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# Exploring the anti-biofilm activity of cinnamic acid derivatives in *Candida albicans*

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ABSTRACT

Some compounds, characterized by phenylethenyl moiety, such as methyl cinnamate and caffeic acid phenethyl ester, are able to inhibit *C. albicans* biofilm formation. On these bases, and as a consequence of our previous work, we synthesized a series of cinnamoyl ester and amide derivatives in order to evaluate them for the activity against *C. albicans* biofilm and planktonically grown cells.

The most active compounds **7** and **8** showed ⩾50% biofilm inhibition concentrations ($BMIC_{50}$) of 2 μg/mL and 4 μg/mL respectively, against *C. albicans* biofilm formation; otherwise, **7** showed an interesting activity also against mature biofilm, with $BMIC_{50}$ of 8 μg/mL.



*Candida albicans* (*C. albicans*) is the most common fungal pathogen in clinical settings and it is responsible of infections that can affect the skin and the mucosa or cause life-threatening systemic disease.[1,2] The mortality among patients with invasive candidiasis is as higher than 40%, even when patients receive antifungal therapy.[3]

One of the major problem related to the treatment of *C. albicans* infections is therapeutic failure, especially due to the onset of therapeutic resistance phenomena, which are very often associated with the biofilm formation.[4]

Biofilms are defined as complex microbial communities encased in a matrix of extracellular polymeric substances, that develops when a community of microorganisms irreversibly adheres to an inert or living surface. Contact with a solid surface triggers the expression of a panel of enzymes, which catalyze the formation of sticky polysaccharides that promote the surface colonization and the microbial cells protection. This adherent community is considered an important virulence factor because it is difficult to eradicate and often responsible for treatment failures.[5] Indeed, the biofilm represents a physical barrier that prevents drugs from entering and expressing their activity.

In *C. albicans* the morphogenesis and the biofilm formation are controlled by a complex mechanism of communication termed *quorum sensing* (QS), a process that is sensitive to the cell density in the biofilm population. The QS is based on the exchange and sensing of low molecular weight signal compounds,[6] in particular, three substances have been identified as QS molecules: farnesol, phenylethyl alcohol and tryptophol.[7] However, the antifungal resistance of *C. albicans* biofilm is complex and it involves not only the physical barrier due to the polysaccharide layer, but a number of different mechanisms, such as over-expression of efflux pumps, genetic changes of drug targets, persister cells, biofilm-host immune system interaction.[8]

Azoles currently used for the treatment of systemic infections (*e.g.* fluconazole, itraconazole) have little effect against *Candida* biofilms, even at high doses and in combination with caspofungin.[9] For these reasons, new therapies are urgently needed to treat the wide variety of *Candida* biofilm infections in the medical setting.

In literature there are few compounds able to inhibit *C. albicans* biofilm formation,[10] among them there are methyl cinnamate and caffeic acid phenethyl ester (CAPE), characterized by phenylethenyl moiety.[11] Based on these evidences, in our previous work we tested, against *C. albicans* planktonic and biofilm cells, a series of caffeic and cinnamic acid derivatives (Chart 1).

By referring to the structure of CAPE and its biological properties,[12–14] we synthesized several ester and amide compounds with simple chains or several more complex groups. Furthermore, we

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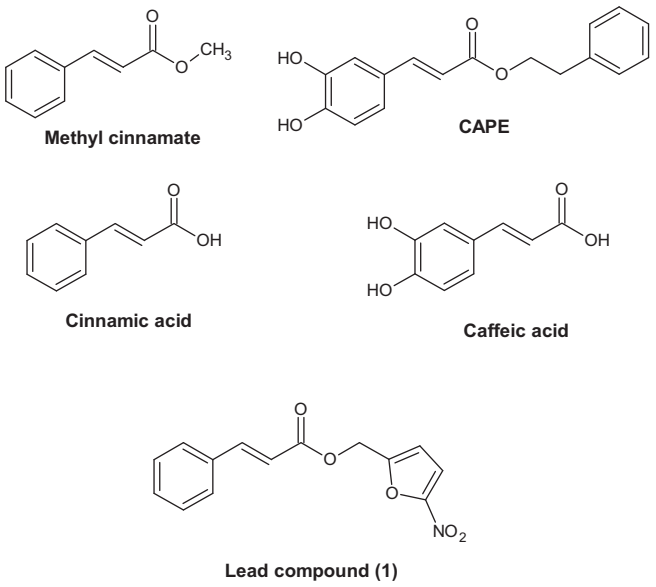



**Chart 1.** Compounds characterized by a phenylethenyl moiety and our lead compound **1**.

have removed the two hydroxylic groups, typical of caffeic acid derivatives, saturated or removed the ethenyl double bond of the acylic moiety. As a result of that work, we identified a new cinnamate derivative ((5-nitrofuran-2-yl)methyl-(2*E*)-3-phenyl-prop-2-enoate (**1**) showing a good inhibition activity against *C. albicans* biofilm. It was able to inhibit the biofilm formation and to reduce the metabolic activity of preformed biofilm, better than the reference drug fluconazole.

Our basic SAR study has showed that the conjugation of the carbonyl group with an unsaturated system seems to be relevant for the anti-biofilm activity; furthermore, the presence of the hydroxylic groups appears to be not necessary.[15]

In this paper we report the synthesis and the activity evaluation, on *C. albicans* biofilm, of a new series of cinnamoyl ester and amide derivatives that have been designed with the idea of preserving the cinnamoyl moiety and removing the hydroxyl groups of caffeic acid, in order to obtain an improved chemical stability and a simplified reaction work up.

We also investigated the importance of the nitro group, present in our reference compound (**1**), forasmuch as, it is known from the literature that compounds able to induce the production of reactive oxygen species (ROS) possess a potential antibiofilm activity.[16,17] The ROS are usually generated by *in vivo* electron transfer processes that involve specific chemical functionalities including aromatic nitro compounds.[18] In this context, we have synthesized several ester and amide derivatives combining the carboxylic function of cinnamic acid with different groups, including nitro heterocyclic groups, azole and indole rings (Chart 2).

We also synthesized some molecular hybrids between cinnamic acid and other compound endowed with own antifungal activity. In particular, we have chosen molecular fragment of anti-biofilm compounds as miconazole (**7**) and molecules that are active against planktonic cells as fluconazole (**8**).[19]

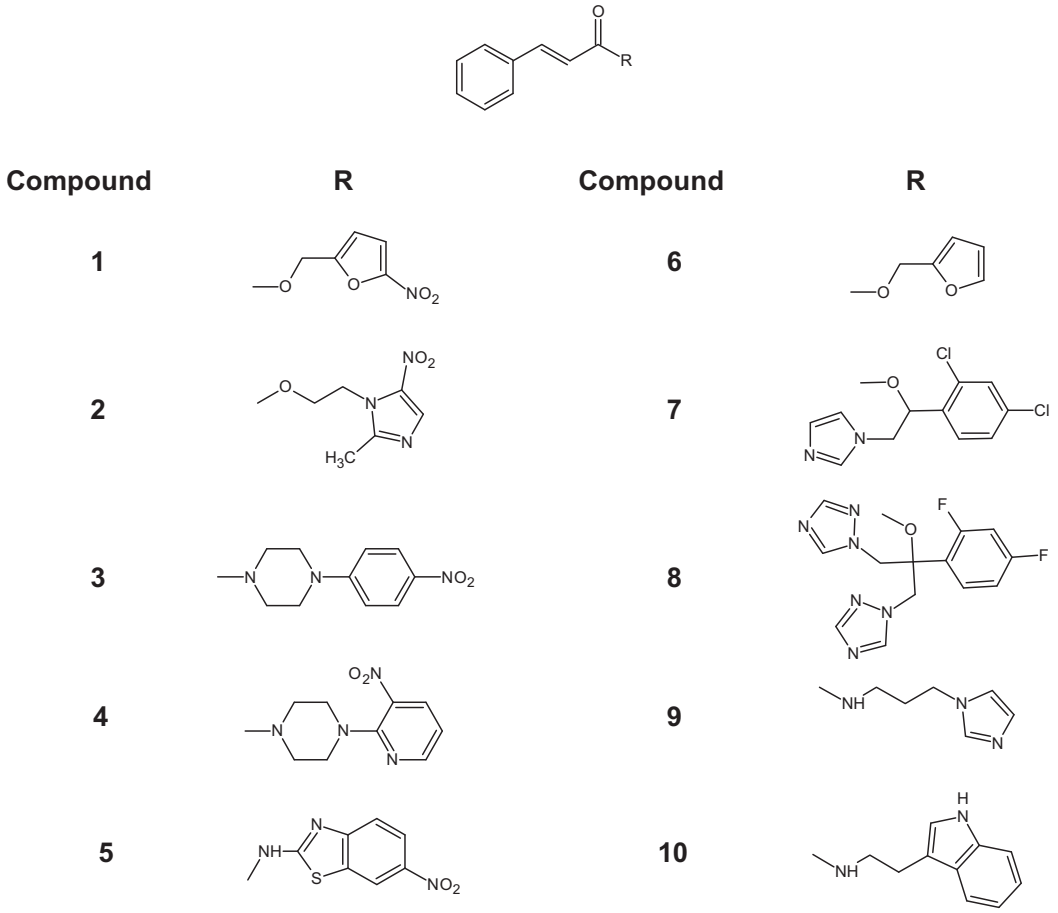
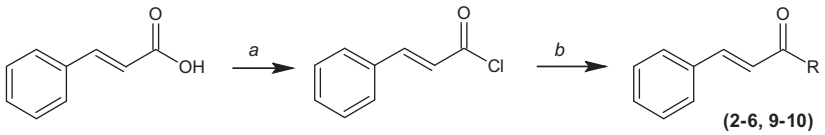



**Chart 2.** Studied cinnamic acid derivatives **1**–**10**.




**Scheme 1.** General procedure for the synthesis of **2**–**6**, **9**–**10**. (a) $SOCl_2$, 2 h, reflux; (b) opportune alcohol or amine, TEA, DCM, 12 h, reflux (**2**–**6**, **9**) or r.t. (**10**).

Furthermore, we synthesized the derivative **10**, a hybrid between cinnamic acid and tryptamine, because it is demonstrated that compounds, such as indole and 3-indolylacetonitrile, are able to reduce biofilm formation and virulence, by the regulation of NRG1, a transcriptional factor that influences filamentation and biofilm formation in *C. albicans*.[20]

The synthetic procedures of the studied compounds are illustrated in the Schemes 1 and 2; briefly, the cinnamic acid was activated to cinnamoyl chloride by the treatment with $SOCl_2$, then the opportune alcohol or amine were added to synthesize the corresponding ester (**2**, **6**) or amide (**3**–**5**, **9**, **10**).

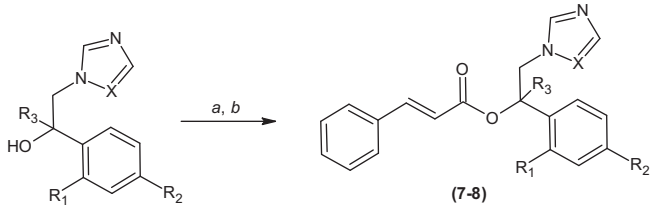
The compounds **2**, **5** and **10** were synthesized modifying the procedures reported in literature.[21–23]

For the synthesis of the compound **2**, the metronidazole was obtained by extraction with dichloromethane after shattering commercial tablets in warm water[24]; whereas, the 1-(3-nitropyridin-2-yl)piperazine, necessary to synthesize compound **4**, was prepared as we have previously reported.[25]

Attempts to synthesize esters **7** and **8** using the above illustrated scheme were unsuccessful, then it was necessary to preventively activate the alcoholic function by NaH before the reaction with cinnamoyl chloride.

The detailed synthetic procedures, the analytical and spectroscopic data of new synthesized compounds are reported in the supplementary material and are in agreement with the proposed structures.

Cinnamic acid, its derivatives **2**–**10** and parent alcohol or amine compounds, have been screened to evaluate the activity against planktonically growing *C. albicans* cells and against *C. albicans* biofilm formation and preformed. For the *in vitro* antifungal and antibiofilm experiments, described in detail in the supplementary material, *C. albicans* ATCC 10231 was used. This strain is sensitive to fluconazole on planktonic cells ($MIC_{50}$ 0.5 μg/mL) and it is resistant in the different phases of biofilm formation ($BMIC_{50}$ 128 μg/mL and >128 μg/mL respectively on biofilm formation and mature biofilm). The minimal inhibitory concentration (MIC) was calculated and expressed as the lowest drug concentration at which a significant decrease in turbidity (>50%) was detected in comparison with the control in the absence of drug. The 50% inhibitory concentration ($BMIC_{50}$) were defined as the concentrations causing 50% inhibition of either biofilm formation or preformed biofilms due to drug treatment.




**7**. $R_1$=$R_2$=Cl; $R_3$=H; X=CH

**8**. $R_1$=$R_2$=F; $R_3$=1H-1,2,4-triazole; X=N

**Scheme 2.** General procedure for the synthesis of **7**, **8**. (a) NaH, $CH_3CN$, 2 h, r. t. (**7**) or 50 °C (**8**); (b) cinnamoyl chloride, 12 h, r. t. (**7**) or 50 °C (**8**).

The results of the microbiological assays are summarized in Table 1, where the $MIC_{50}$ and $BMIC_{50}$ values of the synthesized cinnamic derivatives **2**–**10** are compared to the earlier published compound **1**, cinnamic acid and parent alcohol and amine compounds.

In most cases the presence of the cinnamic moiety produces an increase of the antibiofilm activity, particularly for the biofilm formation, as can be observed for compounds **2**, **6**, **7**, **8** and **10** with respect of the corresponding amines or alcohols. The only exception is presented by the compound **5**, for which was observed an increase of the $BMIC_{50}$ values compared to the corresponding amine.

The activities of compounds **2**–**5** on mature biofilm, on biofilm formation, as well on planktonic cells, range from 32 to >128 μg/mL. These data indicate that the replacement of the nitrofuran moiety of the reference compound **1** with other nitroheterocyclic groups, as nitroimidazole (**2**), nitropyridine and (**4**) nitrobenzothiazole (**5**), or nitrophenyl group (**3**), resulted in a significant reduction of the antibiofilm activity. Furthermore, the removal of the nitro group from the compound **1** to obtain the furan derivative **6** did not produce significant changes against biofilm in formation but it reduced the activity on preformed biofilm, as the $BMIC_{50}$ increased from 64 to 128 μg/mL, and more markedly on planktonic cells, as the $MIC_{50}$ increased from 2 to 64 μg/mL.

Taken together, the activity data for compound **2**–**5** compared to **1** indicate that the presence of nitrofuran moiety appears to be critical to obtain the anti-biofilm activity. These data also suggest that the antibiofilm activity of the nitrofuran moiety could be due to a combined action on planktonic cells and on the biofilm, especially in the mature phases.

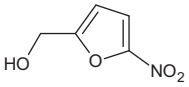
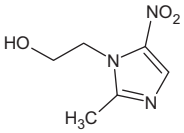
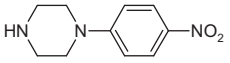
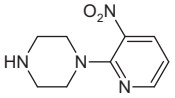
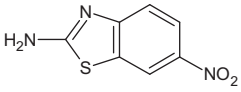
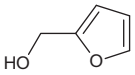
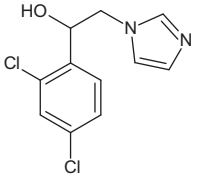
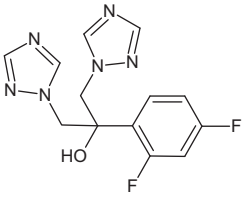
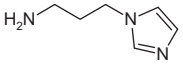
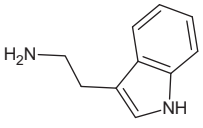
It is noteworthy that in this study we have identified other new interesting anti biofilm compounds. Cinnamic acid not showed antibiofilm activity, as reported in Table 1, but combining it with molecular fragments that are present in antifungal drugs, new compounds have been obtained with antibiofilm activities.

In particular, the most active derivative **7**, containing a molecular fragment of miconazole, has shown inhibition of biofilm in formation at the concentration of 2 μg/mL and reduction in metabolic activity of preformed biofilm at 8 μg/mL, lower than the lead compound **1** (16 and 64 μg/mL, respectively) and the reference drug fluconazole (128 and >128 μg/mL, respectively).

Finally, the esterification of fluconazole with cinnamic acid, to produce the hybrid compound (**8**), it has led to a significant increase in the activity on biofilm formation ($BMIC_{50}$ 4 μg/mL vs 128 μg/mL) and in minor extent on preformed biofilm. On the other hand, the hybrid of cinnamic acid with tryptamine (**10**) have produced an enhancement of the anti-biofilm activity, selectively against biofilm in formation, with the reduction of $BMIC_{50}$ value from 128 μg/mL to 8 μg/mL.

In conclusion, the synthesis of amide and ester derivatives of cinnamic acid has allowed to identify new compounds endowed of antibiofilm activity, particularly effective in the early phases of biofilm formation. The cinnamic moiety does not seem to act as a simple lipophilic carrier but instead appears to be responsible for antibiofilm activities; in fact, in most cases, quite active esters or amides were obtained from amines or alcohols which do not have activity on biofilm (compounds **1**, **2**, **3** and **6** vs parent alcohols or amides). Furthermore, the cinnamic group enhances

**Table 1**
Antifungal activity of compounds **1**–**10**, cinnamic acid and the parent compounds against *C. albicans* ATCC 10231 biofilm and planktonic cells.

| Ester or amide compound | $BMIC_{50}$ (μg/mL) | | $MIC_{50}$ (μg/mL) Planktonic cells | Parent alcohol or amine |
|---|---|---|---|---|
| | **Mature biofilm** | **Biofilm formation** | | |
| **1** | 64<br>64 | 16<br>64 | 2<br>16 |  |
| **2** | >128<br>>128 | 32<br>>128 | 128<br>>128 |  |
| **3** | >32<br>>128 | >128<br>>128 | 32<br>>128 |  |
| **4** | >128<br>>128 | >128<br>>128 | >128<br>>128 |  |
| **5** | >128<br>64 | >128<br>64 | >128<br>>128 |  |
| **6** | 128<br>>128 | 16<br>>128 | 64<br>>128 |  |
| **7** | 8<br>64 | 2<br>64 | 2<br>16 |  |
| **8** | >32<br>>128 | 4<br>128 | 64<br>0.5 |  |
| **9** | >128<br>>128 | >128<br>>128 | >128<br>>128 |  |
| **10** | >128<br>>128 | 8<br>128 | >128<br>>128 |  |
| **Cinnamic acid** | >128 | >128 | >128 | |

The inhibition of biofilm formation and destruction of pre-formed biofilm by different compounds were evaluated by measuring the metabolic activity of cells within the biofilm (XTT assay). The BMIC end point for biofilm is based on the lowest drug concentration producing a decrease of 50% metabolic activity relative the untreated growth control. MIC end point for planktonic cells is based on lowest drug concentration that prevented 50% of growth with respect to the untreated control. At least two experiments were performed on two separate dates for each compound tested in triplicate. The results were expressed as median.

the weak antibiofilm activity of the 1-(2,5-dichlorophenyl)-2-(1H-imidazol-1-yl)ethanol, the parent alcohol of ester **7**, and confers good antibiofilm activity to fluconazole, the parent alcohol of ester **8**.

The activity data obtained for these cinnamic derivatives also indicate that the approach of combining into a hybrid, two molecular fragments characterized by their own activities, could be advantageously exploited to develop new compounds with antibiofilm activity.

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## A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2016.10.091. These data include MOL files and InChiKeys of the most important compounds described in this article.

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