS and MES in water; valinomycin in acetone.



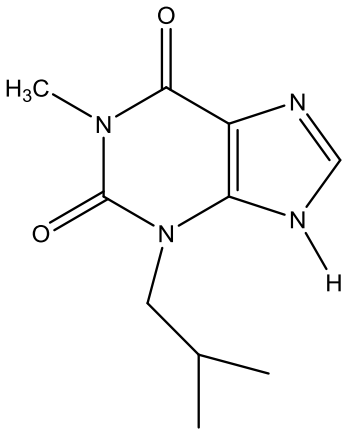
Compound 1 (Coumarin Derivative)



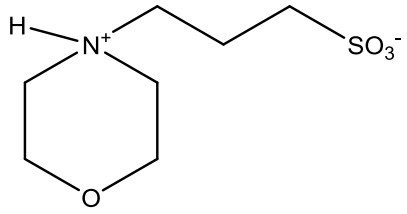
Compound 2 (Angelicin)



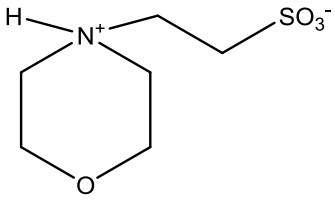
Db-cAMP (Bucladesine) sodium salt



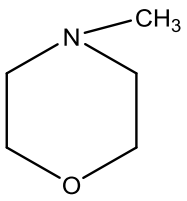
IBMX (3-Isobutyl-1-methylxanthin)



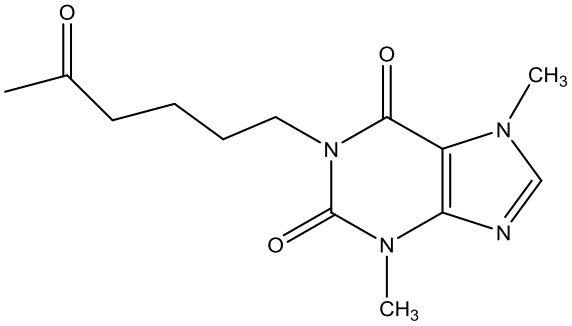
MOPS
(Morpholinopropylsulfonic acid)

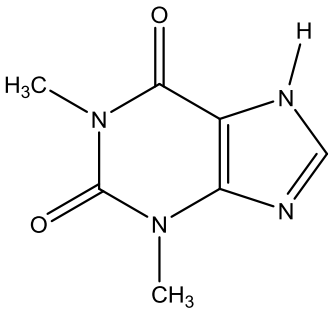


MES
(Morpholinoethan sulfonic acid)



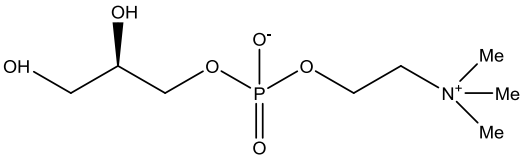
MM
(*N*-Methylmorpholine)



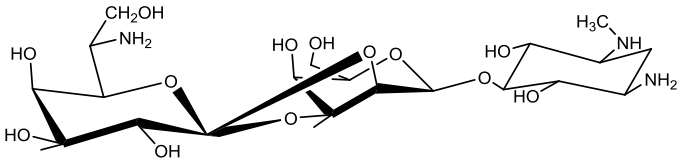


Pentoxifyline

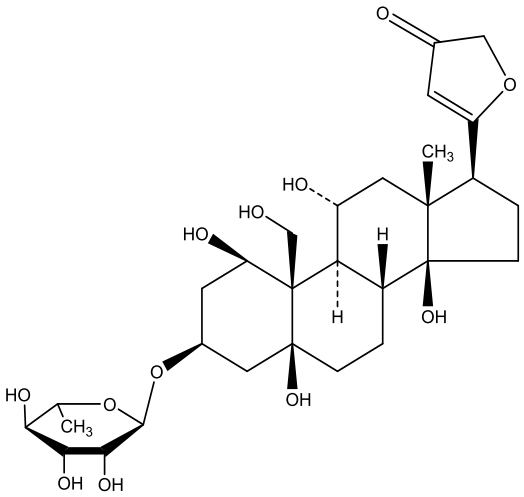
Theophylline



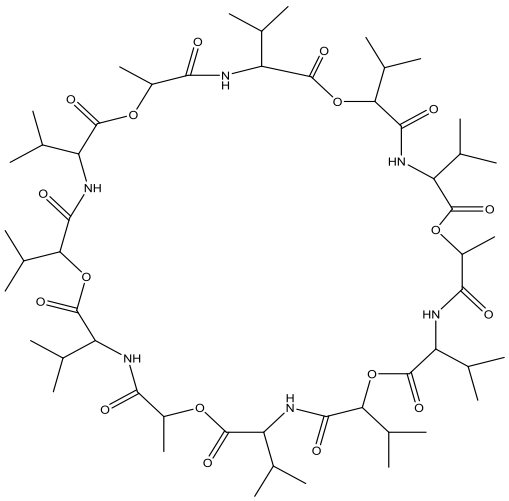
Glycerol phosphorylcholine



Hygromycin



Ouabain



Valinomycin

Ion toxicity test

To evaluate the degree of cell membrane permeability as a result of antifungal agents, fungal cell toxicity in the presence of different concentrations of sodium and calcium solutions was determined [8,9]. Ca^{2+} and Na^{+} added to the medium as $CaCl_2$ and NaCl solutions of $CaCl_2$ and NaCl were sterilized separately and added to the medium at room temperature. Angelicin, compound 1, and Amphotericin B were used in the first well concentration of equal to 250, 125, 70 (µg/ml), respectively. To read the results of ranges of inhibition due to higher concentrations of cations, medium observation is necessary to be done by microscope (especially for Ca^{2+} containing wells), as precipitation hinders naked-eye observation. In this experiment, an Olympus CK_2 inverted microscope with a magnification of 40 was used.

pH estimation using NMR

Chemicals: Growth medium RPMI; contained 0.8 g/L sodium phosphate dibasic, and fortified by the addition of potassium phosphate to a final concentration of 15 mM. Glycerol phosphorylcholine was obtained from sigma and used for calibration purpose [10].

Preparation of cell suspension for NMR

For studies involving glucose, cells were grown to mid-log phase in RPMI (with 15 mM K_3PO_4) medium in plates as a steady culture at 25°C for 20 h. Likewise, SDA was used to pre-grow cells. Cultures were then cooled on ice to 4°C with gentle agitation, and cells were harvested by centrifugation and washed twice with fresh RPMI (+Pi) medium [11]. Finally, the cells were

suspended in RPMI (+Pi) containing D_2O (20%). At the times 20 and 40 min.

The solutions of methanol (to make 15% v/v in the cell suspension) or compound 2 and 1 in methanol (770 and 57 µg/ml cell suspension respectively), and glucose (10.1 mg) were added to the NMR tube, respectively.

^{31}P-NMR spectroscopy: NMR spectra were obtained at 202.3 MHz using a Varian Unit-500 MHz spectrometer operating in the fourier-transform mode. Each spectrum was acquired with 45° pulses at a repetition rate of 1.60 s and 1050-10,000 scans were accumulated. Peak position of inorganic phosphate dissolved in the culture medium used as an internal reference, while the samples containing the test solutions were adjusted and plotted according to this reference. Temperature was ambient throughout the measurements. Cells were transferred to 5-mm NMR tubes and made to a density of about 2×109 cells/ml in RPMI (with 15 mM K_3PO_4 added) medium with final concentration of 20% (v/v) D_2O. D-Glucose was added to the medium in a concentration of 86 mM in the specified groups. The inorganic phosphate was used for calibration of the chemical shifts of orthophosphates for the estimation of changes in intra and extracellular pH.

RESULTS

Effect on ergosterol and ergosterol-related targets: One of the early objectives of this study is to determine the similarity of action between the coumarin antifungals and the antifungal drugs that are on the market. For this purpose, the interaction studies carried out show that there is an antagonistic relationship between ketoconazole and compound 1 (Fig. 1) [12]. This interaction has an FIC value of 250 for *C. neoformans* and 3.9 for *S. cerevisiae*. In both cases, the antagonism is evident.

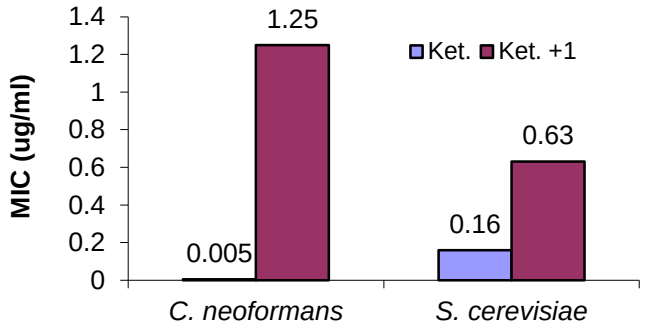


Fig. 1. Interaction between ketoconazole and compound 1. Medium, RPMI 1640, with MOPS buffer, inoculum 5×10^3 (CFU/ml), temperature 37ºC, incubation period 48 h.

Since the fungal cell adjust its interior pH by outwards pumping of protons through the action of membrane bound H^+-ATPase, damage to the cell membrane could mean inwards penetration of H^+ and consequent cytotoxicity due to intracellular pH change. Azole drugs cause a general disturbance by decreasing ergosterol and increasing concentration of lanosterol in the fungal cell membrane. However, ketoconazole did not show any pH-dependency in its activity. Therefore gradient-forced penetration of protons into the fungal cell should not be happening.

At this point the mechanism of pH-dependent activity of coumarin compounds becomes of interest. In order to investigate the above phenomenon further, reviewing the possible mechanisms could be quite helpful. Since the intracellular pH of fungal cell is about 7.4, there is an inward gradient of protons which helps co-transport of nutrients like amino acids into the cell. Therefore, any decrease in the intracellular pH or increase in the extracellular pH could be harmful for the fungal cell. Since many kinds of nutrients cannot be imported into the cell. The pH-dependency of omeprazole (a H^+-ATPase inhibitor) action (Fig. 2) and synergism of compound 1 with other agents affecting ATPase enzymes (Fig. 3 and 4), like ouabain and Na_3 VO_4, or cell membrane potential, like hygromycin band valinomycin, is indicative of the fact that coumarins may change the cell membrane potential possibly through inhibition of H^+-ATPase.

Since the main system for controlling cellular pH and membrane potential in fungal cells is the action of H^+-ATPase, its blockage can lead to serious interruptions in cell functions and cause abnormalities such as sensitivity to weak acids. The acetate loading sensitivity of C. albicans in the presence of angelicin and compound 1 (Fig. 5), supports this idea.

One of the side effects of inhibiting H^+-ATPase in the fungal cell would be lower acid secreting capacity, which translates into higher medium pH. Measurement of medium acidification can be easily carried out by fast responding pH meters. However, a better method is to use NMR to examine the peak positions of phosphorous in the extra and intracellular environment. Since the peak position of phosphorous is pH-dependent (Fig. 6), it can be used to track down the changes in pH values in vivo and even estimate the pH value.

By looking at Figure 7, it becomes clear that the chemical shift of Pi (int) peak in C. albicans cells treated with angelicin is moved upfield. This trend in the displacement of peak position is corresponding to a more acidic cytoplasm. The results of glucose induced medium acidification is shown in Fig 8. In this case, the cytoplasmic acidity of the angelicin treated group is again more than the control group, as represented by an upfield shift of Pi (int) peak. In addition, acidification of the

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medium is much less than the control group, as indicated by a less upfield shift in the Pi (ext) peak position. These experiments all support the hypothesis that H^+-ATPase is inhibited and the cell cannot pump out the protons generated by metabolism, thus causing intracellular pH to drop. Polyphosphate peak in Fig 8 (PP4-n) shows a big shift too. Since most of the polyphosphates is located in the vacuole, this is indicative of a change in the vacuole pH. In fact, there is special H^+-ATPase located in the vacuole membrane, which help in adjusting intracellular pH. This enzyme pumps protons into the vacuole and the resulting cotransport will transfer some nutrients into the vacuole. Since the pH of the vacuole has been changed, it is possible that vacuolar H^+-ATPase has also been inhibited by coumarins. Fig 9 shows that compound 1 has an even larger inhibitory effect on medium acidification by *C. albicans.*

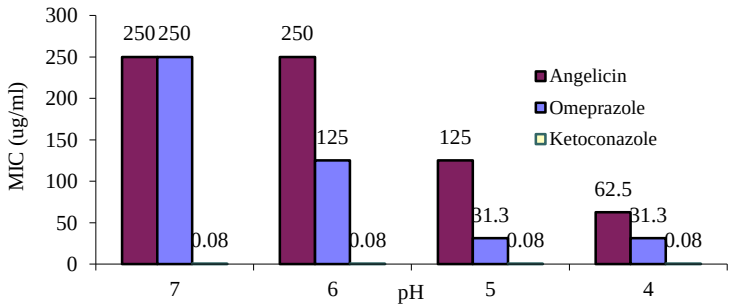

Fig. 2. Variation of MIC value of angelicin with pH against *C. albicans* in RPMI 1640 without addition of MOPS grown at 37ºC for 24 h, inoculum 5×10^3 (CFU/ml). Ketoconazole values are 10×MIC.

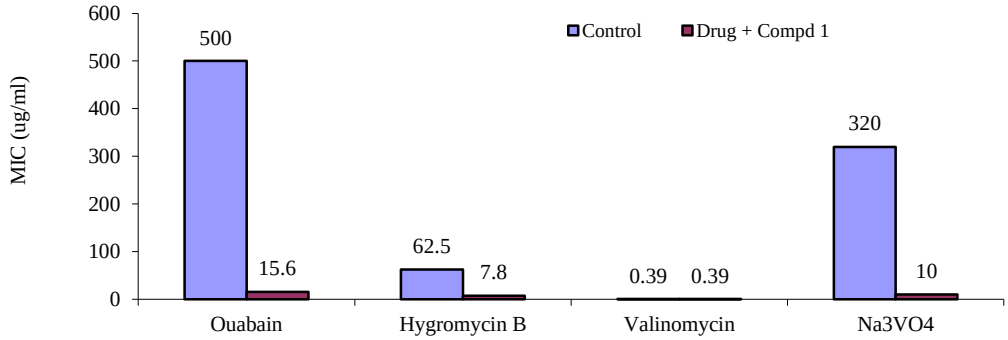

Fig. 3. Variation of MIC values (g/ml) of selected ATPase modulating compounds in broth dilution test against C. albicans with and without co-incubation with compound 1. Medium, RPMI 1640, pH=7, concentration of compound 1, 3.9 (g/ml); temperature 37ºC; incubation period 24 h; inoculum, 5×10^3 (CFU/ml).

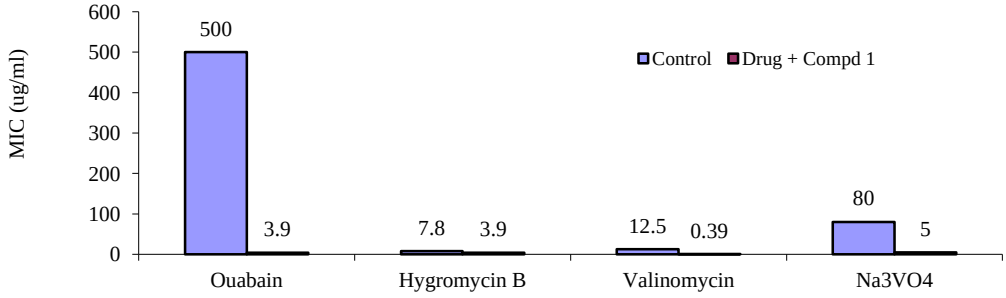

Fig. 4. Variation of MIC values (g/ml) of selected ATPase modulating compounds in broth dilution test against *C. neoformans* with and without co-incubation with compound 1. Medium, RPMI 1640, pH=7, concentration of compound 1, 3.9 (g/ml); temperature 37ºC; incubation period 48 h; inoculum, 5×10^3 (CFU/ml).

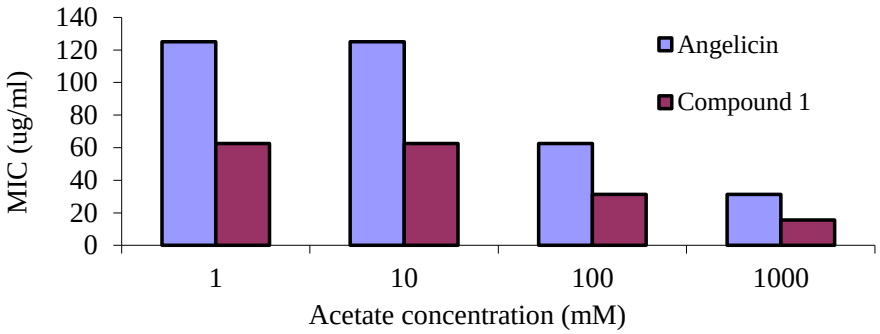

Fig. 5. The acid loading sensitivity of *C. albicans* to angelicin and compound 1 in the presence of different concentrations of sodium acetate. Medium RPMI, pH = 7, incubated at 37ºC for 24 h, inoculum 5×10^3 CFU/ml.

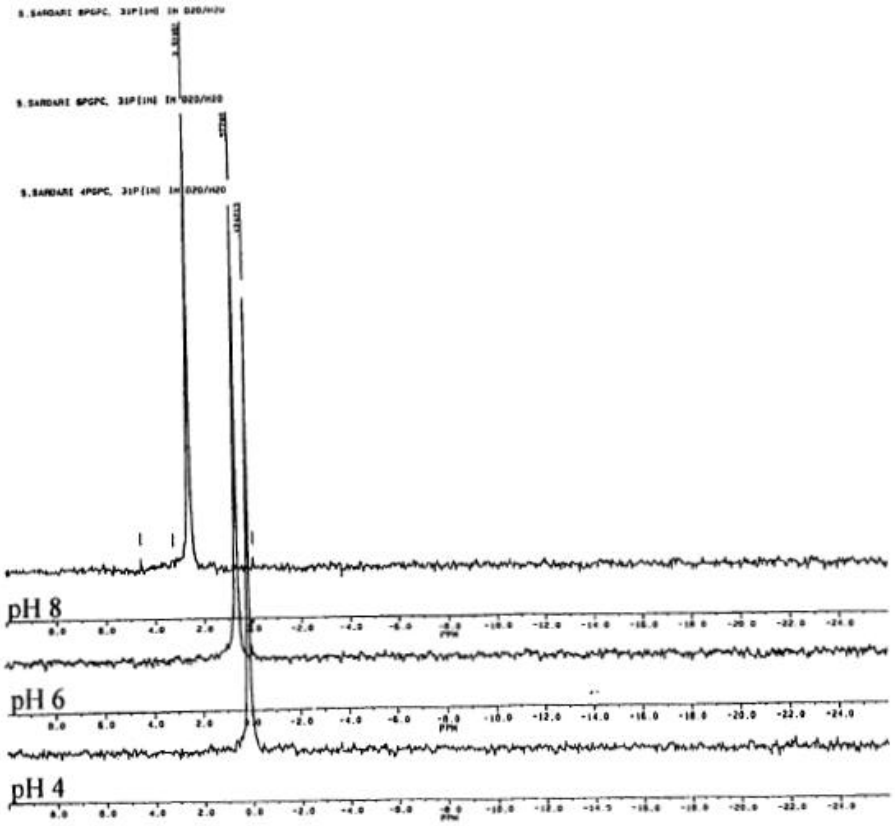

Fig. 6. ^{31}P-NMR of glycerol phosphorylcholine in RPMI (+P) at different pH values.

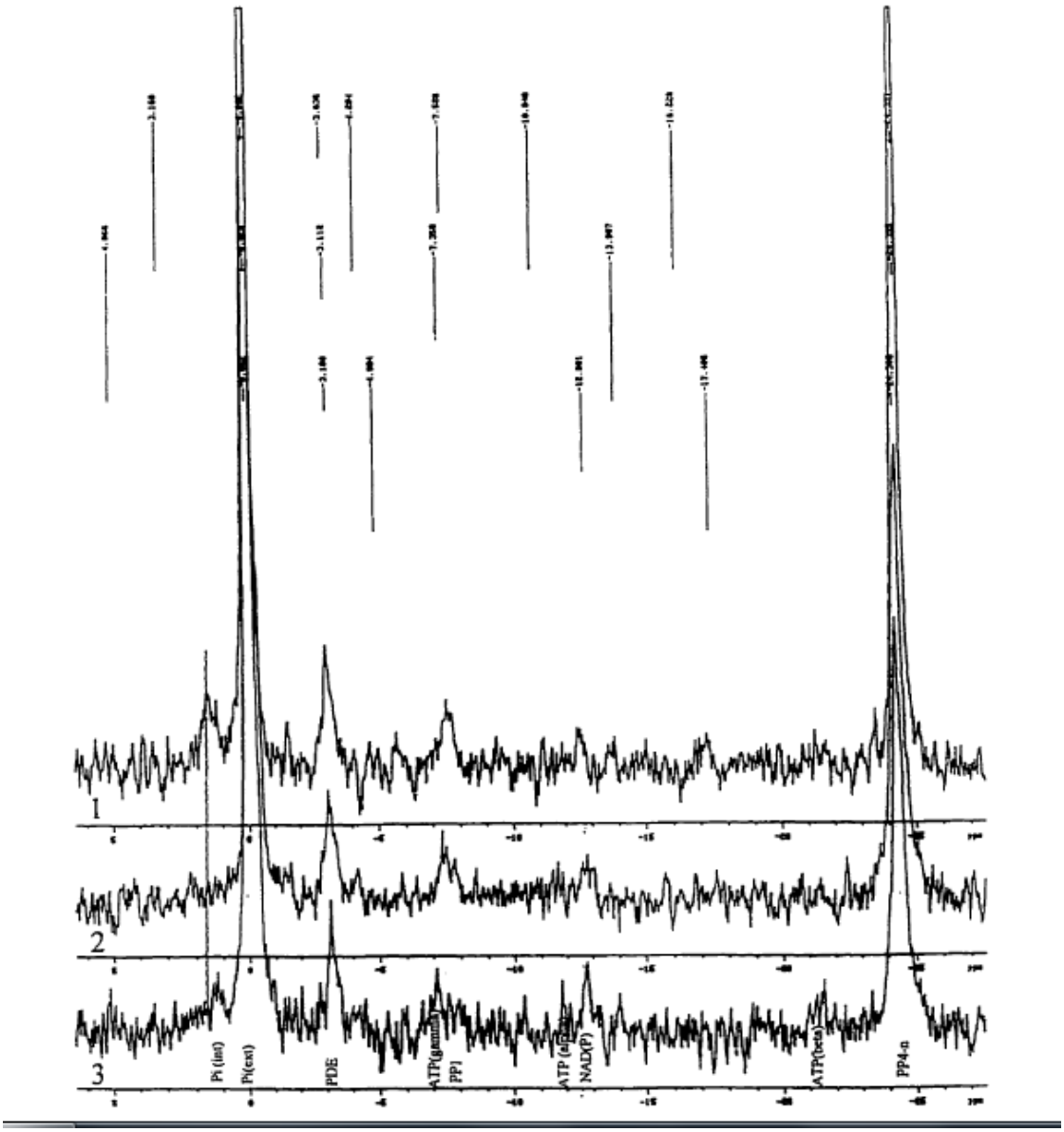

Fig. 7. ^{31}P-NMR spectra of C. *albicans* cells grown in RP1v11 (+P) at 25° C, (1) control group, (2) solvent control (methanol), (3) angelicin administered group. Pi(int), inorganic phosphate intracellular; Pi(ext), inorganic phosphate extracellular; PDE, phosphodiester peak from cell wall components; ATP (γ), the gamma phosphorous of ATP; PPI, terminal phosphorous of polyphosphates; ATP (α), alpha phosphorous of ATP; NAD(P), phosphorous peak of NADP; ATP(β), beta phosphorous of ATP; PP4-n, long chain vacuolar polyphosphate. The vertical line cutting the spectra is aligned with the chemical shift of control group spectrum.

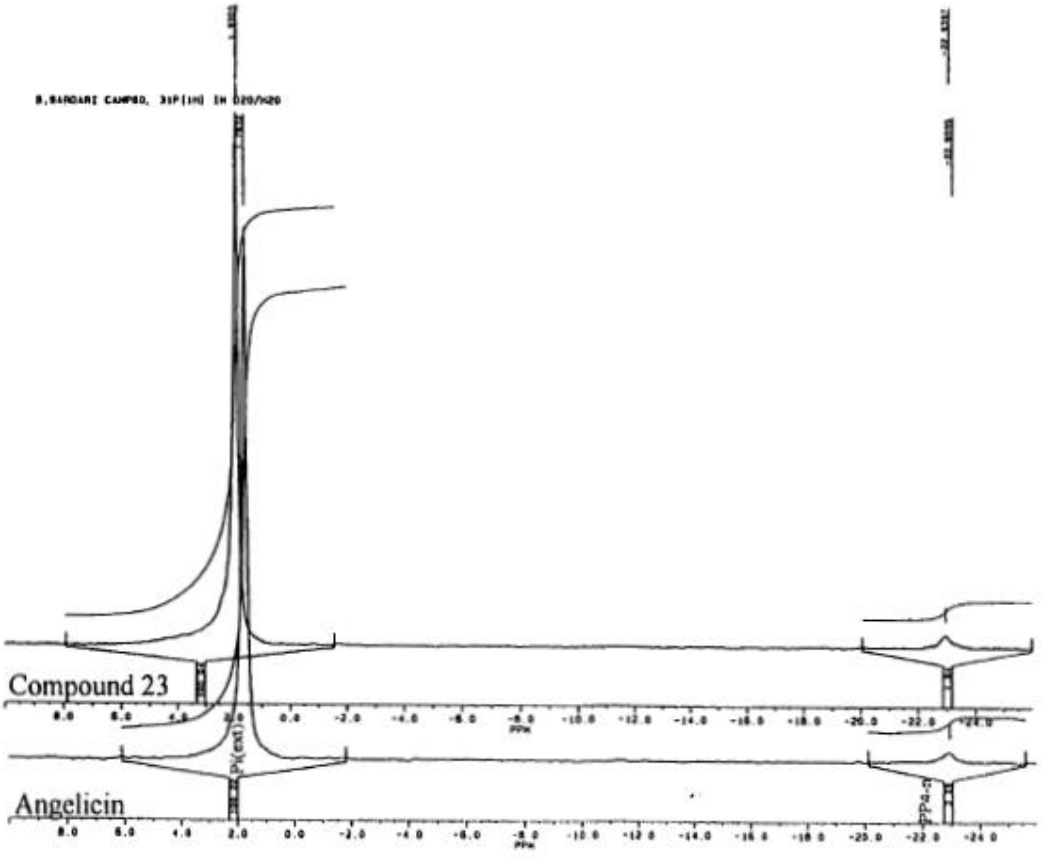

Fig. 9. ^{31}P-NMR of *C. albicans* treated with angelicin and compound 1. Growth condition: medium RPMI (+P), temperature 25° C, incubation period 20 h.

DISCUSSION

Diagnosing the mechanisms of action of known drugs or new agents is an ongoing process in drug development. In many cases including known drugs, novel actions that are not identified before may be found and this could lead to refining of their therapeutic profile [13-16]. One example is the azole antifungals, which show calmodulin modulating activity in addition to the known ergosterol biosynthesis inhibition [17-20].

Coumarins are shown to have many different bioactivities. Coumarin and its major human metabolite, 7-hydroxycoumarin, either as a single agent or in combination with immune modulators, have demonstrated significant antitumor activity without any noticeable symptomatic side effects [21,22]. Among the bioactivities of coumarins,

photoreactivation has been the subject of many studies. However, only a few reports have studied other cellular processes which could involve in the cell inhibition caused by coumarins. It has been demonstrated that coumarin affect neither the cytoskeleton arrangement nor induce depolymerization of microtubules [23-27]. In ion toxicity tests the number of colonies and growth level in each colony showed a significant decrease together with higher levels of Ca^{2+} and Na^{+} for increasing concentrations of AmB, angelicin (not so strong for Na^{+}, and DMSO. This is a sign of membrane perturbance. Altered sensitivities to NaCl and $CaCl_2$ suggest a change in ion permeability as a result of the alteration of the membrane sterol composition [28,29]. Yeasts have various mechanisms which allow them to grow with high Ca^{2+} concentrations. For example, vacuoles possess a potent Ca^{2+} uptake system driven by an H^{+}-ATPase and serve as an intracellular store for Ca^{2+}. Therefore, an increased sensitivity to this ion can be indicative of inhibited vacuolar H^{+}-ATPase or large-scale membrane damage leading to high Ca^{2+} influx [30-33]. A mutant of *S. cerevisiae* which has a disrupted ERG3 gene coding for sterol C-5 desaturase, an enzyme of the ergosterol biosynthetic pathway. Has been identified. This mutant is more sensitive to high Ca^{2+} of the medium [34]. Membrane ergosterol presumably serves as a nonspecific barrier against various chemicals in the environment [35-38]. To our knowledge, this is the first time the acid dependency and possible ATPase inhibitory activity of coumarins are studied and reported [39,40].

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