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Synthesis and Antifungal Action of New Tricyclazole Analogues

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Melanins are very important pigments for the survival and longevity of fungi, so their biosynthesis inhibition is a new biochemical target aiming at the discovery of selective fungicides. In this work is described the synthesis of new pyrazolo-thiazolo-triazole compounds, analogues of tricyclazole (a commercial antifungal product that acts by inhibiting melanin synthesis), and their biological activity was studied on some dermatophytes and phytopathogens. The compounds poorly inhibited the growth and pigmentation of fungi tested and were less efficient than tricyclazole. Electron microscopy on *Botrytis cinerea* showed that treatment with the most active compound caused abnormally thickened and stratified walls in fungi, whose ultrastructure was, in contrast, generally normal. The fungus treated with tricyclazole, on the other hand, appeared to be drastically altered, so as to become completely disorganized. These results suggest that the new azole compounds employ an action mechanism similar to that of other azoles, but dissimilar to that of tricyclazole.

KEYWORDS: Dermatophytes; phytopathogens; antifungal activity; synthesis tricyclazole analogues; TEM

INTRODUCTION

New approaches are being developed in the search for new fungicides. Melanin biosynthesis inhibition is a new biochemical target aiming at the discovery of selective fungicides. The melanins, dark brown or black pigments, are present in fungal walls, in cytoplasm or as extraparietal polymers, and are produced from various natural phenolic precursors, for example, catechol, 1,8-dihydroxynaphthalene (DHN), and γ-glutaminyl-3,4-dihydroxybenzene (GDHB), or from tyrosine via 3,4-dihydroxyphenylalanine (DOPA) (*1*). Also studied were green melanin-like pigments, the synthetic pathway of which showed some enzymes common to the DHN melanin pathway (*2*). Previous studies have demonstrated that the enzymes responsible for the biosynthetic pathway of the melanins (reductase and dehydratase) are in the cytoplasm, whereas the final oxidative enzymes, both for the DHN melanins and for the GDHB melanins, are localized in the fungal walls (*2*). Due to the importance of these pigments for the survival and longevity of fungi, it could be possible to control the potential pathogenic capacity of some fungi by inhibiting their melanin synthesis.

Among the few commercial compounds resulting from this approach, the most important is tricyclazole (5-methyl-1,2,4-triazolo[3,4-*b*]benzothiazole) patented by Eli Lilly, which controls some phytopathogenic fungi, such as *Magnaporthe grisea*, *Colletotrichum lagenarium*, and *Colletotrichum lindemuthianum*, respectively parasites on rice, cucumber and bean (*2*). Tricyclazole is also active on dematiaceous human pathogenic fungi (*3*) and on a number of other imperfect and ascomycetous fungi (*4*). The mechanism of action of this compound has been studied in depth on *M. grisea* (*5*, *6*), where it prevents penetration of rice epidermis by fungal hyphae. Tricyclazole causes a weakening of fungal walls by inhibiting melanin synthesis in appressorial cells of the fungal pathogen, which loses the ability to penetrate the host epidermis and thus to infect plant tissues (*7*−*9*).

On the same fungus, *M. grisea*, in our laboratories, current research aimed at the synthesis and biological evaluation of pyrazole derivatives as fungicides are in progress (*10*). Continuing this program, our interest has been extended to other compounds, analogues of tricyclazole. Owing to the isosterism of the benzene and pyrazole rings, we focused our attention on pyrazolo[3′,4′:4,5]thiazolo[2,3-*c*]-1,2,4-triazole derivatives (**C**) (**Figure 1**).

The aim was to evaluate the influence of the substitution of the benzene ring with that of pyrazole and the effect of various substituents on pyrazole. The synthetic approach was previously planned by our group (*11*). The effect of the new pyrazolo-thiazolo-triazoles was also compared to that of the most widely studied tricyclazole. The purpose of this work was also to extend the screening program to other important fungi, pathogenic for plants, animals, and humans, to explore the potential of these

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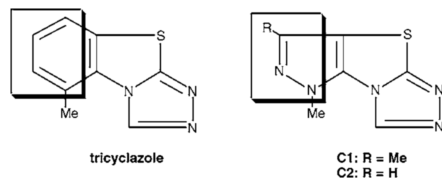

Figure 1. Formulas of tricyclazole and pyrazolo[3′,4′:4,5]thiazolo[2,3-*c*]-1,2,4-triazole derivatives: **C1**, R = Me; **C2**, R = H.

two compounds as fungicides and to understand their possible mode of action.

MATERIALS AND METHODS

Chemicals. Melting points were determined with a Büchi capillary apparatus and are uncorrected. IR spectra were recorded with a Perkin-Elmer Paragon 500 FT-IR spectrometer using potassium bromide pellets. ^{1}H NMR spectra were recorded on a Bruker AC200 spectrometer; chemical shifts (δ) are given in parts per million relative to tetramethylsilane as internal standard. Yields were based on the weight of the products dried in vacuo over phosphorus pentaoxide. Elemental analyses (C, H, N, S) were within $\pm$0.4 of theoretical values. Column chromatography was performed using Merck silica gel (70−230 mesh); for the flash chromatography technique, silica gel (230−400) mesh was employed.

Irradiation of the sample was performed with Pyrex-filtered light from a Helios Italquartz 125 W medium-pressure (λ > 300 nm) mercury lamp.

5-Isothiocyanato-1-methylpyrazole (2). A solution of 5-amino-1-methylpyrazole (**1**) (12 mmol, 1.17 g) dissolved in 20 mL of dichloromethane was added dropwise to a mixture of 6.34 g (60 mmol) of sodium carbonate, 12 mL of water, and 1.84 mL (24 mmol) of thiophosgene. After the addition was completed, the suspension was stirred at room temperature for 3 h; then, 10 mL of dichloromethane and 10 mL of water were added, and the organic phase was separated, dried (sodium sulfate), and evaporated to yield a residue, which was purified by column chromatography (eluent ethyl acetate/petroleum ether 1:1 v/v). This compound was obtained as a colorless oil, 0.98 g, yield 59%: IR (neat) 2037, 1530, 1484 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 3.70 (s, 3H, NMe), 6.20 (s, 1H, CH), 7.37 (s, 1H, CH). Anal. Calcd ($C_5H_5N_3S$) C, H, N, S.

4-(1′-Methylpyrazol-5′-yl)thiosemicarbazide (3). Hydrazine hydrate (99%, 0.49 mL, 10 mmol) was added to a solution of 1.39 g (10 mmol) of 5-isothiocyanato-1-methylpyrazole (**2**) dissolved in 5 mL of ethanol. After the addition was completed, the reaction mixture was stirred at room temperature for 1 h and filtered. The product was washed with 1 mL of ice-cold ethanol. This compound was obtained as colorless crystals (1.22 g, yield 71%), mp 195−196 °C: IR (KBr) 2937, 3160, 1627, 1577 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 3.58 (s, 3H, NMe), 6.05 (s, 1H, CH), 6.35 (br, 3H, NH +NH_2), 7,32 (s, 1H, CH), 9.43 (br, 1H, NH). Anal. Calcd ($C_5H_9N_5S$) C, H, N, S.

2,4-Dihydro-4-(1′-methylpyrazol-5′-yl)-3*H*-1,2,4-triazole-3-thione (4). A solution of 0.34 g (2 mmol) of 4-(1′-methylpyrazol-5′-yl)-thiosemicarbazide (**3**) in a mixture of 10 mL of formic acid and 2 mL of acetic anhydride was stirred at room temperature for 2 h; the solvent was evaporated to yield a residue, which was purified by column chromatography (eluent dichloromethane/methanol/toluene 17:2:1, v/v). This compound was obtained as colorless crystals (0.18 g, yield 50%), mp 195−197 °C: IR (KBr) 3026, 1603, 1551 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 3.91 (s, 3H, NMe), 6.21 (s, 1H, CH), 7.26 (s, 1H, CH), 7.49 (br, 1H, NH), 8.44 (s, 1H, CH). Anal. Calcd ($C_6H_7N_5S$) C, H, N, S.

2,4-Dihydro-4-(1′-methyl-4′-nitrosopyrazol-5′-yl)-3*H*-1,2,4-triazole-3-thione (5). Gaseous ethyl nitrite was bubbled through a solution of 0.54 g (3 mmol) of 2,4-dihydro-4-(1′-methylpyrazol-5′-yl)-3*H*-1,2,4-triazole-3-thione (**4**) in 18 mL of ethanol for 10 min; then a few drops of concentrated hydrochloric acid were added, and the ethyl nitrite bubbling was continued for 30 min. The ethanol was evaporated, and the red solid was collected and washed with petroleum ether to yield 0.60 g of 5, yield 95%, mp 124−127 °C: IR (KBr) 3421, 1606 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 3.62 (s, 3H, NMe), 6.96 (br, 1H, NH), 7.44 (s, 1H, CH), 8.79 (s, 1H, CH). Anal. Calcd ($C_6H_6N_6SO$) C, H, N, S.

5-Methylpyrazolo[3′,4′:4,5]thiazolo[2,3-*c*]-1,2,4-triazole (C2). Hydrazine hydrate (99%, 0.49 mL, 10 mmol) and 5% palladized charcoal (0.15 g) were added to a solution of 0.42 g of 2,4-dihydro-4-(1′-methyl-4′-nitrosopyrazol-5′-yl)-3*H*-1,2,4-triazole-3-thione (**5**) (2 mmol) in methanol (20 mL). After heating under reflux for 5 min, the catalyst was removed, and the filtrate was evaporated to dryness in vacuo to give 2,4-dihydro-4-(4′-amino-1′-methylpyrazol-5′-yl)-3*H*-1,2,4-triazole-3-thione (**6**). The colorless solid was collected, washed with ethyl ether (quantitative yield), and directly reacted as follows.

To a stirred and cooled (0 °C) solution of 2,4-dihydro-4-(4′-amino-1′-methylpyrazol-5′-yl)-3*H*-1,2,4-triazole-3-thione (**6**) in 140 mL of ethanol and 6 mL of 1 M hydrochloric acid, a solution of 0.15 g (2.2 mmol) of sodium nitrite in 5 mL of water was added dropwise, and the reaction mixture was allowed to stand at that temperature for 30 min. After being warmed to room temperature gradually, the mixture was irradiated for 4 h with UV light (λ > 300 nm). After evaporation of the solvent, the crude residue was purified by flash column chromatography (eluent ethyl acetate/petroleum ether 8:2 v/v). Compound **C2** was obtained as colorless crystals, 0.15 g yield 42%, mp 150−153 °C: IR (KBr) 1584, 1519, 1430 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 4.02 (s, 3H, NMe), 7.67 (s, 1H, CH), 8.45 (s, 1H, CH). Anal. Calcd ($C_6H_5N_5S$) C, H, N, S.

Microorganisms. As the two new products **C1** and **C2** are analogues of tricyclazole, which acts on phytopathogenic fungi, our experiments have been concentrated on members of this group of fungi, but we also tested other fungal species, pathogenic for animals and humans, such as some dermatophytes. As tests, the following phytopathogenic fungi were employed: *Magnaporthe grisea* (T.T. Hebert) Yaegashi & Udagawa, ATCC 64413 strain; *Botrytis cinerea* (Pers.) Micheli, ATCC 48339 strain; *Penicillium italicum* (Link)Whem, ATCC 48952 strain, purchased from ATCC (American Type Culture Collection, Rockville, MD); *Trichoderma viride* (Pers.) Tul.; *Trichoderma harzianum* (Pers.)-Tul.; *Alternaria solani* Nees; *Sclerotinia minor* Fuckel; *Thielaviopsis basicol* Went; and *Penicillium crustosum* (Link)Thom., purchased from SIAPA-ISAGRO, Milano, Italy.

The dermatophytes used were *Nannizzia cajetani* Ajello, CBS 495.70 strain; *Epidermophyton floccosum* (Hartz) Langeron and Milochevitch, CBS 358.93 strain; *Trichophyton violaceum* Malmsten, CBS 459.61 strain; and *Trichophyton tonsurans* Malmsten, CBS 483.76 strain, purchased from Centraal Bureau voor Schimmelcultures (CBS), Baarn, The Netherlands; *Trichophyton rubrum* (Castellani) Sabouraud IHME 4321 strain was obtained from the Institute of Hygiene and Epidemiology-Mycology (IHME), Brussels, Belgium.

The cultures were maintained in the laboratory as agar slants on a suitable culture medium, that is, on potato dextrose agar (PDA; Difco, Detroit, MI), for the phytopathogenic fungi, and on Sabouraud dextrose agar (SDA; Difco), for the dermatophytes.

Evaluation of Antifungal Activity. To evaluate biological activity, cultures of each fungus were obtained by transplanting mycelium disks, 10 mm in diameter, from a single culture in stationary phase. These were incubated at 26 $\pm$ 1 °C on the medium suitable for each organism (PDA or SDA), on thin sterile sheets of cellophane, until the logarithmic phase of growth was reached. Then the fungi were transferred to Petri dishes containing the medium supplemented with the compound to be tested. Each compound was dissolved into dimethyl sulfoxide (DMSO), and a proper dilution was aseptically added to the medium at 45 °C to obtain a final concentration of 10, 50, 100, or 200 μg mL^{-1}. The DMSO concentration in the final solution was adjusted to 0.1%. Controls were set up with equivalent quantities (0.1%) of DMSO. The growth rate was determined by measuring daily colony diameter for 7 (for dermatophytes) and 5 (for phytopathogens) days after the transport of the fungus onto dishes containing the substance to be tested. At this time the percentage growth inhibition in comparison with the control was evaluated for each fungus. Three replicates were used for each concentration. The percentage of growth inhibition was expressed as the mean of values obtained in three independent experiments. For comparison, the same concentrations of the commercial fungicide tricyclazole (Beam, Dow AgroSciences) were tested.

Table 1. Percentage of Growth Inhibition by **C1** and **C2** Evaluated 5 Days after Treatment[a]

compd	control	*M. grisea*, μg mL^{-1} 10	50	100	200	control	*B. cinerea*, μg mL^{-1} 10	50	100	200
tricyclazole	0	0	24.1 ± 1.4	62.3 ± 0.9	94.0 ± 1.4	0	0	8.3 ± 0.3	29.6 ± 1.1	77.8 ± 0.8
C1	0	12.2 ± 2.3	13.0 ± 1.7	20.5 ± 0.6	59.1 ± 1.1	0	10.8 ± 1.6	14.6 ± 2.4	28.8 ± 2.0	65.4 ± 1.5
C2	0	3.0 ± 0.4	8.2 ± 0.9	24.0 ± 1.8	43.4 ± 1.3	0	0	4.0 ± 0.7	19.3 ± 1.3	32.2 ± 1.7

[a] Values are means of three trials made in triplicate ± SD.

We looked for antifungal activity by evaluating the capacity of the new substances to inhibit the fungal pigmentation by means of visual and photographic evaluation of the treated cultures, in comparison with untreated control cultures at the same age of growth.

Electron Microscopy. The morphologic aspects induced by treatment with the more active compound (**C1**) at the highest dose (200 μg mL^{-1}), and for comparison also with tricyclazole, on the most sensitive fungus (*B. cinerea*), were studied by transmission electron microscopy (TEM). For TEM, the youngest mycelial cells from untreated colonies and from colonies treated for 24 h with 200 μg mL^{-1} of **C1** or, alternatively, with tricyclazole were chosen and routinely processed as described in Mares et al. (*12*). The hyphae were fixed with 6% glutaraldehyde (GA) in a 0.1 M sodium cacodylate buffer, pH 6.8, for 3 h at 4 °C, washed with the buffer solution, and postfixed overnight with 1% osmium tetraoxide (OsO_4) in the same buffer. Alternatively, the fungi were fixed with 6% GA in a 0.1 M phosphate buffer, pH 7, for 3 h and postfixed with potassium permanganate ($KMnO_4$) 2% for 2 h. The samples were then dehydrated in a graded series of ethanol and embedded in Epon−Araldite resin. Sections were cut with an LKB Ultratome III ultramicrotome, stained with uranyl acetate and lead citrate, and observed with a Hitachi H 800 electron microscope (Hitachi, Tokyo, Japan) at 100 kV, owned by the Electron Microscopic Center of the University of Ferrara, Italy.

RESULTS

Synthesis. 5-Methylpyrazolo[3′,4′:4,5]thiazolo[2,3-*c*]-1,2,4-triazole (**C2**) has been prepared by applying the same synthetic procedure that we had previously investigated (*11*) to obtain **C1**. The synthesis is shown in **Figure 2**.

The synthetic plan involves the use of 5-isothiocyanato-1-methylpyrazole (**2**) obtained from 5-amino-1-methylpyrazole (**1**) by reaction with thiophosgene in the presence of sodium carbonate. Condensation of **2** with hydrazine hydrate in ethanol afforded 4-(1′-methypyrazol-5′-yl)thiosemicarbazide (**3**). Cyclization of **3** with formic acid/acetic anhydride provided the key intermediate 4-(1′-methylpyrazol-5′-yl)-1,2,4-triazole-3-thione (**4**). Nitrosation of **4** and reduction with hydrazine hydrate in the presence of palladized charcoal afforded 4-(4′-amino-1′-methylpyrazol-5′-yl)-1,2,4-triazole-3-thione (**6**). Because **6** was unstable during the usual workup for isolation, it was directly reacted with an equivalent of sodium nitrite in acidic medium. Diazotization of **6** under UV (λ > 300 nm) irradiation provided directly the required 5-methylpyrazolo[3′,4′:4,5]thiazolo[2,3-*c*]-1,2,4-triazole (**C2**) without isolation of the diazo intermediate **7**.

The structures of all compounds were confirmed by analytical and spectral data. In particular, ^{1}H NMR spectra of all compounds agree with the proposed structures. The spectrum of compound **4** shows two resonances at 6.21 and 8.44 attributable to the C−H of pyrazole and triazole rings, respectively. The spectrum of compound **C2** shows instead only one resonance attributable to CH of triazole at 8.45 ppm. Previously the ^{13}C NMR spectra of **C1** further confirmed the proposed structure.

Antifungal Activity. 5,7-Dimethylpyrazolo[3′,4′:4,5]thiazolo-[2,3-*c*]-1,2,4-triazole **(C1)** and 5-methylpyrazolo[3′,4′:4,5]thiazolo[2,3-*c*]-1,2,4-triazole **(C2)** showed various potentials as antifungals, depending on the considered parameter: growth, morphology, or pigmentation.

Growth. At the beginning, the two compounds were tested on *M. grisea*, in comparison with precedent data on other azoles previously tested on this fungus (*10*). Afterward, the screening

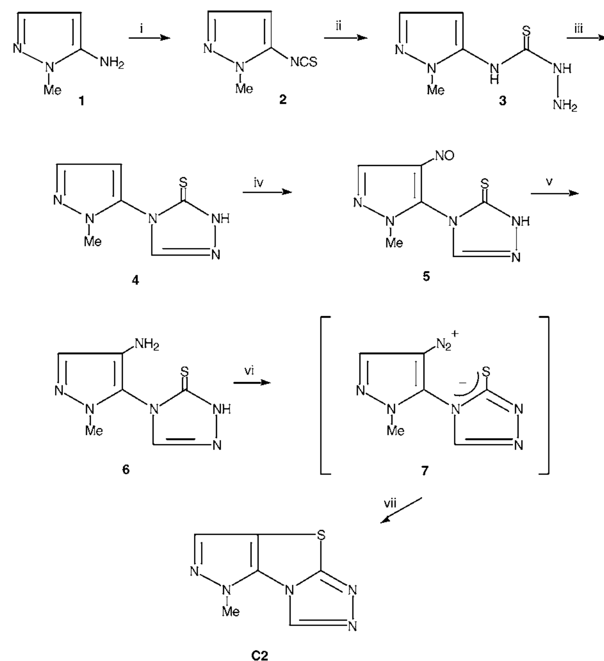

Figure 2. Synthesis of **C2**, 5-methylpyrazolo[3′,4′:4,5]thiazolo[2,3-*c*]-1,2,4-triazole. Reagents: i, thiophosgene; ii, N_2H_4; iii, HCOOH, Ac_2O; iv, ethyl nitrite; v, N_2H_4, 5% Pd/C; vi, $NaNO_2$/AcOH; vii, UV irradiation. For **1**–**7** see text.

Table 2. Percentage of Growth Inhibition Evaluated 5 Days (for Phytopathogens) and 7 Days (for Dermatophytes) after Treatment with **C1**[a]

	tricyclazole 100 μg mL^{-1}	tricyclazole 200 μg mL^{-1}	**C1** 100 μg mL^{-1}	**C1** 200 μg mL^{-1}
phyotpathogens				
M. grisea	62.3 ± 0.9	94.0 ± 1.4	20.5 ± 0.6	59.1 ±1.1
B. cinerea	29.6 ± 1.1	77.8 ± 0.8	28.8 ± 2.0	65.4 ± 1.5
T. harzianum	20.0 ± 0.6	50.2 ± 1.3	0.0	0.0
T. viride	20.2 ± 1.4	60.0 ± 0.9	0.0	0.0
P. italicum	20.1 ± 0.4	70.4 ± 0.5	0.0	0.0
P. crustosum	80.3 ± 0.8	95.0 ± 1.1	0.0	10.0 ±1.5
S. minor	0.0	0.0	0.0	0.0
A. solani	0.0	0.0	0.0	23.4 ± 0.6
T. basicola	40.1 ± 0.9	66.6 ± 1.2	0.0	0.0
dermatophytes				
E. floccosum	14.1 ± 0.7	38.5 ± 2.1	0.0	0.0
T. rubrum	40.3 ± 1.5	69.3 ± 1.8	0.0	0.0
T. tonsurans	32.6 ± 0.8	51.1 ± 0.7	0.0	0.0
T. violaceum	51.4 ± 1.3	80.4 ± 0.4	0.0	0.0
N. cajetani	17.2 ± 1.6	41.0 ± 1.4	0.0	0.0

[a] All values shown are averages of three trials made in triplicate ± SD.

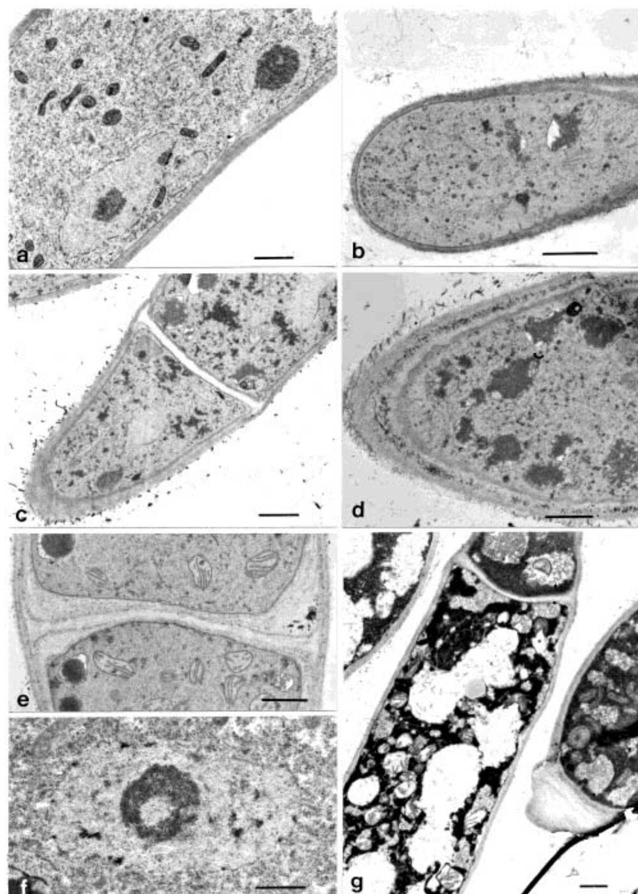

Figure 3. Ultrastructural aspects of *B. cinerea:* (**a**) portion of coenocytic hypha of untreated *B. cinerea* showing nuclei and many mitochondria, GA–OsO_4; (**b**) median section from hyphal tip of untreated *B. cinerea*, GA–$KMnO_4$; (**c**) control hypha showing septum, glycogen granules, and typically organized wall, GA–$KMnO_4$; (**d**) apex of *B. cinerea* treated with **C1** at 200 μg mL^{-1} for 24 h (the wall is made of many layers of different electron opacities), GA–$KMnO_4$; (**e**) anomalous stratification of wall layers at septum level in hypha treated for 24 h with **C1** at 200 μg mL^{-1}, GA–$KMnO_4$; (**f**) detail of *B. cinerea* hypha treated for 24 h with tricyclazole at 200 μg mL^{-1} showing a nucleus with anomalous dense stacks of chromatin, GA–OsO_4; (**g**) hypha of *Botrytis* treated for 24 h with tricyclazole at 200 μg mL^{-1} with wide vacuolization and autophagic aspects, GA–$KMnO_4$. Bars = 1 μm.

was extended to another very important phytopathogen, which causes much damage to crops, that is, *B. cinerea*. The data were compared with those obtained with the fungicide tricyclazole, usually used in agriculture at the dose of 200 μg mL^{-1}.

Table 1 reports the percentages of growth inhibition of *M. grisea* and *B. cinerea* after treatment with **C1**, **C2**, and tricyclazole at the concentrations of 10, 50, 100, and 200 μg mL^{-1}.

The growth of the two fungi treated with **C1** and **C2** was only partially inhibited and, always, higher than that of tricyclazole-treated cultures. Between the two new compounds, the most efficient was **C1**, which, at the lower doses, weakly inhibited both fungi; at 100 and 200 μg mL^{-1} the growth inhibition was feebler than that exerted by tricyclazole, in particular against *M. grisea*.

The **C2** compound always showed less efficiency than **C1**; for this reason the experiments were continued using only the **C1** compound and only at the two highest doses; moreover, the screening was widened to other fungi, pathogens for plants and for animals and humans (**Table 2**).

Table 3. Inhibition of Pigmentation by **C1** and Tricyclazole

	C1[a]	tricyclazole[a]
dermatophytes		
Nannizzia cajetani	−	++
Epidermophyton floccosum	−	−
Trichophyton rubrum	−	−
Trichophyton violaceum	−	+
Trichophyton tonsurans	−	−
DHN phytopathogens		
Alternaria solani	+++	+
Botrytis cinerea	−	+
Magnaporthe grisea	+++	++
Sclerotinia minor	++	++(+)
Thielaviopsis basicola	+	++
GM-P phytopathogens		
Trichoderma viride	+++	+++
Trichoderma harzianum	++	+++
Penicillium italicum	+	+++
Penicillium crustosum	+	+++

[a] −, no depigmentation; +, feeble color variation; ++, good color variation; +++, bleaching.

The data on the growth of five dermatophytes and of another seven phytopathogens were, unfortunately, disappointing, **C1** being totally ineffective on treated fungi, at least relative to this parameter. Exceptions to this state are *P. crustosum* and *A. solani*, the growth of which was inhibited, respectively, by 10.0 and 23.0%, but only when they were treated with **C1** at the highest dose of 200 μg mL^{-1}. On all fungi, tricyclazole inhibited the growth at both doses used, except in the cases of *S. minor* and *A. solani* (growth inhibition = 0.0%). In *P. crustosum* the growth was almost blocked (growth inhibition = 95.0% at 200 μg mL^{-1}).

Morphology. On *B. cinerea*, the fungus whose growth was most inhibited by **C1** treatment, the possible modifications induced by this new compound and by tricyclazole at the highest concentration (200 μg mL^{-1}) were studied by means of the electron microscopy.

The untreated fungus showed conventional morphology with coenocytic hyphae having many nuclei, regular mitochondria (**Figure 3a**), and apices (**Figure 3b**) typical for the ascomicetous fungi. The wall was uniform, with an outer layer made of electron opaque mannoproteins and with an inner layer, more electron transparent, made of interconnected chitin and glucans, in accordance with the ascomycetous wall outline (**Figure 3c**). Glycogen granules and septa were also visible.

After **C1** treatment, the wall was particularly affected in cells that were apparently normal in their ultrastructure. Increased quantities of materials in the intermediate layers, both in the lateral and in the apical portions of the hyphae, were visible. The wall appeared to be clearly stratified, so it was made not only by two single layers external to the plasmatic membrane but by many subsequent layers, recognizable by their different electron opacities (**Figure 3d**).Thus, in the apical dome, alternating electron transparent (chitin) and electron opaque (mannoproteins) zones can be recognized. The same anomalous stratification also was visible at the septa level (**Figure 3e**).

When *B. cinerea* was treated with tricyclazole, the ultractructure of the fungus was drastically altered. The main changes regarded the nucleus, in which anomalous electron dense stacks were visible, perhaps resulting from an unusual breakage of chromatin (**Figure 3f**). Moreover, many hyphae were entirely disorganized, due to the presence of numerous vacuoles, which filled the whole hypha (**Figure 3g**). The presence of these necrotic hyphae could also explain the strong inhibition of the growth observed in tricyclazole-treated mycelium.

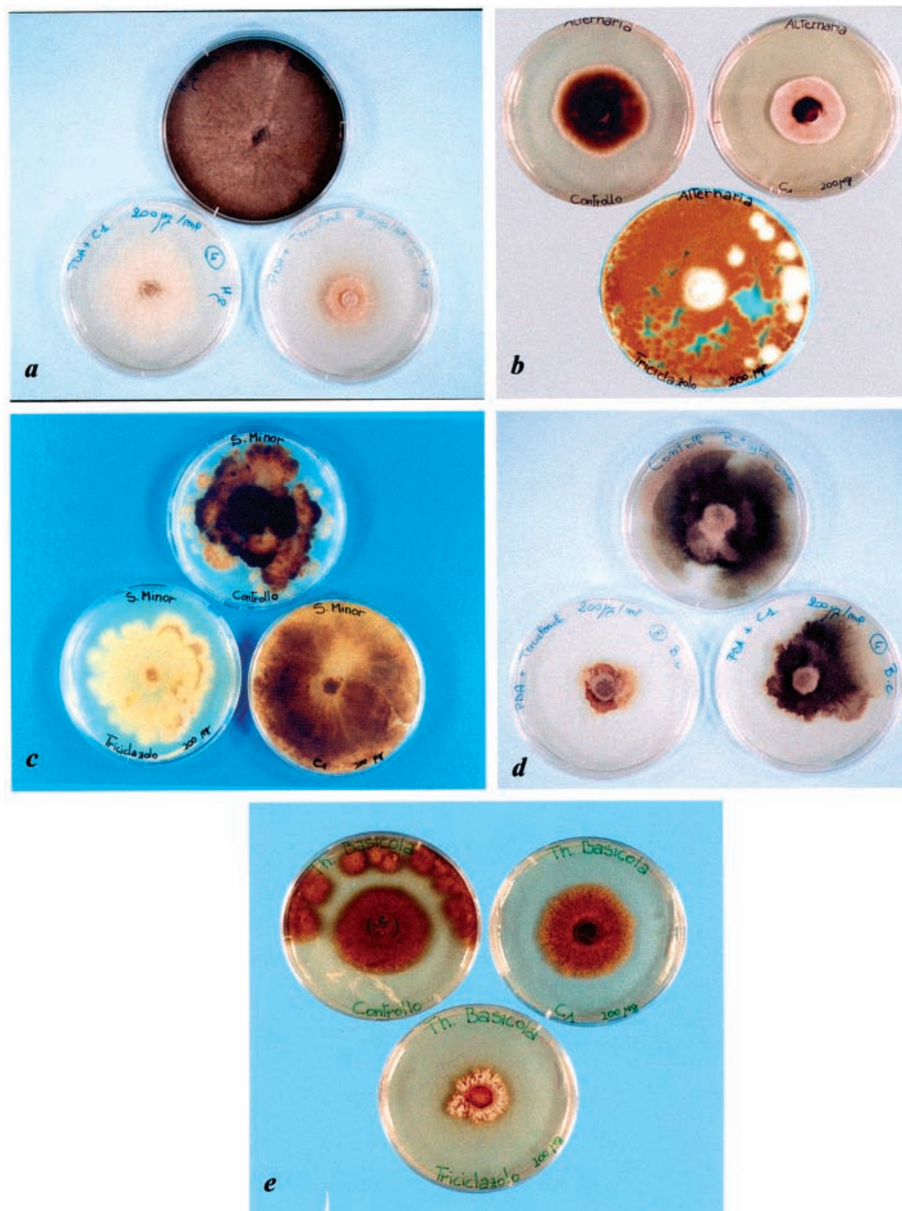

Figure 4. Depigmentation of fungi with DHN melanins after treatment with **C1** and tricyclazole at 200 μg mL^{-1}: (**a**) *M. grisea*; (**b**) *A. solani*; (**c**) *S. minor*; (**d**) *B. cinerea*; (**e**) *T. basicola*.

Pigmentation. Another parameter that we considered in this work was the capacity of the new azoles to inhibit the color of the mycelium. Subsequent visual observations revealed that tricyclazole inhibited the pigmentation of the hyphae, whereas the two new compounds, **C1** and **C2**, were less efficient. Similarly to the results for growth, the capacity to inhibit the pigmentation was greater for **C1** than for **C2**. An important difference in the answer to treatment with the **C1** and with tricyclazole was observed in the two groups of fungi: whereas all dermatophytes were not bleached by the **C1** treatment, all phytopathogenic fungi were depigmented, even if partially (**Table 3**).

Among the five dermatophytes tested, only *N. cajetani*, *T. rubrum*, and *T. violaceum* showed a pigmented control mycelium. After treatment with tricyclazole, only *N. cajetani* and *T. violaceum* showed depigmentation, whereas **C1** was ineffectual.

Among the fungi with DHN melanins (*A. solani*, *B. cinerea*, *M. grisea*, *S. minor*, and *T. basicola*) (*2*), the most sensitive to depigmentation by the **C1** compound was *M. grisea*; treatment with **C1** caused a bleaching of the mycelium even more intense than that caused by tryciclazole at the same dose (**Figure 4a**). In *A. solani*, the homogeneous dark brown color reached complete bleaching with treatment at 200 μg mL^{-1} of **C1**. When the same fungus was treated with tricyclazole, its pigmentation changed from dark to brown (**Figure 4b**). In *S. minor*, treatment with **C1** caused a fading of the culture from dark to light brown, whereas the mycelium treated with tricyclazole became yellow (**Figure 4c**). *B. cinerea* treated with **C1** maintained almost unchanged its color, whereas after tricyclazole treatment, the fungus became yellow-brown (**Figure 4d**). In *T. basicola*, the control mycelium of which was burnt brown, the culture treated with **C1** showed only a slight decrease of the color intensity, whereas those treated with tricyclazole appeared dark yellow (**Figure 4e**).

T. viride, *T. harzianum*, *P. italicum*, and *P. crustosum* are members of fungi with melanin-like pigments (*2*). *T. viride* has an intensely pigmented green mycelium; after treatment with **C1** or with tricyclazole, its surface was a pale green, almost white (**Figure 5a**). *P. italicum* control showed a gray color of the mycelium, which became partially or totally white (**Figure**

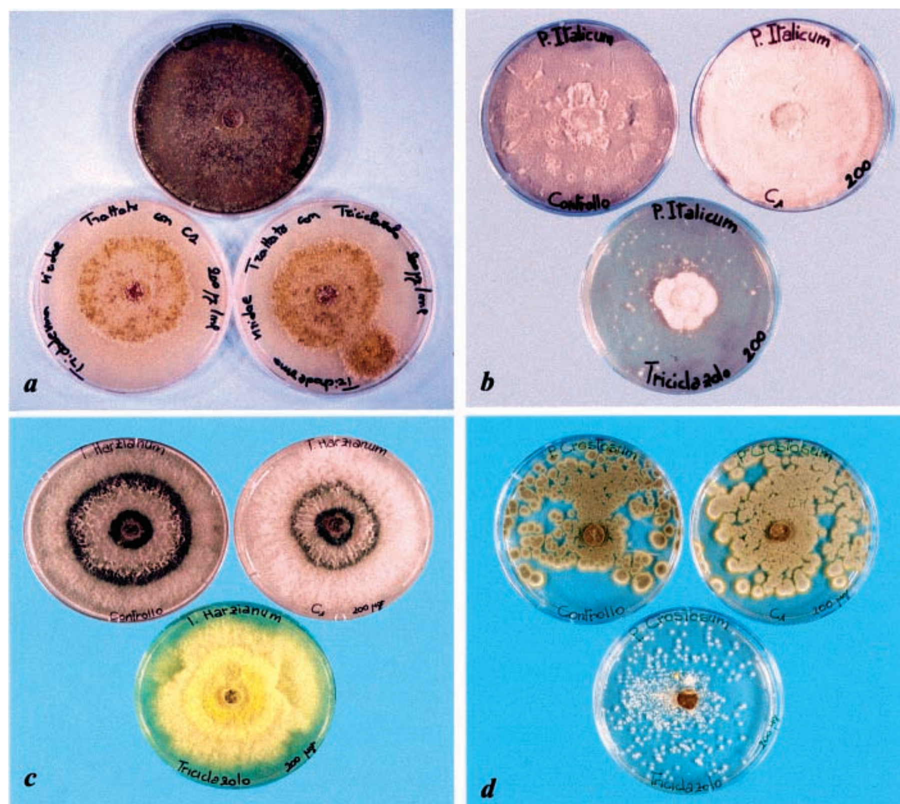

Figure 5. Depigmentation of fungi with GM-P melanins after treatment with **C1** and tricyclazole at 200 μg mL^{-1}: (**a**) *T. viride*; (**b**) *P. italicum*; (**c**) *T. harzianum*; (**d**) *P. crustosum*.

5b) when the fungus was treated, respectively, with **C1** and with tricyclazole. Finally, *T. harzianum* control showed cultures with alternating and concentric green and pale green layers. After **C1** treatment at the dose of 200 μg mL^{-1}, the hyphae, external to the first green ring, were white. With the same dose of tricyclazole, the culture of *T. harzianum* became totally white (**Figure 5c**). *P. crustosum* after **C1** treatment underwent only a feeble decrease of its color intensity, whereas after tricyclazole treatment, the appearance of the culture was white (**Figure 5d**).

DISCUSSION

Due to the importance of tricyclazole and its derivatives, the preparative route to 1,2,4-triazolo[3,4-*b*]benzothiazoles has been extensively studied. The construction of the benzothiazole-triazole condensed ring is generally achieved by reaction of 2-hydrazinobenzothiazole derivatives with triethyl orthoformate (*13*, *14*) or formic acid (*15*, *16*). Wikel and Paget (*17*) also reported the synthesis of analogues of tricyclazole via the reaction of 4-(2-halophenyl)-1-acyl-3-thiosemicarbazides with sodium hydride. The synthetic entry to pyrazolo[3′,4′:4,5]-thiazolo[2,3-*c*]-1,2,4-triazoles (**C**), analogues of tricyclazole, is based on the diazotization of 4-(pyrazol-5′-yl)-1,2,4-triazole-3-thione (**4**) followed by irradiation with UV light (*11*).

The results reported in **Tables 1−3** led to the following considerations: (a) **C1** and **C2** poorly inhibit the growth of the phytopathogens *M. grisea* and *B. cinerea* with a dose-dependent action; (b) **C1** is more effective than **C2**; (c) **C1** is totally ineffectual in inhibiting the growth of all dermatophytes and several phytopathogens; (d) the various fungi have different sensitivities to the same substance (**C1** or tricyclazole), indicating different metabolisms toward them. The growth of **C1**- or **C2**-treated fungi was always greater than that of tricyclazole-treated cultures. On the other hand, this commercial fungicide produces strong growth inhibitions only when it is used at high doses, indicating that it does not influence the growth of the fungi, but rather the pigmentation (*4*). Tricyclazole is an antifungal substance widely used in agriculture, in particular against "rice blast", the disease provoked by *M. grisea*. It shows advantages in comparison with the other fungicides, because it does not need repeated applications; it is systemic in rice for control of the disease and is applied by methods different from the use of dusts and sprays. Many studies, made on *M. grisea*, an anamorph of *Pyricularia oryzae*, have reported that tricyclazole interferes with melanin biosynthesis by inhibiting the reduction of 1,3,8-THN and the scytalone pathway (*6*, *7*, *18*, *19*). The same substance also proved to be very efficient in the discoloration of many phytopathogenic fungi (*4*). The data of our work confirmed that tricyclazole acts by decoloring the mycelium of many phytopathogens, but it is interesting to note that it is active not only on fungi with DHN melanins (*M. grisea*, *B. cinerea*, *A. solani*, *S. minor*, and *T. basicola*) but also on fungi with melanin-like pigments (*T. viride*, *T. harzianum*, *P. italicum*, and *P. crustosum*), in contrast to previously obtained results (*2*). The new azolic derivative **C1**, an analogue to tricyclazole, is less efficient than tricyclazole in the depigmentation of all fungi, except for *A. solani* (**Figure 4b**) and *M. grisea* (**Figure 4a**), owing to a possible metabolic difference of these two fungi compared to all other fungi.

With regard to the dermatophytes, both tricyclazole and **C1** were ineffective in the depigmentation of their mycelia. Although pigmentation of the dermatophytes has been poorly studied and little has been learned about their pigments, Zussman et al. (*20*) suggested that the pigments were formed by the oxidation of tyrosine by tyrosinase and, thus, could belong to the DOPA melanins. Our negative results seem to suggest that the fungi with pigments, which are made from a number of

different compounds derived from tyrosine, are not susceptible to depigmentation by tricyclazole or its analogues. Nevertheless, there is still no conclusive proof that DOPA melanin is formed in any cell wall or in that of the dermatophytes in particular.

The different ultrastructural anomalies observed in *B. cinerea* after treatment with **C1** and tricyclazole lead to the supposition that the two substances can have different modes of action.

In particular, **C1** seems to have an action mechanism like that of other known azoles. The differences here observed between control and **C1**-treated hyphal wall components could be explained on the basis of the altered assembly of its components. It can be hypothesized that, in the apical dome (that is, the place of wall origin) (*21*), the normal assembly of principal parietal components (chitin, β-glucans, and mannoproteins) is altered by the **C1** treatment, so that a coordinate assembly of the various components is absent, whereas an unbalanced sequence of alternating electron transparent (chitin) and opaque (glucans-mannans) layers is present. This altered wall succession of its component can be brought back to a direct action of the 5,7-dimethylpyrazolo[3′,4′:4,5]thiazolo[2,3-*c*]-1,2,4-triazolo **(C1)** with membrane sterol, in accordance with the mechanism that it apparently shares with other azoles (*12*, *22*), interfering with the normal production of ergosterol.

Moreover, like other azoles, **C1** seems to be fungistatic, not fungicidal. In fact, treatment with **C1** does not provoke the death of *B. cinerea*, as occurs after treatment with tricyclazole at the same dose. This substance, proved by Zeun and Buchenauer (*23*) on the same fungus, retarded production of *B. cinerea* sclerotia and provoked a strong inhibition of the melanin layer of its wall, causing also deep alterations of the morphology of sclerotia seen at TEM. Therefore, it is evident that tricyclazole has an action mechanism different from that of **C1**; it is able to alter wall permeability, causing an increase in water entry and, thus, an increase in pressure, as was demonstrated by other authors, and in particular by Howard and Ferrari (*5*) on *M. grisea.* This hypothesis is in accord with observations of our work, where tricyclazole causes deep alterations in *B. cinerea* hyphae, resulting in complete disorganization of their cells (**Figure 3g**).

Therefore, the similarity of the molecular structure and of general hyphae decoloration lead us to hypothesize that **C1** has a mechanism of action similar to, but not the same as, that of tricyclazole. Further studies to clarify the mechanism of action of this new azolic compound are in progress.

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