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Specific Antidermatophytic Activity of Trifluoromethylthiolated Cinnamate Derivatives: A New Approach to the Therapy of Superficial Fungal Infections of the Skin.

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Abstract: Dermatophytosis is the most widespread fungal skin infection worldwide and directly affects patients' quality of life. They are challenging to manage because of the need for prolonged treatment and the development of resistance to antifungal agents. This article studies the antifungal activity of trifluoromethylthiolated cinnamate derivatives on *Candida* species and dermatophytes as well as possible mechanisms of action, irritability, and cytotoxicity tests. These molecules show activity against dermatophyte fungi with minimum inhibitory concentrations (MIC) as low as 0.39 $\mu\text{g mL}^{-1}$. In particular, chloroaromatic derivatives demonstrated the best inhibition profile (1.56 - 6.25 $\mu\text{g mL}^{-1}$). Importantly, our series of molecules were not active against *Candida* spp., being selective for dermatophytes. A study of the mechanism of action suggests that our SCF₃ cinnamates do not bind directly to ergosterol or the cell wall. The physicochemical parameters and the absence of irritability resulting from the HET-CAM test demonstrate that compound **8** can be considered a future candidate for the therapy of dermatophytosis.

Introduction

Dermatophytosis is the most common form of mycoses, affecting the skin, nails, and hair, caused by yeasts and filamentous fungi. It affects around 25% of the world's population. Dermatophytosis is characterized when the etiologic agent isolated is a keratinophilic filamentous fungus, also known as tinea or ringworm.^[1, 2]

The most common clinical manifestations are erythematous, circular surface skin lesions and nail infections with discoloration, scaling, thickening, and separation from the nail bed. However, these symptoms presented by skin lesions can be easily confused with some other skin diseases, such as rosacea, psoriasis, lupus erythematosus, or impetigo, making clinical diagnosis difficult.^[3] Although cutaneous manifestations are prevailing, invasive dermatophytosis can also occur. The latter condition is attributed to immunocompromised individuals, the use of

immunosuppressants, HIV infection, and solid organ transplantation.^[4]

Dermatophytosis directly and significantly affects patients' quality of life. Economic, social, physiological, and even psychological aspects, such as low self-esteem, are involved in these infections.^[5, 6] Recently, cases of tinea faciei have emerged due to the COVID-19 pandemic, as prolonged use of masks causes an area of occlusion, heat, and sweating in a tropical climate.^[7, 8] Dermatophytosis is treated with topical and/or systemic (oral) antifungal agents. The main classes of antifungals used are allylamines, triazoles, and imidazoles. However, the long-term nature of the treatment and certain side effects mean that patients do not adhere well to the treatment, and, as a result, recalcitrant infections are reported. Discontinued treatment and prolonged duration also trigger the selection of resistant strains, which become even more frequent.^[9]

Numerous fluorinated compounds were synthesized and studied in the pharmaceutical sciences and widely applied to the therapy of various pathologies; it includes antineoplastic agents, anesthetics, anti-inflammatory agents, and antidepressants.^[10] Examples of molecules containing one or more fluorine atoms in their structure that are commonly used in medicinal chemistry are vinflurine (antineoplastic), eflornithine (facial hirsutism in women), prozac (antidepressant), 5-fluorouracil (antineoplastic), and fluoroquinolones (antibiotics).^[11] Discovered in the 1960s, the fluoroquinolones are remarkably effective antibiotics with excellent pharmacokinetic properties and a broad-spectrum antimicrobial activity. However, adverse effects and development of resistance have led researchers to design new generations of fluoroquinolones for even more effective treatments.^[12]

Furthermore, fluoroquinolones have been the subject of in-depth studies for their antifungal activity, as well as for their synergistic relationship in combination with other antifungal agents.^[13, 14] Another example is Flucytosine, a prodrug of 5-fluorouracil first synthesized in 1957 as an antitumor agent, which was subsequently developed for its antifungal activity and is now used in the treatment of systemic candidiasis and cryptococcosis in combination with amphotericin B.^[15]

The trifluoromethylthio group (SCF₃) is an emerging motif that has become extraordinarily popular as a fluorine-containing motif in drug design due to its strong electron-withdrawing power and its high lipophilicity. In this context, the present study aims to evaluate the spectrum of activity of trifluoromethylthiolated cinnamate derivatives on dermatophyte filamentous fungi and *Candida* sp. This study prompted the investigation of mechanisms of action and irritability tests. The main objective is to find new molecules with antifungal potential to combat fungal resistance and improve the therapeutic options available.

Methodology

Synthesis and characterization of molecules

(Z)-alkyl 3-aryl-2-((trifluoromethylthio)methyl)acrylates (**1-15**, and **17**) and (Z)-3-phenyl-2-((trifluoromethylthio)methyl)acrylonitrile (**16**) were prepared by direct nucleophilic trifluoromethylthiolation of the corresponding Morita-Baylis-Hillman carbonates following the procedure reported by one of us (Figure 1).^[16] All these compounds are known for which analytical data are identical to those reported in the literature: compounds **1** CAS [1636889-37-6], **2** CAS [1636889-45-6], **3** CAS [1636889-47-8], **4** CAS [1636889-41-2], **5** CAS [1636889-50-3], **6** CAS [1636889-38-7], **7** CAS [1636889-39-8], **8** CAS [1636889-40-1], **9** CAS [1636889-48-9], **10** CAS [1636889-49-0], **11** CAS [1636889-42-3], **12** CAS [1636889-43-4], **13** CAS [1636889-44-5], **14** CAS [1636889-51-4], **15** CAS [1636889-53-6], **16** CAS [1636889-58-1], **17** CAS [2301899-05-6].

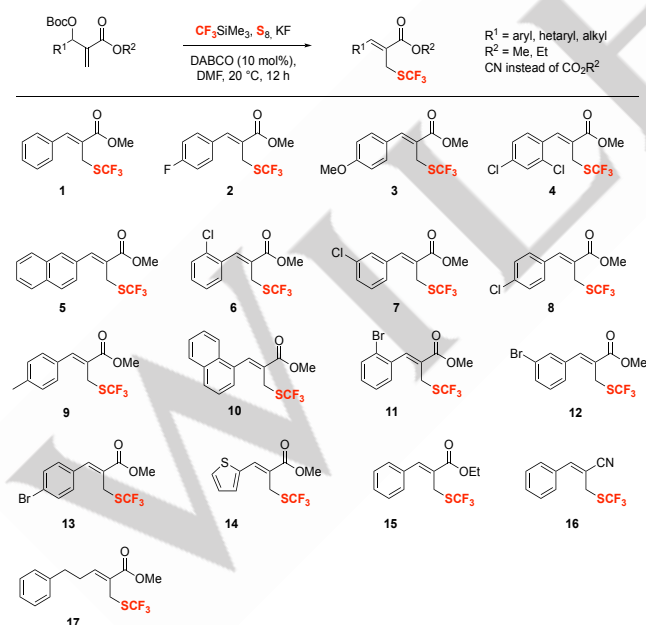


Figure 1. Synthesis of allylic trifluoromethylthio derivatives **1-17**.

In vitro antifungal susceptibility tests

Fungal strains: Yeast species of the genus *Candida* (*C. albicans* - ATCC 24433, *C. krusei* - CK02, *C. glabrata* - CG09, and *C. tropicalis* - CT72A) and dermatophytic filamentous fungi (*Microsporium canis* - MCA01, *Microsporium gypseum* - MGY01,

Trichophyton mentagrophytes - TME16, and *Trichophyton rubrum* - TRU45) were selected for the evaluation of antifungal activity. These strains are stored in the mycology collection of the Micology Laboratory of the Universidade Federal do Rio Grande do Sul (UFRGS), in Porto Alegre, Brazil. For *Candida* sp. the isolate resistant, susceptible dose-dependent and multidrug resistant was defined based on Kuriyama *et al.*^[17] and CLSI breakpoints.^[18,19] As for dermatophytes, the resistance (in the sense of reduced susceptibility) was established according to the increase in minimal inhibitory concentration (MIC) values for some clinical strains in relation to the majority, considering the following resistance threshold concentrations: terbinafine - MIC ≥1 μg mL⁻¹, griseofulvin - MIC ≥4 μg mL⁻¹, and ketoconazole - MIC ≥8 μg mL⁻¹.

Antifungal agents: Terbinafine (TBF) and anidulafungin (AND), purity ≥97%, were supplied by Cristalia (Sao Paulo, Brazil); griseofulvin (GSF), purity ≥97%, was acquired from Wallace Pharmaceuticals (Mumbai, India); ketoconazole (KTZ), purity ≥96%, was obtained from All Chemistry (São Paulo, Brazil); itraconazole (ITZ), purity ≥97%, was supplied by Cassará laboratory (Dist. PHARMOS, Buenos Aires, Argentina); miconazole (MCZ), purity ≥97%, was supplied by Valdequímica Chemical Products (Sao Paulo, Brazil); voriconazole (VRC), purity ≥98%, was supplied by Pfizer (Sao Paulo, Brazil) and amphotericin B (AMB), purity ≥98%, was supplied by Inlab (Sao Paulo, Brazil). The preparation of stock and work solutions followed the recommendations of the CLS.^[18] The commercial antifungals were used as reference substances for comparison with the synthetic compounds in the tests conducted.

Determination of minimal inhibitory concentration (MIC): The series of synthetic fluorinated compounds (**1-17**) were evaluated for MIC determination. The MIC was obtained through susceptibility testing using the broth microdilution technique described in protocols M27-A3^[17] for yeast and M38-A2^[20] for filamentous fungi. A suspension of fungal cells was prepared in sterile saline (0.85%) from fresh colonies previously grown on sabouraud dextrose agar (SDA; Kasvi, Brazil) for *Candida* spp. and potato dextrose agar (PDA; Kasvi, Brazil) for dermatophytes. The cell density of each inoculum was adjusted to obtain a concentration of 0.5 - 2.5 × 10³ CFU mL⁻¹ for *Candida* spp. and 1 - 3 × 10³ CFU mL⁻¹ for dermatophytes in the wells of polystyrene plates (96 wells) containing the already microdiluted molecules (concentrations of 50 to 0.09 μg mL⁻¹). The susceptibility tests were performed with RPMI 1640 culture medium (containing L-glutamine, without sodium bicarbonate, buffered at pH 7.0 with MOPS 0.165 mol L⁻¹; Sigma-Aldrich). The plates were incubated at 35 ± 2 °C and 30 ± 2 °C (for *Candida* spp. and dermatophytes, respectively). Plate reading was performed after 2 and 4 days of incubation (*Candida* spp. and dermatophytes, respectively). The MIC was assumed as the lowest concentration of the antifungal agent capable of complete visual growth inhibition. In addition to the MIC reading, the minimum effective concentration (MEC) was also conducted for dermatophytes. MEC was considered the lowest concentration of the antifungal agent capable of causing

the growth of small, rounded, compact hyphal forms compared to the hyphal control growth.

Determination of minimal fungicidal concentration (MFC):

The determination of the MFC was carried out after visual reading of the susceptibility test plates for *Candida* spp. and dermatophytes. Aliquots of 10 μL from each well corresponding to MIC, 2 x MIC, and 4 x MIC, from the visual reading, were taken and deposited on the surface of SDA (for yeast) and PDA (for dermatophytes). Aliquots were spread with a Drigalski loop and incubated at 35 ± 2 °C and 30 ± 2 °C. After incubation, the plates were read to determine the number of colonies (CFU mL^{-1}). The MFC was defined as the lowest concentration that yielded up to three colonies.^[21]

Hen's Egg Test on the Chorioallantoic Membrane (HET-CAM)

White fertile eggs Lohmann (Lohmann selected Leghorn, LSL) were used in the HET-CAM test. The eggs were kept in incubation for 10 days ($38\text{--}39$ °C and humidity between 55 and 60%). On the 10th day, the egg shell (around the airspace) was carefully removed with a rotary tool (Dremel, WI). Afterwards, 0.3 mL of each substance was added in each egg, respectively (negative control: 0.9% saline; positive control: 0.1 M NaOH, **7** and **8** solutions). The irritant effect was observed in times of 30 seconds, 2 and 5 minutes after application of each substance. The result of the irritation score (IS) was given according to the equation 1, on a scale from 0 to 4.9 denoted nonirritant (or practically no irritation) and 5.0 to 21 denoted irritant (moderate/severe or extreme irritation).^[22]

Equation 1:

$$IS = \left(\left(\frac{(301 - \text{Hemorrhage Time})}{300} \right) \times 5 \right) + \left(\left(\frac{(301 - \text{Lysis Time})}{300} \right) \times 7 \right) + \left(\left(\frac{(301 - \text{Coagulation Time})}{300} \right) \times 9 \right)$$

Cytotoxicity

The 3T3 cell line was routinely cultured in 75 cm^2 flasks using DMEM supplemented with 10% heat inactivated fetal bovine serum (FBS, Sigma, St Louis, USA), 100 U mL^{-1} penicillin (Gibco, Paisley, UK), and 100 mg mL^{-1} streptomycin (Gibco, Paisley, UK). The cells were maintained at 37 °C in a humidified 5% CO_2 –95% air atmosphere. The cells were fed every 2–3 days, and sub-cultured once 70–80% confluence was reached. The cytotoxicity was evaluated through the MTT reduction and neutral red (NR) uptake assays as previously describe.^[23] The cells were seeded at a density of 100,000 cells per well in 96 well plates. Triton X-100 1% (Sigma-Aldrich, St. Louis, USA) was used as a positive control. Negative control cells were incubated in culture medium. Cytotoxicity was evaluated by incubating the cells with 1, 2, 6.25, 16, 32, and 64 $\mu\text{g mL}^{-1}$ of synthetic fluorinated compounds **7** and **8** for 24 h at 37 °C. As solutions for the compounds **7** and **8** were made up in DMSO, a solvent control of 0.1% DMSO was used.

Mechanisms of action

Ergosterol binding assay: Strains MCA01, MGY01, TME16, and TRU45 were previously cultured in SDA at 32 °C for approximately seven days. After this period, the test was performed according to the broth microdilution technique. Concentrations of 25, 50, and 100 $\mu\text{g mL}^{-1}$ of ergosterol were added to the culture media in the presence of compounds **7** and **8**. Amphotericin B was used as a control. Plates were incubated at 32 °C and read after seven days. This test evaluates the ability of molecules to bind to the ergosterol present in the fungal cell membrane.^[24]

Protective effect of sorbitol: This test is performed according to the broth microdilution technique. Molecules **7** and **8** were evaluated for their ability to cause damage to the fungal cell wall. 0.8 M of D-sorbitol was added to the culture medium as an osmotic protector. Strains MCA01, MGY01, TME16, and TRU45 were used in this test. Plates were incubated at 32 °C and read after seven days.^[24]

Statistical analysis

The results of cytotoxicity are presented as mean \pm standard error of the mean (SEM) from at least three independent experiments. Normality of the data distribution was assessed by the Kolmogorov-Smirnov normality test. Significance was accepted at $p < 0.05$. Statistical comparisons between groups were performed by one-way ANOVA followed by Bonferroni post-hoc.

Results and Discussion

The biological activity resulting from the performance of the tested derivatives **1-17** allowed us to analyze the structure-activity relationship (SAR). First, we were pleased to see that most of the tested compounds demonstrated selective antifungal action with the exception of compounds **2** ($R^1=4\text{-F-phenyl}$) and **3** ($R^1=4\text{-OMe-phenyl}$) that showed poor activity on both dermatophytic fungi and yeasts (Table 1). The highest activities were found for compounds **7** ($R^1=3\text{-Cl-phenyl}$) and **8** ($R^1=4\text{-Cl-phenyl}$), inhibiting all dermatophytes at low MICs. Indeed, compound **8** showed a MIC at 1.56 $\mu\text{g mL}^{-1}$ and a fungicidal effect from the concentration of 12.5 $\mu\text{g mL}^{-1}$ for resistant *T. mentagrophytes*. The susceptibility profile obtained for dermatophytes showed that molecule **17** ($R^1=\text{phenethyl}$) was the most active, performing the lowest MIC of 0.39 $\mu\text{g mL}^{-1}$ for *M. gypseum*, and *T. rubrum*. However, its performance for *T. mentagrophytes* was not satisfactory compared to the other molecules. The structure of **17** differs to other compounds as it features a two-methylene spacer between the phenyl and the allyl SCF_3 motif that are no longer conjugated, which may explain its different behaviour. The chlorine atom at the *meta* or *para* position of the aromatic ring was essential for the activity of **7** and **8** while the *ortho* position (compound **6**) considerably reduced the activity for all tested isolates.

Table 1. MIC/MEC/MFCs for dermatophytes and MIC/MFC to *Candida* sp. ($\mu\text{g mL}^{-1}$) for synthetic compounds (1-17) and antifungal drugs.

Compounds and antifungal drugs	Dermatophytes				<i>Candida</i> sp.			
	MCA01	MGY01	TME16 ^[b]	TRU45	ATCC 24433	CG09 ^[a]	CK02 ^[b]	CT72A ^[b]
1	>50/-/50	25/-/50	>50/-/50	25/-/50	50/50	50/50	50/50	50/50
2	25/-/50	12.5/-/25	25/-/50	12.5/-/25	12.5/12.5	12.5/12.5	25/25	12.5/12.5
3	50/-/50	50/-/50	50/-/50	50/-/50	50/50	25/25	25/50	25/25
4	>50/-/50	>50/-/50	>50/-/50	>50/-/50	>50/>50	>50/>50	>50/>50	>50/>50
5	>50/-/50	>50/-/50	>50/-/50	>50/-/50	>50/>50	>50/>50	>50/>50	>50/>50
6	8.33/-/12.5	20.83/-/25	50/-/6.25/	6.25/-/6.25	$\geq 50/\geq 50$	$\geq 50/\geq 50$	$\geq 50/\geq 50$	$\geq 50/\geq 50$
7	1.56/-/3.12	1.56/-/3.12	8.33/-/12.5	2.08/-/3.12	$\geq 50/\geq 50$	$\geq 50/\geq 50$	$\geq 50/\geq 50$	$\geq 50/\geq 50$
8	1.56/-/3.12	1.56/-/3.12	6.25/1.56/12.5	3.12/-/3.12	$\geq 50/\geq 50$	$\geq 50/\geq 50$	$\geq 50/\geq 50$	$\geq 50/\geq 50$
9	5.20/-/-	3.12/-/6.25	50/1.56/-	5.20/-/12.5	$\geq 50/\geq 50$	$\geq 50/\geq 50$	$\geq 50/\geq 50$	$\geq 50/\geq 50$
10	50/25/-	50/-/-	50/-/-	50/6.25/50	50 \geq 50	50 \geq 50	50 \geq 50	50 \geq 50
11	8.33/3.12/12.5	6.25/-/12.5	50/-/50	5.20/-/12.5	$\geq 50/\geq 50$	$\geq 50/\geq 50$	$\geq 50/\geq 50$	$\geq 50/\geq 50$
12	6.25/3.12/12.5	6.26/-/12.5	50/1.56/50	3.12/-/6.25	$\geq 50/\geq 50$	$\geq 50/\geq 50$	$\geq 50/\geq 50$	$\geq 50/\geq 50$
13	2.08/-/3.12	2.08/-/3.12	50/3.12/ 50	3.12/-/6.25	$\geq 50/\geq 50$	$\geq 50/\geq 50$	$\geq 50/\geq 50$	$\geq 50/\geq 50$
14	50/12.5/-	50/25/-	50/25/-	50/12.5/-	$\geq 50/\geq 50$	$\geq 50/\geq 50$	$\geq 50/\geq 50$	$\geq 50/\geq 50$
15	16.6/-/25	50/-/50	50/1.56/50	12.5/-/25	$\geq 50/\geq 50$	$\geq 50/\geq 50$	$\geq 50/\geq 50$	$\geq 50/\geq 50$
16	8.33/3.12/12.5	6.25/3.12/12.5	16.66/3.12/25	10.41/3.12/25	$\geq 50/\geq 50$	$\geq 50/\geq 50$	$\geq 50/\geq 50$	$\geq 50/\geq 50$
17	1.56/-/3.12	0.39/-/0.78	50/6.25/-	0.39/-/0.78	$\geq 50/\geq 50$	$\geq 50/\geq 50$	$\geq 50/\geq 50$	$\geq 50/\geq 50$
TBF	0.06	0.12	0.12	0.12	-	-	-	-
GSF	1 (S)	1 (S)	>32 (R)	1 (S)	-	-	-	-
KTZ	0.06	0.12	1	0.12	0.5(S)	32 (R)	1(S)	0.25 (S)
FCZ	-	-	-	-	2(S)	8 (DDS)	-	1 (S)
ITZ	-	-	-	-	-	32 (R)	0.5 (DDS)	0.25 (DDS)
MCZ	-	-	-	-	-	32 (R)	8 (R)	32 (R)
VRC	-	-	-	-	-	-	-	0.06 (S)

[a]: Multidrug-resistant fungal isolates; [b]: resistant fungal isolates; MIC: minimal inhibitory concentration; MEC: minimal effective concentration; MFC: minimal fungicidal concentration; MCA01: *Microsporum canis*; MGY01: *Microsporum gypseum*; TME16: *Trichophyton mentagrophytes*; TRU45: *Trichophyton rubrum*; ATCC 24433: *Candida albicans*; CK: *Candida krusei*; CG: *Candida glabrata*; CT: *Candida tropicalis*. TBF: terbinafine; KTZ: ketoconazole; FCZ: fluconazole; ITZ: itraconazole; MCZ: miconazole; VRC: voriconazole; R: resistant; S: susceptible; DDS: dose-dependent susceptibility.

Furthermore, the presence of two chlorine atoms on the aromatic ring at *ortho* and *para* positions in compound **4** was detrimental to the activity. Consequently, chlorine as a bulky *ortho* substituent decreases the activity possibly through a change in the conformation of the molecule, which cause a lower binding affinity to the active site. Chlorine substitution by fluorine at the *para* position as in **2** ($R^1=4\text{-F-phenyl}$) or by bromine at the *meta* or *para* positions as in **12** ($R^1=3\text{-Br-phenyl}$) and **13** ($R^1=4\text{-Br-phenyl}$) derivatives did not increase the activity. The positive role of an electron-withdrawing substituent on the phenyl was evidenced by comparison with the inactive compound **1** ($R^1=\text{phenyl}$). Conversely, electron-donating phenyl substituents proved to be detrimental to the activity as in **3** ($R^1=4\text{-OMe-phenyl}$) and/or the selectivity as with **9** ($R^1=4\text{-Me-phenyl}$), which demonstrated good activity except for *T. mentagrophytes*. Changing the phenyl by a

naphthyl group in compounds **5** ($R^1=2\text{-naphthyl}$) and **10** ($R^1=1\text{-naphthyl}$) resulted in a loss of activity. A similar observation was done by replacement of the phenyl in **1** by a thiophene in **14**. In addition, we replaced the methyl ester of **1** with the ethyl ester of **15** and the nitrile of **16** in order to increase the lipophilicity; however, no substantial increase in activity was observed. Our family of molecules featuring halogen atoms, both a SCF_3 and a chloroaromatic motives are potent candidates for the therapy of dermatophytosis. This contributes to the already bright development of halogenated compounds that are well described in the literature to present a broad range of biological activities.^[25-27] Of particular interest, fluorine is the smallest halogen, the most electronegative element of the periodic table and the carbon-fluorine bond is stronger and more stable than any other bond to carbon.^[28] Molecules containing fluorine atoms in their structure

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are usually more lipophilic and, consequently, more lipid-soluble. This lipophilicity allows fluorinated molecules to be easily integrated into membranes and to present significantly enhanced permeability.^[14,29]

The trifluoromethylthiolated cinnamate derivatives evaluated in our study showed substantial antifungal activity and high selectivity since the most active compounds have selective activity on the dermatophytic fungi versus yeasts. Such high selectivity of allylic SCF₃ derivatives on filamentous fungi has not been previously described in the literature. Some commercially available antifungals have a different spectrum of action, such as caspofungin, which has more significant activity against *Candida* species and reduced activity for some filamentous.^[30,31] However, most antifungals and new molecules under evaluation generally show activity extended to yeast and filamentous fungi. A previous study involving derivatives of allylic selenocyanate, with a similar structure to trifluoromethylthiolated cinnamate, demonstrated excellent activity against *Candida* species and, although less active, also showed activity against *Fusarium* species.^[32,33] The SCF₃ compound **8** and its selenocyanate analog have the trifluoromethylthio/selenocyanate groups as the only structural difference.^[32] Therefore, the trifluoromethylthio group may be related to the selectivity found in the activity between yeasts and dermatophytes. Our trifluoromethylthiolated cinnamate derivatives were submitted to the SwissADME program available online.^[34] The pharmacokinetic parameters of allylic SCF₃ on SwissADME alerted to the probable inhibition of the cytochromes P450 (CYPs) superfamily enzymes CYP2C9, CYP2C19, and CYP1A2. On the other hand, allylic selenocyanate derivatives did not show this tendency to inhibit CYPs.^[32] This characteristic may reinforce the hypothesis of the selectivity in the activity of allylic trifluoromethylthio derivatives since the CYP2C19 inhibition is suggested for all molecules (1-17). CYPs are present in plants, humans, animals, and fungi. These enzymes are responsible for protecting against xenobiotics and other environmental stresses.^[35] Some CYPs, such as CYP450, are found in mammalian cells and the yeast of the *Candida* genus.^[36] Some classic antifungals are reported to inhibit the CYP fungal (e.g. Fluconazole, Voriconazole, Itraconazole) responsible for inhibiting the enzyme 14 α -demethylase (CYP51), which demethylates lanosterol in the biosynthesis pathway of ergosterol.^[37] Therefore, we hypothesized that allylic trifluoromethylthio molecules interact with some CYP involved in biosynthetic processes crucial to the survival of dermatophytes but dispensable to yeasts. In this way, yeasts can use some alternative enzyme system to detoxify the action of the molecules.^[38] The screening of mechanisms of action shows that the exogenous ergosterol binding assay did not increase the MIC greater than three dilutions for molecules **7** and **8**, as seen in the control of amphotericin B (Table 2).

Table 2. MICs of ergosterol binding assay to **7**, **8** and amphotericin B against dermatophyte strains.

Compound/fungal strain	Reading ($\mu\text{g mL}^{-1}$)			
	E(-)	MIC ₁	MIC ₂	MIC ₃
AMB				
MCA01	0.5	1	2	8
MGY01	1	1	4	8
TME16	0.5	0.5	4	8
TRU45	0.5	1	8	16
7	E(-)	MIC₁	MIC₂	MIC₃
MCA01	1.56	1.56	1.56	1.56
MGY01	3.12	3.12	3.12	3.12
TME16	6.25	6.25	12.5	12.5
TRU45	3.12	3.12	3.12	3.12
8	E(-)	MIC₁	MIC₂	MIC₃
MCA01	0.781	0.390	0.781	0.780
MGY01	3.12	3.12	3.12	1.56
TME16	3.12	3.12	3.12	3.12
TRU45	3.12	3.12	3.12	3.12

MCA01: *Microsporum canis*; MGY01: *Microsporum gypseum*; TME16: *Trichophyton mentagrophytes*; TRU45: *Trichophyton rubrum*; E(-): MIC of the compounds tested in the absence of ergosterol in the medium; MIC₁: MIC of compounds with the presence of 25 $\mu\text{g mL}^{-1}$ of sorbitol in the culture medium; MIC₂: MIC of compounds with the presence of 50 $\mu\text{g mL}^{-1}$ of sorbitol in the culture medium; MIC₃: MIC of compounds with the presence of 100 $\mu\text{g mL}^{-1}$ of sorbitol in the culture medium; AMB: amphotericin B.

These results also suggest that the molecules do not bind directly on the ergosterol present in the fungal cell membrane. To the protective effect of sorbitol test, the molecules **7** and **8** even shown do not act on the fungal cell wall since MICs did not increase in the presence of sorbitol (Table 3) such as the control anidulafungin. Therefore, these derivatives may have activity on some important metabolic route for filamentous fungi, not being directly related to damage caused to structures such as the membrane or fungal cell wall.^[39]

Table 3. MICs in presence and in absence of sorbitol to **7**, **8** and anidulafungin against dermatophytes.

	AND		7		8	
	S(+)	S(-)	S(+)	S(-)	S(+)	S(-)
MCA01	0.25	1	3.12	1.56	0.781	0.781
MGY01	0.5	2	6.25	6.25	3.12	6.25
TME16	0.5	4	6.25	6.25	3.12	3.12
TRU45	0.5	2	6.25	3.12	6.25	3.12

S(+): medium containing 0.8 M of sorbitol; S(-) medium without sorbitol; AND: anidulafungin.

The specificity presented by this library of molecules is unprecedented and very significant since there is currently no antifungal drug able to inhibit only filamentous fungi. This finding is very promising and assigns SCF₃ molecules as potential future treatment in the specific therapy of dermatophytosis without causing damage to existing yeasts in our microbiota, maintaining the balance of the microbiome and immune system.^[40-42] Other genera of filamentous fungi will be studied forward to assess their spectrum of action.

Cytotoxicity results show that molecule **7** reduced by approximately 50% the cell viability of fibroblasts from 2 $\mu\text{g mL}^{-1}$ (Figure 2-A).

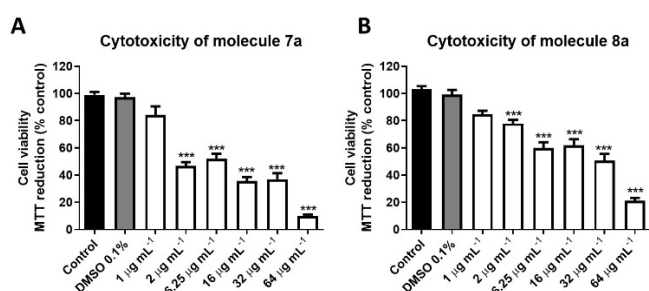


Figure 2. Cytotoxicity assay of mouse embryonic fibroblast (3T3 cell line) with 1, 2, 6.25, 16, 32, and 64 $\mu\text{g mL}^{-1}$ of synthetic fluorinated compounds **7** and **8**. 0.1% DMSO: solvent control. Control: cells incubated in culture medium. ***statistical difference in relation to the control ($p < 0.05$).

Molecule **8**, on the other hand, showed a reduction of approximately 25% in cell viability at 2 $\mu\text{g mL}^{-1}$, whereas a 50% reduction in viability was observed at 32 $\mu\text{g mL}^{-1}$ (Figure 2-B). However, this concentration of 32 $\mu\text{g mL}^{-1}$ is ten times higher than the average of the MICs obtained for the dermatophytes tested. The position of the chlorine atom on the aromatic ring may be involved in the toxicity, with the *para*-chloro in molecule **8** being less toxic than the *meta*-chloro in molecule **7**. A study shows that the drug fluconazole tested on 3T3 cell lines showed inhibition of 50% of cell viability with approximately 200 $\mu\text{g mL}^{-1}$,^[43] and the concentration necessary for the inhibition of 90% of the growth of dermatophytes (8–64 $\mu\text{g mL}^{-1}$).^[44]

The irritability test (HET-CAM) showed that both molecules tested at a concentration of 50 $\mu\text{g mL}^{-1}$ presented an irritation score lower than 4.9, considered non-irritating (Figure 3).

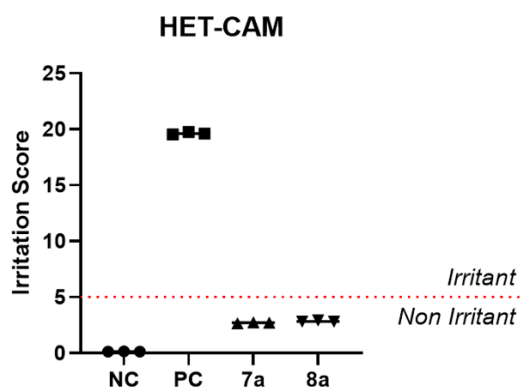


Figure 3. Hen's Egg Test on the Chorioallantoic Membrane (HET-CAM) for molecules **7** and **8** tested at a concentration of 50 $\mu\text{g mL}^{-1}$. NC: negative control. PC: positive control. The symbols ● (NC), ▲ (**7**), and ▼ (**8**) showed no statistical difference between them, but they were statistically different from the symbol ■ (PC) ($p < 0.05$).

Molecules **7** and **8** did not show a statistical difference between them. Furthermore, the lack of irritability for **7** and **8** molecules at concentrations six times higher than MICs in the HET-CAM test also shows the possibility of their use for topical treatment. The HET-CAM test has been used as an alternative to the *in vivo* Draize rabbit eye test. The fundamental process can also be applied to preliminary assessments for skin irritation.^[45, 46] The control drug, fluconazole, is available for most topical and oral

administration and has a low potential for irritability. The molecules **7** and **8** also demonstrated a low irritability potential, reaffirming the possibility of these derivatives as a new alternative for the therapy of dermatophytosis. Thus, derivative **8**, due to its low MIC (1.56–6.25 $\mu\text{g mL}^{-1}$) and reduced toxicity and absence of irritability, can be considered the hit of the tested derivatives.

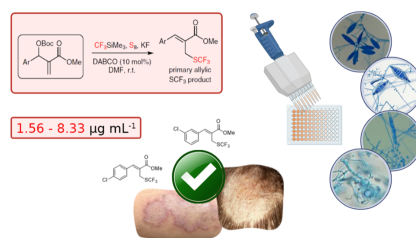
Conclusion

The antifungal activity found for the SCF₃ molecules tested in this work was very encouraging, considering the low MIC obtained for derivative **8** and the favorable physicochemical and cytotoxicity parameters of such compounds. The selectivity of these molecules enables the future development of a specific and selective antifungal for the treatment of dermatophytosis, which can preserve beneficial yeasts present in our microbiota. In addition, the potential topical use can be explored given the absence of irritability at concentrations up to six-times higher than MICs.

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A series of trifluoromethylthiolated cinnamate derivatives have demonstrated promising potential applications as topical antidermatophytic agents. In particular, **two derivatives have** a lower propensity for cytotoxicity at low concentrations and a low irritant potential profile, even at concentrations 6-30 times higher than the Minimum Inhibitory Concentration (MIC: 1.56 - 8.33 μg mL⁻¹)