Synthetic Pyrazole Derivatives as Growth Inhibitors of Some Phytopathogenic Fungi

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The present study was carried out to investigate the antifungal activity of pyrazole/isoxazole-3-carboxamido-4-carboxylic acids, 4-oxo-5-substituted pyrazolo[3,4-*d*]pyrimidine-6-thiones, and *N*-alkyl/aryl-*N'*-(4-carbethoxy-3-pyrazolyl)thioureas against *Pythium ultimum*, *Botrytis cinerea*, and *Magnaporthe grisea*. The results on growth inhibition showed differences in the sensitivity of the three fungi to the tested substances, and in general *P. ultimum* was shown to be the most sensitive. On all phytopathogens the best results within the pyrazole/isoxazolecarboxamide series are given by the compounds with the carboxamide and carboxylic groups in positions 3 and 4; the presence of these groups seems to be critical for biological activity in this series of compounds. Among the pyrazolopyrimidines the derivative supplied with the benzylic group was the most active on the three fungi and in particular against *P. ultimum.* Several compounds belonging to the thiourea series are able to inhibit selectively *M. grisea* at 50 and 10 μg mL^{-1}, doses at which the reference commercial compound tricyclazole had low or no effect.

KEYWORDS: Antifungals; pyrazole-3-carboxamido-4-carboxylic acids; isoxazole-3-carboxamido-4-carboxylic acids; 4-oxo-5-substituted pyrazolo[3,4-*d*]pyrimidine-6-thiones; *N*-alkyl/aryl-*N'*-(4-carbethoxy-3-pyrazolyl)thioureas; *Pythium ultimum*; *Botrytis cinerea*; *Magnaporthe grisea*

INTRODUCTION

With the successful introduction of carboxamides as fungicides with systemic activity by the U.S. Rubber Co. in 1966, a new chapter in the history of plant protection began. For the first time it was possible to control important crop diseases in barley and wheat (*1*).

Within this chemical class of fungicides, variation of mechanism of action can be observed. Carboxamides benodanil, boscalid, carboxin, fenfuram, flutolanil, mepronil, oxycarboxin, thifluzamide, furametpyr, and penthiopyrad are fungitoxic because they inhibit the succinate dehydrogenase complex, in the respiratory electron chain, leading to inhibition of aspartate and glutamate synthesis (*2–5*). The biochemical mode of action of diclocymet and carpropamid appears to be based on inhibition of dehydratase in melanin biosynthesis (*3, 4*). The inhibition of ATP production is proposed as the mode of action of the thiophene carboxamide silthiofam; the target of the thiazole derivative ethaboxam is unknown (*3, 4*).

Pyrazole and isoxazole fungicides have attracted attention from many industrial companies. This led to the commercial introduction of hymexazole, rabenzazole, and pyraclostrobin. Hymexazole is used to control soil-borne diseases caused by *Pythium*, *Fusarium*, *Aphanomyces*, *Corticium*, and *Typhula* spp. (*2, 3*). Rabenzazole inhibits the synthesis of β-tubulin during mitosis of fungi (*2, 6*). Pyraclostrobin, a strobilurin-mitochondrial electron transport inhibitor, shows broad spectrum activity on anthracose-inducing fungi and on *Alternaria*, downyn mildew, *Cercospora* leaf spot, rust, powdery mildew, *Septoria*, *Phytophthora*, *Rhizoctonia*, and *Pythium* (*3–5*) (**Figure 1**).

Molecules with both pyrazole and carboxamide moieties led to the synthesis of several antifungals (*7–10*). Furametpyr (*3, 4*), 5-chloro-*N*-(1,3-dihydro-1,1,3-trimethylisobenzofuran-4-yl)-1,3-dimethylpyrazole-4-carboxamide, is a fungicide used to control rice sheat blight (*11–13*). Penthiopyrad, *N*-[2-(1,3-dimethylbutyl)-3-thienyl]-1-methyl-3-(trifluoromethyl)-1*H*-pyrazole-4-carboxamide, shows activity on strobilurin and DMI resistant diseases (gray mold, powdery mildew, apple scab, rusts, *Rhizoctonia*, *Botrytis*) (*3–5*) (**Figure 1**).

Within the framework of investigation on biologically active heterocycles, we found that pyrazole and isoxazole derivatives are interesting for their biological properties as antifungal agents (*14–22*).

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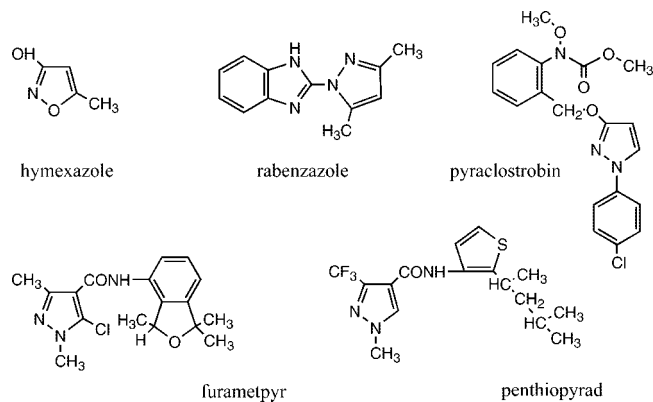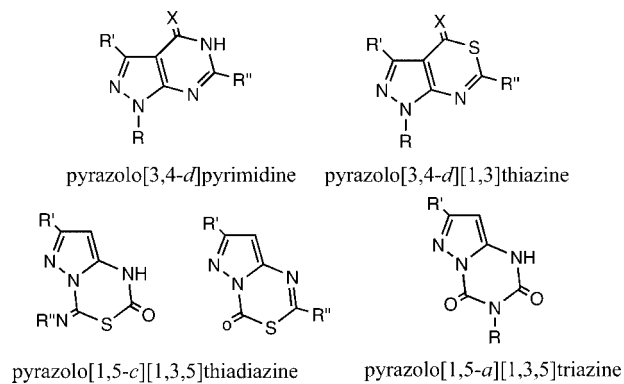**10332** *J. Agric. Food Chem.,* Vol. 55, No. 25, 2007

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Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

a: R= 3-trifluoromethylphenyl
b: R= 4-bromophenyl
c: R= 4-chlorophenyl
d: R= 3-chlorophenyl
e: R= 2-chlorophenyl
f: R= 4-nitrophenyl
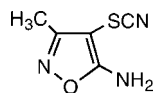g: R= ethyl
h: R= butyl
i: R= benzyl
j: R=cyclohexyl

Figure 6

Among pyrazoles, pyrazolo[3,4-*d*]pyrimidine-4(5*H*)-thiones were effective in controlling *Pythium ultimum* and *Rhizoctonia solani* (*23*). In particular, the activity of trifluoromethyl derivatives was comparable to that of the reference commercial fungicides captafol and mancozeb (*24, 25*). Pyrazolo[3,4-*d*]pyrimidine-4(5*H*)-thione, pyrazolo[3,4-*d*][1,3]thiazin-4-one/thione, and pyrazolo[1,5-*c*][1,3,5]thiadiazin-4-one/thione derivatives were screened for antifungal activity against the causal agent of rice blast disease, *Magnaporthe grisea*; some of these compounds caused a remarkable inhibition of fungal growth (*17*). Among these derivatives 7-methyl-2-phenylpyrazolo[1,5-*c*][1,3,5]thiadiazin-4-one inhibited the fungus even at the lowest dose tested (10 μg mL^{-1}), at which the reference commercial fungicide tricyclazole was completely ineffective.

Against the same fungus, more recently even pyrazolo[1,5-*a*][1,3,5]triazine-2,4-dione, pyrazolo[1,5-*c*][1,3,5]thiadiazin-2-one, and pyrazolo[3,4-*d*][1,3]thiazin-4-one/thione derivatives were tested; several compounds showed greater inhibition than tricyclazole, providing potential new chemicals for control of *M. grisea* infections (*20*) (**Figure 2**).

Among isoxazoles, 5-aminoisoxazole-4-thiocyanates were effective in controlling a large number of phytopathogens including *Botrytis cinerea* (*14*) and dermatophytic fungi (*15, 16*) (**Figure 3**).

The present study was carried out to investigate the antifungal activity of pyrazole-3-carboxamido-4-carboxylic acids **1n**, **1p**, **1q**, **1r**, **2n**, and **2s** in comparison with isoxazole-3-carboxamido-4-carboxylic acids **1m** and **2m** whose synthesis has already been
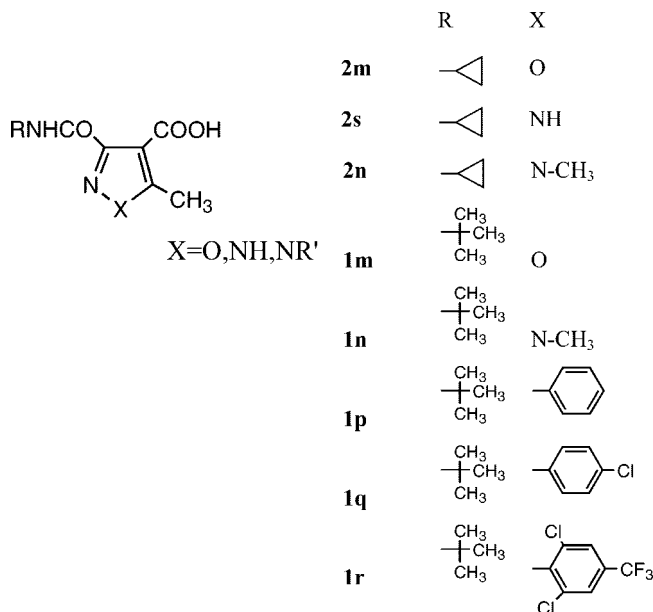
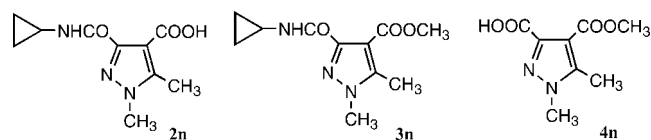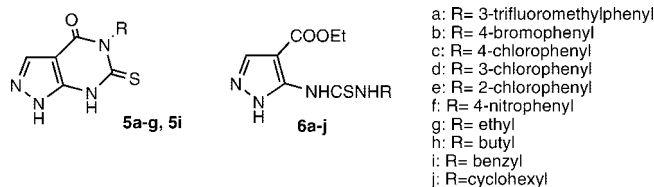accomplished by us (*26*); the compounds are referred to here as in a previous paper (**Figure 4**) where they showed a low herbicidal activity, being efficient only at high doses, in the millimolar range (*28*).

Since pyrazole-4-carboxylic acid esters were already reported to be fungicides (*27*), the corresponding 4-ester of the pyrazole-3-carboxamide (**3n**) and of pyrazole-3,4-dicarboxylic acid (**4n**) here are tested because it is expected that they also exhibit similar activity (**Figure 5**).

Moreover, a series of 4-oxo-5-substituted pyrazolo[3,4-*d*]pyrimidine-6-thiones (**5a–g,i**) was synthesized and tested as potential antifungal agents (**Figure 6**).

An additional purpose was to extend the screening program to *N*-alkyl/aryl-*N*′-(4-carbethoxy-3-pyrazolyl)thioureas (**6a–j**), prepared according to the previously reported procedures (*28*), to explore the potential of this further class of compounds as growth fungal inhibitors. In fact, as results from literature, the =C=S moiety is as pharmacophore provided with antimycotic activity (*29*) (**Figure 6**).

As tests *P. ultimum*, *B. cinerea*, and *M. grisea* were selected because they are important phytopathogenic fungi.

MATERIALS AND METHODS

Chemicals. Melting points were determined with a Buchi 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 spectrom-

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eter; 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 pentoxide. Elemental analyses (C, H, N, S) were within ±0.4 of theoretical values. For the flash chromatography technique, silica gel (230–400 mesh) was employed.

Synthesis. Pyrazole/isoxazole-3-carboxamido-4-carboxylic acids (**1**–**4**) and N-alkyl/aryl-N′-(4-carbetoxy-3-pyrazolyl)thioureas (**6a**–**j**) were synthesized in the laboratories of the Dipartimento di Scienze Farmaceutiche, University of Ferrara, as described previously (*26, 28*).

Synthesis of 4-Oxo-5-substituted Pyrazolo[3,4-d]pyrimidine-6-thiones (**5a**–**g**,**i**). A suspension of the pertinent N-alkyl/aryl-N′-(4-carbethoxy-3-pyrazolyl)thiourea (**6**) (0.6 g) in 4% aqueous sodium hydroxide (4 mL) was refluxed until no more of the starting material could be detected by TLC (1–2 h). After cooling, the solution was neutralized with 4% hydrochloric acid and then extracted with ethyl acetate (3 × 20 mL). After drying over anhydrous magnesium sulfate, the solvent was removed and the white solid residue was purified by flash column chromatography.

By using this procedure the following compounds were obtained:

5a *(R = 3-trifluoromethylphenyl):* yield 75%; mp 255 °C (purified by flash column chromatography; eluent ethyl acetate/petroleum ether, 8:2); ^{1}H NMR (DMSO-d_6) δ 7.55–7.77 (m, 4H, Ph), 8.62 (s, 1H, CH), 13.50 (s, 1H, NH), 13.77 (s, 1H, NH); IR (KBr, cm^{-1}) ν_{max} 3160, 2945, 1712, 1619, 1542. Anal. Calcd for $C_{12}H_7F_3N_4OS$: C, 46.15; H, 2.26; N, 17.94; S, 10.27. Found: C, 46.01; H, 2.32; N, 17.72; S, 10.45.

5b *(R = 4-bromophenyl):* yield 73%; mp 300–305 °C (purified by flash column chromatography; eluent ethyl acetate/petroleum ether, 8:2); ^{1}H NMR (DMSO-d_6) δ 7.18–7.64 (m, 4H, Ph), 8.60 (s, 1H, CH), 13.44 (s, 1H, NH), 13.75 (s, 1H, NH); IR (KBr, cm^{-1}) ν_{max} 3140, 2949, 1685, 1609, 1542. Anal. Calcd for $C_{11}H_7BrN_4OS$: C, 40.88; H, 2.18; N, 17.34; S, 9.92. Found: C, 40.75; H, 2.35; N, 17.48; S, 9.78.

5c *(R = 4-chlorophenyl):* yield 70%; mp 290–293 °C (purified by flash column chromatography; eluent ethyl acetate/petroleum ether, 8:2); ^{1}H NMR (DMSO-d_6) δ 7.25–7.51 (m, 4H, Ph), 8.60 (s, 1H, CH), 13.44 (s, 1H, NH), 13.75 (s, 1H, NH); IR (KBr, cm^{-1}) ν_{max} 3142, 2938, 1708, 1612, 1544. Anal. Calcd for $C_{11}H_7ClN_4OS$: C, 47.40; H, 2.53; N, 20.10; S, 11.50. Found: C, 47.62; H, 2.43; N, 19.98; S, 11.42.

5d *(R = 3-chlorophenyl):* yield 65%; mp 310–315 °C (purified by flash column chromatography; eluent ethyl acetate/petroleum ether, 8:2); ^{1}H NMR (DMSO-d_6) δ 7.21–7.49 (m, 4H, Ph), 8.60 (s, 1H, CH), 13.50 (s, 1H, NH), 13.75 (s, 1H, NH); IR (KBr, cm^{-1}) ν_{max} 3137, 2914, 1730, 1613, 1585, 1551. Anal. Calcd for $C_{11}H_7ClN_4OS$: C, 47.40; H, 2.53; N, 20.10; S, 11.50. Found: C, 47.25; H, 2.62; N, 20.02; S, 11.33.
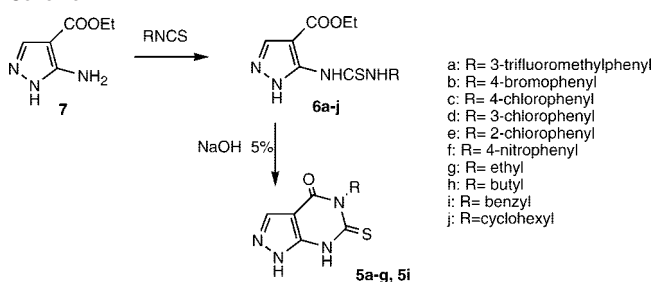
5e *(R = 2-chlorophenyl):* yield 82%; mp 264–269 °C (purified by flash column chromatography; eluent ethyl acetate/petroleum ether, 8:2); ^{1}H NMR (DMSO-d_6) δ 7.41–7.59 (m, 4H, Ph), 8.63 (s, 1H, CH), 13.53 (s, 1H, NH), 13.82 (s, 1H, NH); IR (KBr, cm^{-1}) ν_{max} 3138, 2936, 1700, 1612, 1542. Anal. Calcd for $C_{11}H_7ClN_4OS$: C, 47.40; H, 2.53; N, 20.10; S, 11.50. Found: C, 47.54; H, 2.61; N, 20.02; S, 11.36.

5f *(R = 4-nitrophenyl):* yield 78%; mp 328–333 °C (purified by flash column chromatography; eluent methylene chloride/methanol/toluene, 17:2:1); ^{1}H NMR (DMSO-d_6) δ 7.55–8.33 (m, 4H, Ph), 8.63 (s, 1H, CH), 13.56 (s, 1H, NH), 13.80 (s, 1H, NH); IR (KBr, cm^{-1}) ν_{max} 3278, 1718, 1615, 1589, 1542. Anal. Calcd for $C_{11}H_7N_5O_3S$: C, 45.67; H, 2.44; N, 24.21; S, 11.09. Found: C, 45.49; H, 2.59; N, 24.09; S, 10.94.

5g *(R = ethyl):* yield 70%; mp 254–258 °C (purified by flash column chromatography; eluent ethyl acetate/petroleum ether, 8:2); ^{1}H NMR (DMSO-d_6) δ 1.17 (t, 3H, J = 7.0 Hz, Me), 4.41 (q, 2H, J = 7.0 Hz, CH$_2$), 8.53 (s, 1H, CH), 13.23 (s, 1H, NH), 13.70 (s, 1H, NH); IR (KBr, cm^{-1}) ν_{max} 3151, 1703, 1623, 1546. Anal. Calcd for $C_7H_8N_4OS$: C, 42.84; H, 4.11; N, 28.55; S, 16.34. Found: C, 42.66; H, 4.24; N, 28.37; S, 16.23.

5i *(R = benzyl):* yield 72%; mp 253 °C (purified by flash column chromatography; eluent ethyl acetate/petroleum ether, 8:2); ^{1}H NMR (DMSO-d_6) δ 5.65 (s, 2H, CH$_2$), 7.20–7.30 (m, 5H, Ph), 8.56 (s, 1H, CH), 13.40 (s, 1H, NH), 13.77 (s, 1H, NH); IR (KBr, cm^{-1}) ν_{max} 3390, 1702, 1627, 1543. Anal. Calcd for $C_{12}H_{10}N_4OS$: C, 55.80; H, 3.90; N, 21.69; S, 12.41. Found: C, 55.96; H, 4.02; N, 21.48; S, 12.23.

Scheme 1

a: R= 3-trifluoromethylphenyl
b: R= 4-bromophenyl
c: R= 4-chlorophenyl
d: R= 3-chlorophenyl
e: R= 2-chlorophenyl
f: R= 4-nitrophenyl
g: R= ethyl
h: R= butyl
i: R= benzyl
j: R=cyclohexyl

Fungal Growth Conditions and Evaluation of Antifungal Activity. As tests, the following phytopathogenic fungi were employed: *P. ultimum* Trow, ATCC 58812 strain, *B. cinerea* (Pers.) Micheli, ATCC 48339 strain, and *M. grisea* (T. T. Hebert) Yaegashi & Udagawa, ATCC 64413 strain; all strains were purchased from the American Type Culture Collection (ATCC), Rockville, MD. The fungi were maintained at 4 °C as agar slants on potato dextrose agar (PDA; Difco, Detroit, MI).

To evaluate the ability of the compounds to inhibit fungal growth, 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 ± 1 °C on PDA (pH 5.6 ± 0.2) on thin sterile sheets of cellophane (BeP Italia, Gorizia, Italy) until the logarithmic phase of growth was reached and then 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, and 100 μg mL^{-1}. The DMSO concentration in the final solution was adjusted to 0.1%. Control media contained equivalent quantities (0.1%) of DMSO. The growth rate was determined by measuring daily the colony diameter for 5 days after the transport of the fungus onto dishes containing the substance to be tested. Three replicates were used for each concentration. Results were expressed as percentage of growth in untreated controls and are the means of at least three independent experiments. As positive controls for comparison the same concentrations (10, 50, and 100 μg mL^{-1}) of standard commercial fungicide specific for each fungus were also tested: pyraclostrobin (Fluka) for *P. ultimum*, benodanil (Fluka) for *B. cinerea*, and tricyclazole (Beam, Dow AgroSciences) for *M. grisea*.

The concentrations causing 50% inhibition (IC$_{50}$) of *in vitro* activity for selected compounds were obtained by analysis of inhibition curves of the activity values (%) versus the logarithm of inhibitory concentration. All calculations were performed by using the nonlinear regression curve fitting Graph Pad Prism computer program (San Diego, CA). At least five doses in the inhibitory range were considered.

RESULTS AND DISCUSSION

Synthesis. The preparative route to the target products **6a**–**j** and **5a**–**g**,**i** is outlined in **Scheme 1**. N-Alkyl/aryl-N′-(4-carbethoxy-3-pyrazolyl)thioureas **6** were obtained by reaction of 3-amino-4-carbethoxypyrazole (**7**) with the appropriate isothiocyanate, according to previously reported procedures (*28*). High yields of **6** have been obtained in the reaction of **7** with aryl isothiocyanates, but in the reaction of **7** with ethyl, butyl, and cyclohexyl isothiocyanate, the N′-(3-pyrazolyl)thioureas **6g**,**h**,**j** were isolated after purification by silica gel chromatography as byproducts. Cyclization of **6** with 4% aqueous sodium hydroxide provided the required 4-oxo-5-substituted pyrazolo[3,4-d]pyrimidine-6-thiones (**5a**–**g**,**i**) (**Scheme 1**).

Biological Activity. The ability of these 28 compounds to inhibit the growth of *P. ultimum*, *B. cinerea*, and *M. grisea* was evaluated at concentrations of 10, 50, and 100 μg mL^{-1} in comparison with their untreated controls and with the reference compounds for each fungus: pyraclostrobin, benodanil, and tricyclazole, respectively.

Results, summarized in **Tables 1**–**3**, were expressed as percent of growth in untreated controls, taken at the fifth day from the

Table 1. Percentage Inhibition Rate after 5 Days of Treatment with New Compounds **1–4** on *P. ultimum*, *B. cinerea*, and *M. grisea* and Relative Standard Specific Fungicides[a]

Compound		R	*P.ultimum* $\mu g\ mL^{-1}$			*B. cinerea* $\mu g\ mL^{-1}$			*M. grisea* $\mu g\ mL^{-1}$		
			10	50	100	10	50	100	10	50	100
2m	(structure)		46,0±1.2	46,6±2.6	53,5±2.0	3,8±0.4	+	+	+	2,3±0.9	8,97±0.5
2s	(structure)	-H	3,2±1.0	32,9±2.2	39,9±1.4	+	10,6±0.8	19,2±0.1	3,6±0.2	5,9±0.6	9,0±1.5
2n		-CH₃	+	6,6±0.5	11,4±0.7	3,0±0.2	9,9±1.3	19,8±0.1	+	4,3±1.0	8,6±0.5
3n	(structure)	-CH₃	4,8±0.2	4,3±0.3	7,7±0.2	+	4,0±0.1	3,2±1.1	+	+	3,4±0.7
4n	(structure)	-CH₃	+	+	+	+	+	3,3±0.2	0	+	8,4±0.4
1m	(structure)		9,7±1.1	15,8±0.9	40,2±2.3	4,3±0.7	13,3±0.3	18,2±1.0	+	+	+
1n		-CH₃	+	12,5±0.6	41,3±3.1	+	9,6±0.3	61,9±2.2	0,9±0.1	4,1±0.5	5,8±0.4
1p	(structure)	(phenyl)	12,1±1.2	82,3±3.1	83,4±2.7	39,5±1.7	43,1±1.8	60,5±2.6	0,6±0.1	3,8±0.6	13,9±0.5
1q		(4-Cl-phenyl)	47,2±1.5	69,3±2.7	69,3±1.9	45,6±1.4	49,3±0.7	49,9±0.9	20,9±1.2	31,5±1.6	33,2±1.4
1r		(2,6-dichloro-4-CF₃-phenyl)	1,9±0.3	37,9±1.1	54,7±0.9	17,3±0.4	22,6±1.2	27,5±0.7	+	20,6±0.4	24,4±1.5
pyraclostrobin	(structure)		100±0	100±0	100±0						
benodanil	(structure)					6,3±0.3	91,2±2.5	97,6±2.0			
tricyclazole	(structure)								0	24,0±0.6	62,0±1.8

[a] Growth was evaluated as described in Materials and Methods either in the absence or in the presence of pyrazole derivatives at concentrations ranging from 10 to 100 $\mu g\ mL^{-1}$. Each sample was carried out in triplicate, and values were expressed as percentage of untreated controls.

transplant of the fungi; an example graph showing the growth lines of a fungus untreated and treated with one of the most active compound and the specific reference compound during the 5 days of the experiment is shown in **Figure 7**.

For the most active substances were carried out also the IC₅₀, the concentrations causing 50% inhibition of *in vitro* activity (**Table 4**).

P. ultimum. Considering *P. ultimum* the reference compound is pyraclostrobin. For the cyclopropylcarboxamides (**Table 1**) the replacement of the carboxyisoxazole moiety with a carboxypyrazole resulted in a loss of activity (**2m** > **2s** > **2n**).

The methyl ester **3n** showed lower inhibition whereas **4n** was completely ineffective when compared to the corresponding carboxamide **2n**.

When in position 3 there is a *tert*-butylcarboxamide group, the efficacy of the pyrazole-3-*tert*-butylcarboxamide **1n** (R = CH₃) and isoxazole-3-*tert*-butylcarboxamide **1m** is comparable.

Interesting is the insertion of a substituent in position 1 of pyrazole. The presence of a phenyl, 4-chlorophenyl, or 2,6-dichloro-4-trifluoromethylphenyl group at position 1 (com-
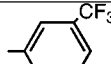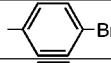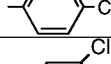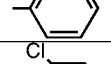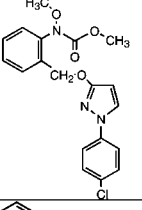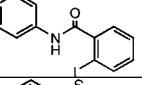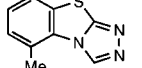
pounds **1p**, **1q**, and **1r**) significantly improved the antimycotic activity of the compounds. The phenyl derivative **1p** was able to exert an inhibition of fungal growth higher than 80% at concentrations of 50 and 100 $\mu g\ mL^{-1}$; remarkable is also the inhibition near 50% exerted by the 4-chlorophenyl derivative **1q** at the lowest dose tested (10 $\mu g\ mL^{-1}$).
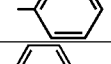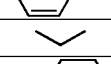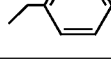
The pyrazolo[3,4-*d*]pyrimidine derivative **5a** [5-(3-trifluoromethyl)phenyl] was able to exert a dose-dependent inhibition of fungal growth (**Table 2**).

The replacement of the (3-trifluoromethyl)phenyl group with a 4-bromophenyl, 4-chlorophenyl, 4-nitrophenyl, or 3-chlorophenyl group resulted in a gradual decrease of activity (**5b** > **5c** > **5f** > **5d**) or in a total inefficacy with 2-chlorophenyl (**5e**) and ethyl (**5g**) groups. Instead, the presence of a benzyl group in position 5 (compound **5i**) significantly improved the activity.

The efficacy of the thioureas **6e**, **6c**, **6d**, **6f**, and **6j** (R = 2-chlorophenyl, 4-chlorophenyl, 3-chlorophenyl, 4-nitrophenyl, and cyclohexyl) is comparable at the highest dose varying from 60.8% to 70%. Remarkable is the inhibition brought

Table 2. Percentage Inhibition Rate after 5 Days of Treatment with New Compounds **5** on *P. ultimum*, *B. cinerea*, and *M. grisea* and Relative Standard Specific Fungicides[a]

Compound	R	*P.ultimum* $\mu g\ mL^{-1}$			*B. cinerea* $\mu g\ mL^{-1}$			*M. grisea* $\mu g\ mL^{-1}$		
		10	50	100	10	50	100	10	50	100
5a	CF₃ phenyl	17.2±2.2	34.5±1.3	57.8±0.8	0.5±0.4	1.0±0.3	7.2±0.6	+	1.4±0.2	4.1±1.3
5b	Br phenyl	+	22.5±1.5	61.8±2.8	+	+	+	+	18.0±1.3	20.0±1.7
5c	Cl phenyl	1.7±0.5	12.2±1.8	37.8±1.0	+	+	31.7±0.7	+	+	+
5d	Cl phenyl	2.2±0.2	4.1±1.0	10.8±0.3	+	8.5±0.5	27.3±0.5	+	1.0±0.4	5.0±1.1
5e	Cl,Cl phenyl	+	+	+	40.0±1.5	63.8±2.5	66.2±3.0	+	+	+
5f	NO₂ phenyl	4.6±0.4	8.7±0.6	13.7±0.2	+	+	2.1±0.1	+	+	+
5g	cyclopropyl	+	+	+	+	+	+	+	3.6±0.4	4.1±0.3
5i	benzyl	44.1±1.4	64.6±2.3	78.0±2.9	13.5±1.0	23.4±1.1	31.9±0.9	0.5±0.1	23.4±1.2	44.4±2.0
pyraclostrobin		100±0	100±0	100±0						
benodanil					6,3±0.3	91,2±2.5	97,6±2.0			
tricyclazole								0	24,0±0.6	62,0±1.8

[a] Growth was evaluated as described in Materials and Methods either in the absence or in the presence of pyrazole derivatives at concentrations ranging from 10 to 100 $\mu g\ mL^{-1}$. Each sample was carried out in triplicate, and values were expressed as percentage of untreated controls.

by the 2-chloropheyl derivative **6e** and cyclohexyl derivative **6j** at the lowest concentration tested (**Table 3**).

Nevertheless, all compounds gave an inhibition rate inferior to that of the reference compound pyraclostrobin at the same doses (**Tables 1–3**). The compounds selected for IC$_{50}$ were **1p**, **1q**, and **5i** (**Table 4**); they showed an antifungal activity in the micromolar range, but it is not comparable with that of the reference compound pyraclostrobin.

B. cinerea. For this fungus the reference compound is benodanil.

Among the first set of compounds, the cyclopropyl derivatives **2m**, **2s**, and **2n** were marginally effective at all tested doses (**Table 1**).

On the contrary the presence of the *tert*-butyl group improved the antifungal activity of the pyrazole derivatives (**1n–r**) but not that of the isoxazole derivative (**1m**). Middling inhibitions (around 50%) resulted from the 4-chlorophenyl derivative **1q** at all doses; remarkable (45.6% at 10 $\mu g\ mL^{-1}$) and higher to that one of benodanil (equal to 6.34% at 10 $\mu g\ mL^{-1}$) was the inhibition at the lowest concentration.

Among the pyrazolo[3,4-*d*]pyrimidines, only **5e** (2-chlorophenyl) was able to exert a good inhibition of growth at all of the concentrations; in particular, noteworthy is the inhibition brought

at the lowest rate tested (**Table 2**). The benzyl, 4-chlorophenyl, and 3-chlorophenyl derivatives **5i**, **5c**, and **5d** showed only low activities at 100 $\mu g\ mL^{-1}$, about 30% inhibition, whereas they were as ineffective at the lower doses as were the other compounds belonging to the same series.
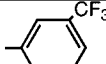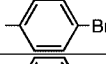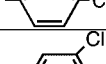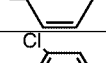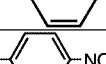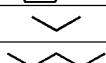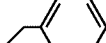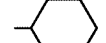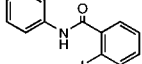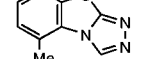
The efficacy of the thioureas **6d** (R = 3-chlorophenyl) and **6i** (R = benzyl) at the highest dose is comparable, but only **6d** showed remarkable inhibition at the lowest dose tested (**Table 3**).

The 4-chlorophenyl, 4-nitrophenyl, and 2-chlorophenyl derivatives **6c**, **6f**, and **6e** caused an inhibition of 46.2%, 37.8%, and 36.0%, respectively.

The compounds selected to evaluate the IC$_{50}$ were **5e** and **6d**; the last showed a value even lower than that of benodanil, suggesting a good activity against *B. cinerea* (**Table 4** and **Figure 7**).

M. grisea. For *M. grisea* the specific reference compound tricyclazole here is used at concentrations of 10, 50, and 100 $\mu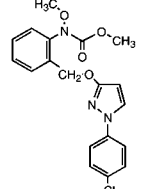 g\ mL^{-1}$, a range in which all new compounds were tested. At these doses tricyclazole exerts an increasing inhibition effect, even if it does not achieve the fungal 100% inhibition, that is reached in the normal agricultural practice where, as a rule, it is used at 200 $\mu g\ mL^{-1}$ (*2*). The mechanism of action of this

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Table 3. Percentage Inhibition Rate after 5 Days of Treatment with New Compounds **6** on *P. ultimum*, *B. cinerea*, and *M. grisea* and Relative Standard Specific Fungicides[a]

Compound	R	*P.ultimum* μg mL^{-1}			*B. cinerea* μg mL^{-1}			*M. grisea* μg mL^{-1}		
		10	50	100	10	50	100	10	50	100
6a	CF$_3$ phenyl	23.0±0.5	24.2±1.4	28.0±1.7	+	+	23.2±0.7	29.4±0.9	46.3±1.4	51.0±3.2
6b	Br phenyl	+	2.0±0.3	3.1±0.2	+	+	16.3±1.1	16.1±0.9	27.1±0.6	27.2±1.4
6c	Cl phenyl	38.0±2.2	52.9±3.1	61.5±3.4	+	21.0±2.5	46.2±1.1	21.4±0.6	33.6±0.9	36.7±2.6
6d	Cl phenyl	32.9±1.7	38.1±2.2	60.8±2.0	56.0±1.2	56.8±0.8	76.2±1.5	16.6±0.4	18.0±0.3	26.0±1.3
6e	Cl phenyl	66.5±2.8	68.7±0.7	70.0±3.1	4.3±0.5	30.9±1.2	36.0±2.4	48.7±2.0	60.9±2.8	67.0±1.4
6f	NO$_2$ phenyl	27.2±1.0	36.0±1.5	63.4±0.8	13.2±0.2	33.0±1.6	37.9±0.5	13.0±0.4	15.6±1.2	17.7±1.7
6g	propyl	+	+	20.0±0.8	+	+	+	13.8±0.6	18.1±1.3	28.9±1.2
6h	butyl	+	+	10.4±0.4	4.7±0.5	15.1±1.1	32.4±2.7	+	8.6±1.4	10.0±0.1
6i	benzyl	3.7±0.1	34.6±2.1	38.3±1.5	0	26.0±0.5	32.0±1.6	15.8±0.8	35.6±1.9	41.4±1.6
6j	cyclohexyl	44.4±2.7	59.0±2.9	63.9±1.6	+	+	+	29.8±0.8	26.7±1.4	36.0±1.6
pyraclostrobin		100±0	100±0	100±0						
benodanil					6,3±0.3	91,2±2.5	97,6±2.0			
tricyclazole								0	24,0±0.6	62,0±1.8

The R column shows common structure with COOEt and NHCSNHR groups on pyrazole ring.

[a] Growth was evaluated as described in Materials and Methods either in the absence or in the presence of pyrazole derivatives at concentrations ranging from 10 to 100 μg mL^{-1}. Each sample was carried out in triplicate, and values were expressed as percentage of untreated controls.

compound has been studied on *M. grisea*, where it prevents penetration of plant epidermis by weakening the fungal walls by inhibiting pigmentation of the hyphae (*30*). In our previous work some pyrazole derivatives showed two different mechanisms of action: one similar to that of other azoles, by inhibiting hyphal growth, and the other similar to that of tricyclazole, by inhibiting pigmentation of the mycelium (*17*). For this reason on this fungus we evaluated both inhibition of growth and depigmentation.

Under the experimental conditions employed, the compounds belonging to pyrazole/isoxazolecarboxamides (**1** and **2**) and their esters (**3n** and **4n**) in general showed a low efficiency (**Table 1**).

Only the 4-chlorophenyl derivative **1q** was able to exert a 20.9% inhibition of fungal growth at a concentration of 10 μg mL^{-1}, a dose at which tricyclazole was completely ineffective, and at 50 μg mL^{-1} caused an inhibition equal to 31.5%, that is slightly higher than that of the reference compound.

Even the results of the pyrazolopyrimidine derivatives on *Magnaporthe* are scanty: in the series only the pyrazolo[3,4-*d*]pyrimidine **5i** (R = benzyl) was active, and its action is comparable to that of tricyclazole at 50 μg mL^{-1} (**Table 2**).

On the contrary, the thiourea **6e** (R = 2-chlorophenyl) showed a good activity higher than the one of the reference compound at all of the doses (**Table 3**). Noteworthy for all of the compounds of this series, except **6h** (R = butyl), is the ability of inhibit the growth of *M. grisea* even at the lowest dose. The compounds selected for IC$_{50}$ were **6a** and **6e**, both showing values lower than that of tricyclazole, in particular, **6e** (**Table 4**).

A parameter that we considered in this work was the capacity of the new azoles to inhibit the color of the mycelium. Subsequent visual observation showed that tricyclazole inhibits the pigmentation of the hyphae, whereas all of the new compounds **1**–**6** were ineffective.

In conclusion, the results on fungal growth inhibition (**Tables 1**–**3**) and IC$_{50}$ evaluation (**Table 4**) showed differences in the sensitivity of the three fungi to the substances tested, *P. ultimum* being in general the most inhibited.

Within the compounds of the pyrazole/isoxazolecarboxamide series on all fungi compounds **1p** and **1q** were found to give the best results. The presence of the carboxamide and carboxylic groups in positions 3 and 4 seems to be critical for biological activity. In our previous work carboxamides **1** and **2** were tested
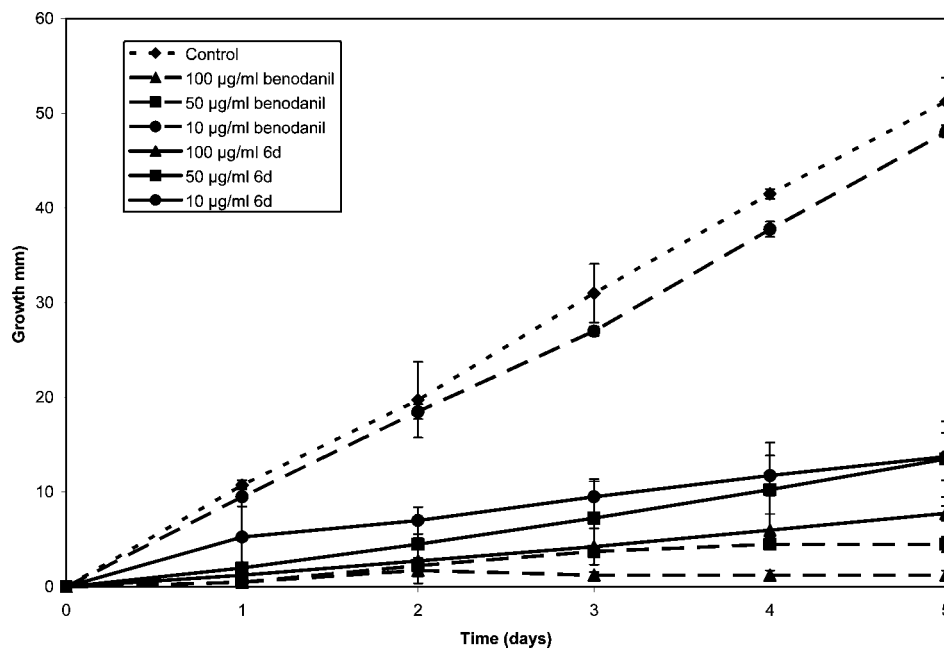
Pyrazole Derivatives as Inhibitors of Phytopathogenic Fungi

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Figure 7. *B. cinerea* treated with **6d** and benodanil.

Table 4. IC$_{50}$ of Selected Compounds

P. ultimum		B. cinerea		M. grisea	
compound	IC$_{50}^{a}$ (μM)	compound	IC$_{50}^{a}$ (μM)	compound	IC$_{50}^{a}$ (μM)
1p	79	**5e**	83	**6a**	224
1q	38	**6d**	31	**6e**	48
5i	66				
pyraclostrobin	≪10	benodanil	66	tricyclazole	474

^{a} The concentrations causing 50% inhibition (IC$_{50}$) of in vitro activity were obtained by analysis of inhibition curves of the activity values (%) versus the logarithm of inhibitory concentration. All calculations were performed by using the nonlinear regression curve fitting Graph Pad Prism computer program (San Diego, CA).

as possible herbicides and showed in general a low ability to act as photosynthetic electron inhibitors and only at millimolar concentrations (*28*); thus, their use as agrochemicals seems to be not satisfactory. Among the same compounds tested as antifungals the most interesting is **1q**, which is the only one with both herbicidal (IC$_{50}$ = 0.95 mM on isolated chloroplasts) and antifungal activity (IC$_{50}$ = 38 μM on *P. ultimum*). But, whereas the herbicidal activity is evinced at millimolar concentration, the antifungal activity is evinced in the micromolar range, at doses lower (about 25 times) than those of the herbicidal. From these preliminary data we think that its use as an antifungal could be not precluded, because the effect against the fungus is substantial at concentrations at which it is ineffective against plants.

Important among the pyrazolopyrimidines is the activity of the derivative supplied with the benzylic group **5i**, which resulted as the most active on the three fungi and in particular against *P. ultimum*.

Noteworthy is the activity of most of the thiourea series, which are able to selectively inhibit the growth of *M. grisea* at 10 μg mL^{-1}, at a dose of treatment 20 times lower to that of the reference compound tricyclazole. The high inhibitory ability at low concentrations and, at the same time, the lacked change of mycelial pigmentation after treatment let us suppose that these new thiourea molecules have a mechanism of action different from that of tricyclazole that inhibit the melanin biosynthesis (*31*).

Against *B. cinerea* the pyrazolopyrimidine derivative **5e** (R = 2-chlorophenyl) and thioureidic derivative **6d** (R = 3-chlorophenyl) are to be underlined because they showed a good activity at 50 and 100 μg mL^{-1}, but, especially at 10 μg mL^{-1}, they showed inhibitions much higher than those of the reference compound benodanil.

These results can be interesting for the future possibility of using in agricultural practices chemical synthetic substances against two widespread phytopathogenic fungi (*M. grisea* and *B. cinerea*) at doses much lower than those currently used and, thus, more ecocompatible. Screening in depth on the compound phytotoxicity will be made for the most active compound in each group.

In spite of the different responses given by the fungi, the present results are encouraging for further studies to clarify the mechanism of action of the most active compounds.

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