JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Development of New Fungicides against *Magnaporthe grisea*: Synthesis and Biological Activity of Pyrazolo[3,4-*d*][1,3]thiazine, Pyrazolo[1,5-*c*][1,3,5]thiadiazine, and Pyrazolo[3,4-*d*]pyrimidine Derivatives

CHIARA B. VICENTINI,*,† GIUSEPPE FORLANI,§ MAURIZIO MANFRINI,† CARLO ROMAGNOLI,# AND DONATELLA MARES‡

Dipartimento di Scienze Farmaceutiche, Dipartimento di Biologia, and Dipartimento delle Risorse Naturali e Culturali, Università di Ferrara, I-44100 Ferrara, Italy; and Dipartimento del Museo di Paleobiologia e dell'Orto Botanico, Università di Modena e Reggio Emilia, Italy

Some pyrazolo[3,4-*d*]pyrimidin-4(5*H*)-thione, pyrazolo[3,4-*d*][1,3]thiazin-4-one/thione, and pyrazolo-[1,5-*c*][1,3,5]thiadiazine-4-one/thione derivatives were synthesized and screened for antifungal activity against the causal agent of rice blast disease, *Magnaporthe grisea*. In all cases a remarkable inhibition of fungal growth was found in the range from 10 to 200 μg mL^{-1}. Several compounds were able to control mycelium growth at a rate of 10 μg mL^{-1}, a concentration at which the reference compound tricyclazole was completely ineffective. At least in the case of the most active substance, at the same dose the growth of seedlings or cultured cells of rice was substantially unaffected. Results allowed definition of structural requirements either to maintain or to enhance mycotoxic activity.

KEYWORDS: Rice; blast; *Magnaporthe grisea*; antifungals; pyrazolo[3,4-*d*][1,3]thiazine; pyrazolo[1,5-*c*]-[1,3,5]thiadiazine; pyrazolo[3,4-*d*]pyrimidine

INTRODUCTION

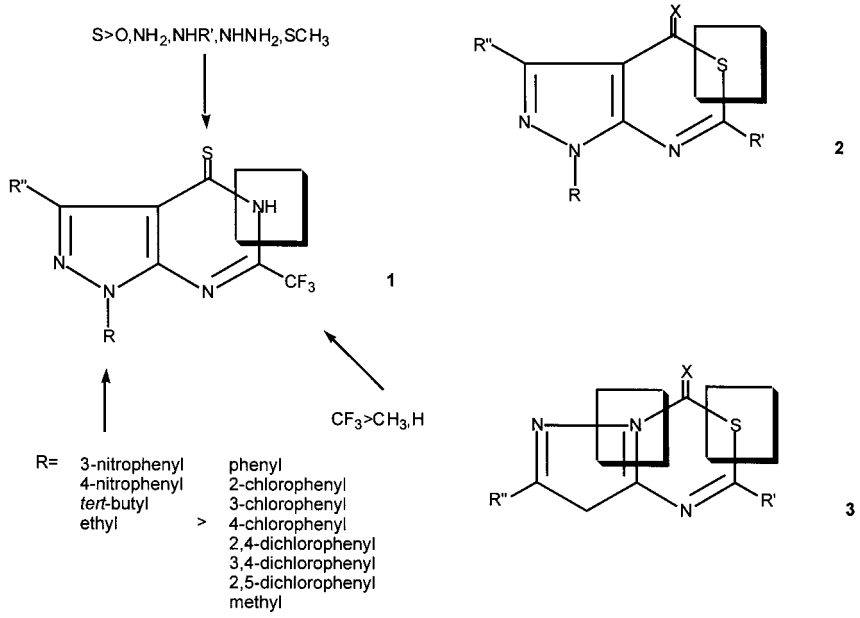
Plant diseases are estimated to cause yield reduction of almost 20% in the major food and cash crops worldwide (*1*). Fungicides remain vital for effective control of fungal plant pathogens; thus, researchers are continuously developing a diverse range of products with novel modes of action (*2*). Rice is the major human staple crop for almost half of the world's population, particularly in East and Southeast Asia, and is of great economic value in northern Italy. The filamentous fungus *Magnaporthe grisea* (T.T. Hebert) Yaegashi & Udagawa, an anamorph of *Pyricularia grisea* (Cooke) Sacc., can cause disease in many species of the grass family. The disease in rice, rice blast, is considered to be its main fungal disease because of its wide distribution and destructiveness under favorable conditions (*3*). Plant infection is brought about by the action of specialized cells called appressoria, generating enormous turgor pressure, which is translated into an invasive force that allows a narrow penetration hypha to break plant cuticle (*4*). With this aim, the fungus requires and utilizes melanin-derived pressure; thus, melanin biosynthesis inhibition has been shown to be a promising biochemical target for the discovery of new selective fungicides. As to the commercial compounds resulting from this approach, the most important is tricyclazole, patended by Eli Lilly (*5*). Tricyclazole causes a weakening of fungal walls and prevents the penetration of plant epidermis by fungal hyphae. During the past few years a number of other compounds that inhibit several enzymes in melanin biosynthesis have been described with a putative ability of preventing blast disease (e.g. refs *6* and *7*). However, because of such an action, these compounds are ineffective in controlling rice blast if applied after pathogen penetration (*8*). Consequently, the development of new fungicides able to inhibit fungal growth would be of greatest agronomical value.

Within the framework of a program directed to investigate the antifungal activity of nitrogen heterocycles, the synthesis of pyrazolo[3,4-*d*]pyrimidin-4(5*H*)-thiones has previously been reported (*9*). A screening for antifungal activity showed that some compounds of this series have a wide spectrum of activity against phytopathogenic fungi of different taxa and are particularly effective in controlling *Pythium ultimum* and *Corticium solani* (*9*). With this background, the antifungal activity of derivatives of pyrazolo[3,4-*d*]pyrimidin-4(5*H*)-thiones was investigated. The first stage was to prepare 6-trifluoromethyl derivatives (*10*−*12*): this was prompted by the reported efficacy of the trifluoromethyl group in some pesticides (*13*). In particular three lead compounds (R = 3-nitrophenyl, 4-nitrophenyl, and *tert*-butyl) showed EC_{50} and MIC values comparable or only slightly inferior to those of the reference commercial fungicides captafol and mancozeb against *Sclerotinia minor*, *C. solani*, and *P. ultimum* (*10*, *11*, *14*). After these results, the introduction of

* Author to whom correspondence should be addressed [fax (39) 0532-291296; e-mail vcc@unife.it].
† Dipartimento di Scienze Farmaceutiche, Università di Ferrara.
§ Dipartimento di Biologia, Università di Ferrara.
‡ Dipartimento delle Risorse Naturali e Culturali, Università di Ferrara.
# Università di Modena e Reggio Emilia.

10.1021/jf0202436 CCC: $22.00
Published on Web 07/12/2002

Scheme 1



new substituents in the heterocyclic system was planned. The purpose was to realize the replacement of the 4-thione of pyrazolopyrimidines with various substituents in order to investigate the influence of substitution at C_4 on antifungal activity. By combining the obtained results (*9*−*12*, *14*), we concluded that key structural requirements for the antifungal activity of pyrazolo[3,4-*d*]pyrimidines (**1**) were (i) a thione function at position 4, (ii) a trifluoromethyl group at position 6, and (iii) a 3- or 4-nitrophenyl or a *tert*-butyl group at position 1 (**Scheme 1**).

In this paper we report the synthesis and evaluation of biological activity against *M. grisea* of pyrazolo[3,4-*d*]pyrimidin-4(5*H*)-thione derivatives (**1**; **Scheme 1**) and of the analogues pyrazolo[3,4-*d*][1,3]thiazin-4-one/thione (**2**) and pyrazolo[1,5-*c*][1,3,5]thiadiazine-4-one/thione (**3**). The widely used fungicide tricyclazole was adopted as a reference compound. The sensitivity of rice cells and seedlings to the most active substance was also tested.

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 pentoxide. Elemental analyses (C, H, N, S) were within ±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.

Syntheses. Compounds **1a**−**d** (*11*), **5** (R = *t*-Bu; R′ = Ph, CH_2Ph; R″ = Me) (*15*), **6** (R′= 4-ClPh, 4-BrPh; R″ = Me) (*16*), and **2i**, **2k**, **2m**, and **3a** (*15*) were prepared according to the methods previously reported by us.

***N*-(5-Pyrazolyl)carboxamides (4).** *Method A.* A solution of the appropriate 5-aminopyrazole (10 mmol) in trifluoroacetic anhydride (5 mL) was heated at 100 °C for 1 h. After cooling, the solution was evaporated to give a crude solid, which was purified by column chromatography or by recrystallization from the indicated solvent. By using this procedure the following compounds were obtained.

4 *(R = tert-butyl; R′ = CF_3; R″ = methyl):* yield 71%; mp 92−95 °C (purified by column chromatography, eluent 1:1 ethyl acetate/petroleum ether); IR (KBr, cm^{-1}) ν_{max} 3158, 2992, 1752, 1676, 1571; ^{1}H NMR ($CDCl_3$) δ 1.65 (s, 9H, *t*-Bu), 2.33 (s, 3H, Me), 6.30 (s, 1H, CH), 10.31 (br, 1H, NH).

4 *(R = phenyl; R′ = CF_3; R″ = methyl):* yield 74%; mp 180−185 °C (ethyl ether/petroleum ether); IR (KBr, cm^{-1}) ν_{max} 2980, 2830, 1740, 1600, 1570, 1510; ^{1}H NMR ($CDCl_3$) δ 2.27 (s, 3H, Me), 6.47 (s, 1H, CH), 7.27−7.54 (m, 5H, Ph), 8.47 (br, 1H, NH); ^{1}H NMR (DMSO-d_6) δ 2.25 (s, 3H, Me), 6.37 (s, 1H, CH), 7.46−7.50 (m, 5H, Ph), 11.61 (s, 1H, NH).

4 *(R = 3,4-dichlorophenyl; R′ = CF_3; R″ = methyl):* yield 71%; mp 81−84 °C (purified by column chromatography, eluent 1:1 ethyl acetate/petroleum ether); IR (KBr, cm^{-1}) ν_{max} 2929, 1741, 1578, 1487; ^{1}H NMR ($CDCl_3$) δ 2.32 (s, 3H, Me), 6.52 (s, 1H, CH), 7.22−7.62 (m, 3H, Ph), 8.02 (br, IH, NH).

4 *(R = 3-nitrophenyl; R′ = CF_3; R″ = methyl):* yield 82%; mp 114−117 °C (ethyl ether/petroleum ether); IR (KBr, cm^{-1}) ν_{max} 3152, 1745, 1540; ^{1}H NMR ($CDCl_3$) δ 2.33 (s, 3H, Me), 6.40 (s, 1H, CH), 7.69−8.31 (m, 5H, Ph + NH).

Method B. A solution of the pertinent acyl chloride (20 mmol) in methylene chloride (10 mL) was added dropwise to a mixture of the appropriate 5-amino-1-*tert*-butylpyrazole (20 mmol) in methylene chloride (100 mL) and sodium hydrogen carbonate (1.68 g, 20 mmol) in water (50 mL). After 24 h of stirring at room temperature, the organic phase was washed with 5% sodium hydrogen carbonate and water and then anhydrified over anhydrous magnesium sulfate. After removal of the solvent, the residue was recrystallized from the indicated solvent to give colorless crystals. By using this procedure the following compounds were obtained.

4 *(R′ = phenyl, R″ = H; R = t-Bu):* yield 55%; mp 200−201 °C (ethyl ether); IR (KBr, cm^{-1}) ν_{max} 3299, 2991, 1660, 1557, 1511; ^{1}H NMR ($CDCl_3$) δ 1.68 (s, 9H, t-Bu), 6.39 (s, 1H, CH), 7.47−7.57 (m, 3H, Ph), 7.60 (s, 1H, CH), 7.71 (br, 1H, NH).

4 *(R′, R″ = phenyl; R = t-Bu):* yield 69%; mp 187−190 °C (ethyl ether); IR (KBr, cm^{-1}) ν_{max} 3227, 2988, 1652, 1552, 1523; ^{1}H NMR ($CDCl_3$) δ 1.70 (s, 9H, t-Bu), 6.69 (s, 1H, CH), 7.25−7.92 (m, 10H, 2Ph), 10.86 (br, 1H, NH).

***N*-(5-Pyrazolyl)thiocarboxamides (5).** Lawesson's reagent (2.02 g, 5 mmol) was added to a solution of the appropriate carboxamide **4** (5 mmol) in hexamethylphosporamide (12.5 mL) at 80 °C. The mixture was kept under stirring at 80 °C until no more of the starting material could be detected by TLC (5−6 h). The solution was then diluted with water and extracted with ethyl acetate. The organic layer was extracted with 1 N sodium hydroxide (3 × 50 mL), and the aqueous layer was acidified to pH 5 with 10% hydrochloric acid and then extracted with ethyl acetate (3 × 50 mL). After drying over anhydrous magnesium

sulfate, the solvent was removed and the solid residue was purified by column chromatography to give a pale yellow crystalline product.

5 *(R = tert-butyl; R′ = CF3; R″ = methyl):* yield 57%; oil (purified by column chromatography, eluent 17:2:1 methylene chloride/methanol/toluene); IR (near, cm^{-1}) ν_{max} 3258, 2984, 1557; ^1H NMR ($CDCl_3$) δ 1.63 (s, 9H, *t*- Bu), 2.03 (s, 3H, Me), 6.30 (s, 1H, CH), 10.50 (br, 1H, NH).

5 *(R = phenyl; R′ = CF3; R″ = methyl):* yield 39%; mp 220−223 °C (purified by column chromatography, eluent 17:2:1 methylene chloride/methanol/toluene); IR (KBr, cm^{-1}) ν_{max} 2791, 1567, 1533, 1502; ^1H NMR ($CDCl_3$) δ 2.36 (s, 3H, Me), 7.03 (s, 1H, CH), 7.43−7.51 (m, 5H, Ph), 9.5 (br, 1H, NH).

5 *(R = 3,4-dichlorophenyl; R′ = CF3; R″ = methyl):* yield 14%; mp 203−204 °C (purified by column chromatography, eluent 1:1 ethyl acetate/petroleum ether); IR (KBr, cm^{-1}) ν_{max} 2924, 1577, 1483; ^1H NMR ($CDC1_3$) δ 2.35 (s, 3H, Me), 6.80 (s, 1H, CH), 7.22−7.59 (m, 3H, Ph), 9.29 (br, 1H, NH).

5 *(R = 3-nitrophenyl; R′ = CF3; R″ = methyl):* yield 40%; mp 178−180 °C (purified by column chromatography, eluent 8:2 ethyl acetate/petroleum ether); IR (KBr, cm^{-1}) ν_{max} 2924, 1535; ^1H NMR ($CDCl_3$) δ 2.35 (s, 3H, Me), 6.64 (s, 1H, CH), 7.66−8.32 (m, 4H, Ph), 9.30 (br, 1H, NH).

5 *(R′ = phenyl, R″ = H; R = t-Bu):* yield 74%; oil (purified by column chromatography, eluent 1:1 ethyl acetate/petroleum ether); IR (near, cm^{-1}) ν_{max} 3377, 2988, 1549, 1470; ^1H NMR ($CDCl_3$) δ 1.67 (s, 9H, t-Bu), 6.37 (s, 1H, CH), 7.46−7.56 (m, 3H, Ph), 7.54 (s, 1H, CH), 8.55 (br, 1H, NH).

5 *(R′, R″ = phenyl; R = t-Bu):* yield 73%; mp 101−104 °C (purified by column chromatography, eluent 17:2:1 methylene chloride/methanol/toluene); IR (KBr, cm^{-1}) ν_{max} 3147, 2982, 1553, 1519; ^1H NMR ($CDCl_3$) δ 1.71 (s, 9H, t-Bu), 6.70 (s, 1H, CH), 7.25−7.90 (m, 10H, 2Ph), 8.58 (br, 1H, NH).

6-Substituted Pyrazolo[3,4-*d*][1,3]thiazin-4-thiones (2a—d,f,h). Thiophosgene (0.41 mL, 5.5 mmol) was added to a suspension of thiocarboxamide **5** (5 mmol) in anhydrous toluene (100 mL), placed in a round-bottom flask equipped with a Vigreaux reflux condenser. The mixture was heated under reflux until hydrogen chloride evolution ceased (∼5 h). After removal of the solvent, the solid residue was purified by column chromatography to give a yellow crystalline product.

2a *(R = tert-butyl; R′ = CF3; R″ = methyl):* yield 19%; mp 133−135 °C (purified by column chromatography, eluent 3:7 ethyl acetate/petroleum ether); IR (KBr, cm^{-1}) ν_{max} 2977, 1563, 1515, 1472; ^1H NMR ($CDCl_3$) δ 1.76 (s, 9H, *t*-Bu), 2.70 (s, 3H, Me).

2b *(R = phenyl; R′ = CF3; R″ = methyl):* yield 28%; mp 120−124 °C (purified by column chromatography, eluent 3:7 ethyl acetate/petroleum ether); IR (KBr, cm^{-1}) ν_{max} 3448, 1559, 1520, 1471; ^1H NMR ($CDCl_3$) δ 2.73 (s, 3H, Me), 7.40−7.81 (m, 5H, Ph).

2c *(R = 3,4-dichlorophenyl; R′ = CF3; R″ = methyl):* yield 48%; mp 138−140 °C (purified by column chromatography, eluent 1:1 ethyl acetate/petroleum ether); IR (KBr, cm^{-1}) ν_{max} 3435, 1554, 1522, 1468; ^1H NMR ($CDCl_3$) δ 2.79 (s, 3H, Me), 7.52−8.13 (m, 3H, Ph).

2d *(R = 3-nitrophenyl; R′ = CF3; R″ = methyl):* yield 78%; mp 105−108 °C (purified by flash column chromathography, eluent 3:7 ethyl acetate/petroleum ether); IR (KBr, cm^{-1}) ν_{max} 3111, 2926, 1537, 1470; ^1H NMR ($CDCl_3$) δ 2.81 (s, 3H, Me), 7.70−8.92 (m, 4H, Ph).

2f *(R = tert-butyl; R′ = benzyl; R″ = methyl):* yield 71%; mp 81−82 °C (purified by flash column chromatography, eluent 0.4:9.6 ethyl acetate/petroleum ether); IR (KBr, cm^{-1}) ν_{max} 1560, 1470, 1220; ^1H NMR (DMSO-d_6) δ 1.60 (s, 9H, *t*-Bu), 2.53 (s, 3H, Me), 4.19 (s, 2H, CH_2), 7.2−7.4 (m, 5H, Ph).

2h *(R = tert-butyl; R′ = phenyl; R″ = methyl):* yield 75%; mp 169−170 °C (purified by flash column chromatography, eluent 0.2:9.8 ethyl acetate/petroleum ether); IR (KBr, cm^{-1}) ν_{max} 1540, 1510, 1470, 1220; ^1H NMR ($CDCl_3$) δ 1.63 (s, 9H, *t*-Bu), 2.71 (s, 3H, Me), 7.50−7.54 (m, 3H, Ph), 8.00−8.20 (m, 2H, Ph).

6-Trifluoromethylpyrazolo[3,4-*d*][1,3]thiazin-4-one (2e). Trichloromethyl chloroformate (0.6 mL, 5 mmol) was added to a suspension of thiocarboxamide **5** (5 mmol) in anhydrous toluene (100 mL), placed in a round-bottom flask equipped with a Vigreaux reflux condenser. The mixture was stirred at room temperature for 30 min and then heated under reflux until hydrogen chloride evolution ceased (∼5 h). After removal of the solvent, the solid residue was purified by column chromatography to give a white crystalline product.

2e *(R = 3-nitrophenyl; R′ = CF3; R″ = methyl):* yield 20%; mp l08−110 °C (purified by column chromatography, eluent 1:1 ethyl acetate/petroleum ether); IR (KBr, cm^{-1}) ν_{max} 2927, 1690, 1537; ^1H NMR ($CDCl_3$) δ 2.71 (s, 3H, Me), 7.70−8.94 (m, 4H, Ph).

Cleavage of the *tert*-Butyl Group from 5 (R = *t*-Bu) and from 2f—h. A solution of **5** (R = *t*-Bu), **2i**, or **2j** (2 mmol) in formic acid (12 mL) was heated under reflux until dealkylation was completed (1 h). The solution was evaporated to give a solid, which was taken up with water (10 mL) and extracted with ethyl acetate (3 × 15 mL). After drying over anhydrous magnesium sulfate, the solvent was removed and the resulting solid was recrystallized from the indicated solvent to give a white crystalline product.

6 *(R′ = CF3; R″ = methyl):* yield 64%; mp 186−188 °C (ethyl ether); IR (KBr, cm^{-1}) ν_{max} 3280, 2775, 1597, 1560, 1491; ^1H NMR ($CDCl_3$) δ 2.33 (s, 3H, Me), 5.6 (br, 1H, NH), 5.19 (s, 1H, CH), 11.00 (br, 1H, NH).

6 *(R′ = phenyl, R″ = H):* yield 94%; mp 191−3 °C (ethyl acetate); IR (KBr, cm^{-1}) ν_{max} 3402, 2922, 1583, 1483; ^1H NMR ($CDCl_3$) δ 7.36−7.88 (m, 5H, Ph), 7.56 (s, 1H, CH), 8.05 (s, 1H, CH), 10.86 (br, 1H, NH).

6 *(R′, R″ = phenyl):* yield 71%; mp 204−206 °C (ethyl ether); IR (KBr, cm^{-1}) ν_{max} 3417, 2918, 1597, 1490; ^1H NMR (DMSO-d_6) δ 7.37−7.64 (m, 11H, CH + 2Ph), 12.18 (s, 1H, NH), 13.29 (s, 1H, NH).

2j *(R′ = benzyl; R″ = methyl):* yield 92%; mp 199−200 °C (toluene); IR (KBr, cm^{-1}) ν_{max} 3200−2700, 1570, 1490, 1460; ^1H NMR (DMSO-d_6) δ 2.59 (br, 3H, Me), 4.14 (br, 2H, CH_2), 7.32 (br, 5H, Ph), 14.21 (br, 1H, NH).

2l *(R′ = phenyl; R″ = methyl):* yield 93%; mp 249−250 °C (ethyl acetate); IR (KBr, cm^{-1}) ν_{max} 3160−2600, 1630, 1560; ^1H NMR (DMSO-d_6) δ 2.63 (br, 3H, Me), 7.58 (m, 3H, Ph), 7.98 (d, J = 7.2 Hz, 2H, Ph).

2-Substituted Pyrazolo[1,5-*c*][1,3,5]thiadiazine-4-ones (3b—d,f,g). Trichloromethyl chloroformate (0.6 mL, 5 mmol) was added to a solution of the pertinent **6** (5 mmol) in anhydrous tetrahydrofuran (50 mL). After 10 h of stirring at room temperature, the solvent was removed and the solid residue was purified by column chromatography.

3b *(R′ = p-bromophenyl; R″ = methyl):* yield 22%; mp 243−245 °C (purified by column chromatography, eluent 3:7, ethyl acetate/petroleum ether); IR (KBr, cm^{-1}) ν_{max} 3433, 1736, 1591, 1575; ^1H NMR ($CDCl_3$) δ 2.48 (s, 3H, Me), 6.62 (s, 1H, CH), 7.65 (d, J = 8.6 Hz, 2H, Ph), 7.85 (d, J = 8.6 Hz, 2H, Ph).

3c *(R′ = p-chlorophenyl; R″ = methyl):* yield 25%; mp 229−231 °C (purified by column chromatography, eluent 3:7, ethyl acetate/petroleum ether); IR (KBr, cm^{-1}) ν_{max} 3447, 1727, 1596, 1583; ^1H NMR ($CDCl_3$) δ 2.48 (s, 3H, Me), 6.61 (s, 1H, CH), 7.50 (d, J = 8.6 Hz, 2H, Ph), 7.93 (d, J = 8.6, 2H, Ph).

3d *(R′= CF3; R″ = methyl):* yield 53%; mp 120−123 °C (purified by flash chromatography, eluent 1:9 ethyl acetate/petroleum ether); IR (KBr, cm^{-1}) ν_{max} 3097, 1708, 1585; ^1H NMR ($CDCl_3$) δ 2.51 (s, 3H, Me), 6.84 (s, 1H, CH).

3f *(R′ = phenyl, R″ = H):* yield 25%; mp 168−171 °C (purified by column chromatography, eluent 3:7, ethyl acetate/petroleum ether); IR (KBr, cm^{-1}) ν_{max} 3350, 1715, 1575; ^1H NMR ($CDCl_3$) δ 6.60 (s, 1H, CH), 7.53−8.08 (m, 5H, Ph), 7.57 (s, 1H, CH).

3g *(R′, R″ = phenyl):* yield 96%; mp 223−225 °C (purified by column chromatography, eluent 3:7, ethyl acetate/petroleum ether); IR (KBr, cm^{-1}) ν_{max} 3350, 1710, 1680, 1580, 1550; ^1H NMR ($CDCl_3$) d 7.11 (s, 1H, CH), 7.46−8.04 (m, 10H, 2Ph).

2-Trifluoromethylpyrazolo[1,5-*c*][1,3,5]thiadiazine-4-thione (3e). Thiophosgene (0.60 mL, 6 mmol) was added dropwise to a heterogeneous mixture of dealkylated thioamide **6** (R′ = CF_3) (6 mmol) in water (45 mL) with vigorous stirring. After 3 h of stirring at room temperature, the precipitate was collected, washed with water, and dissolved in ethyl acetate. After drying over magnesium sulfate, the solvent was removed to give a solid, which was purified by column chromatography.

3e (R′ = CF3; R″ = methyl): yield 45%; mp 75−78 °C (purified by column chromatography, eluent 1:1, ethyl acetate/petroleum ether); IR (KBr, cm^{-1}) ν_{max} 3089, 1593; ^1H NMR ($CDCl_3$) δ 2.54 (s, 3H, Me), 6.89 (s, 1H, CH).

Fungal Growth Conditions and Evaluation of Antifungal Activity. *Magnaporthe grisea* (T.T. Hebert) Yaegashi & Udagawa, ATCC 64413 strain, was purchased from American Type Culture Collection (Rockville, MD) and maintained at 4 °C as agar slants on potato dextrose agar (PDA; Difco, Detroit, MI).

To evaluate biological activity, cultures 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 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, 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 5 days after the transport of the fungus onto dishes containing the substance to be tested. Three replicates were used for each concentration. Percentage inhibition was expressed as the mean of values obtained in three independent experiments. The same concentrations of a commercial fungicide, tricyclazole (Beam, Dow AgroSciences), were also tested. For compound **3a**, the most active as fungicide, concentrations <10 μg mL^{-1} were also used, that is, 5, 2.5, 1, and 0.5 μg mL^{-1}.

Rice Seedlings and Plant Cell Cultures. Rice (*Oryza sativa* L. cv Maratelli) seeds (obtained from Ente Nazionale Risi, Mortara, Italy) were surface-sterilized by treatment for 30 min at room temperature under vacuum in a 1% (v/v) solution of plant preservative mixture (PPM, Plant Cell Technology, Inc., Washington, DC) containing 0.5 mM $MgCl_2$, followed by 16 h in the same solution under shaking (80 rpm). Then they were allowed to germinate in a growth chamber at 25 ± 1 °C under 16-h days (250 μmol m^{-2} s^{-1}) and 8-h nights in Magenta vessels on 50 mL of agarized (0.8 wt %/v) half-strength MS salts (*17*), pH 6.5, containing 0.1% (v/v) PPM. Callus cultures were initiated from excised cotyledons placed in Petri dishes onto agarized (0.8% wt/v) R2 medium (*18*) supplemented with 20 g L^{-1} sucrose and 2 mg L^{-1} 2,4-dichlorophenoxyacetic acid (2,4D). For the first 3 months calli were cultured in complete darkness, with subcultures at 3-week intervals. Then friable callus pieces were transferred into 0.5-L Erlenmeyer flasks containing 100 mL of liquid MS medium supplemented with 30 g L^{-1} sucrose and 2 mg L^{-1} 2,4D, and suspension cultures were incubated under dim light on a rotary shaker (100 rpm). Subcultures were made every 14 days by transferring 25-mL aliquots to 100 mL of fresh medium.

To evaluate the effect of compound **3a** on seedling growth, seeds were germinated aseptically as described on agarized medium to which the compound had been added as a 10 mg mL^{-1} solution in DMSO. The experimental design was a randomized complete block with five replicates. Each block comprised 12 vessels (each containing eight seeds) of the following treatments: six rates (0, 1, 5, 10, 20, and 50 μg mL^{-1}) and the corresponding volumes of DMSO. Destructive harvest was carried out 10 days after germination. Four plants from each treatment were combined; seed teguments were discarded, and the dry weight was determined for each sample following drying in an oven at 90 °C for 48 h. The effect of the same concentrations of compound **3a** on exponentially growing cells was measured as described (*19*). Briefly, cell samples withdrawn from stock cultures in the late exponential phase of growth were used to inoculate 100-mL Erlenmeyer flasks to a density ranging from 1.0 to 1.2 mg mL^{-1} (dry weight) in a final volume of 25 mL. The compound was added just after cell density reached 1.5 mg mL^{-1}. After a further 10 days of incubation, when untreated controls reached the early stationary phase of growth (~4.0 mg mL^{-1}), cells were harvested by vacuum filtration, and the dry weight increase was determined as above. The data, expressed as percentage of controls treated with DMSO alone, were analyzed by using standard statistical procedures for analysis of variance and *t* test. Means were separated by the least-significant difference test. When differences are reported, they are at the 99% confidence level ($P = 0.01$).

RESULTS AND DISCUSSION

Synthesis. The preparative route to the target products is outlined in **Scheme 2**. *N*-(5-Pyrazolyl)carboxamides (**4**) were obtained by acylation of the corresponding 5-aminopyrazoles. Thiation of **4** with Lawesson's reagent provided the thiocarboxamides (**5**). Heating under reflux of equimolar amounts of **5** and thiophosgene or trichloromethyl chloroformate gave satisfactory yields of the target 6-trifluoromethylpyrazolo[3,4-*d*][1,3]thiazin-4-thiones (**2a**−**d**,**f**,**h**) and -ones (**2e**,**g**,**i**), respectively. The 1-*tert*-butyl derivatives (**2f**−**i**) were converted by heating in formic acid into the dealkylated homologues (**2j**−**m**). Following the same procedure the *N*-(1-*tert*-butyl-5-pyrazolyl)thiocarboxamides (**5**, R = *t*-Bu) were converted into the homologues (**6**). As expected, the reaction of **6** with trichloromethyl chloroformate or thiophosgene proceeded smoothly to afford pyrazolo[1,5-*c*][1,3,5]thiadiazine-4-ones (**3a**−**d**,**f**,**g**) and thione (**3e**).

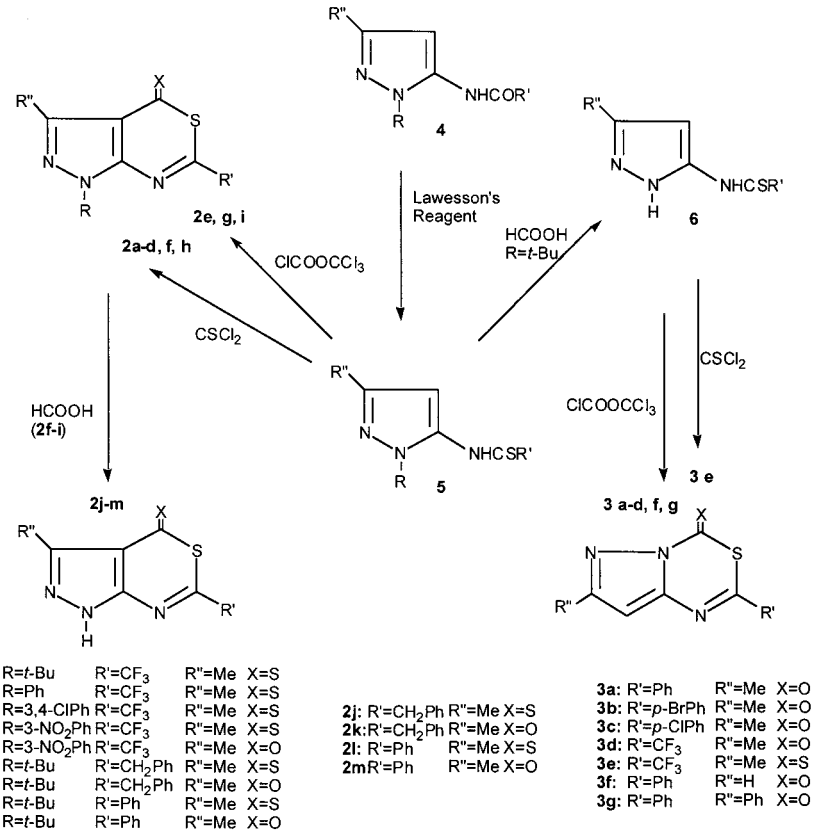
Biological Activity. The ability of these compounds to inhibit the growth of *M. grisea* was evaluated at rates of 10−200 μg mL^{-1}, a range in which the reference compound tricyclazole was found to exert an increasing effect. Results are summarized in **Table 1**. The first set of compounds, pyrazolo[3,4-*d*]-pyrimidine derivatives with R = *tert*-Bu and R = Ph, were marginally effective at all tested doses. On the contrary, the presence of a chlorophenyl or a nitrophenyl group (compounds **1c**,**d**) significantly improved their antifungal activity, which in both cases was higher than that of tricyclazole. Remarkably, at concentrations >50 μg mL^{-1} compound **1c** achieved total control of fungal growth.

Among pyrazolo[3,4-*d*][1,3]thiazine derivatives, compounds **2a**−**d** have exactly the same substituents as the previous group. In this case also, the presence of a *tert*-butyl or a phenyl at position 1 resulted in a lower effectiveness than those of compounds bearing 3,4-chlorophenyl or 3-nitrophenyl groups, so their presence seems to be critical for biological activity. Interestingly, compound **2d** was able to exert a 71% inhibition of fungal growth at concentrations as low as 10 μg mL^{-1}, a dose at which tricyclazole was completely ineffective. The replacement of X = S in compound **2d** with X = O, giving rise to compound **2e**, significantly reduced the inhibitory effect. A similar result was obtained when R′ = CF_3 was changed to a phenyl or a CH_2Ph group in compounds **2h**−**m**. However, the lack of a dose−effect relationship for some compounds of this series may suggest that in the aqueous environment required for fungal growth their solubility (and thus the availability) may be low. The recorded effect may thus be ascribed to the small concentration able to dissolve into the culture medium.

With respect to pyrazolo[1,5-*c*][1,3,5]thiadiazine derivatives, compound **3a** (X = O, R′ = Ph) was found to be the most effective. The activity against *M. grisea* was reduced by the presence at position R′ of either larger (bromophenyl, **3b**; chlorophenyl, **3c**) or smaller (CF_3, **3d**) groups. If at position R″ was H (**3f**) or a phenyl group (**3g**), there was a strong decrease of activity, in particular in **3g**. Contrary to compounds **2**, in this case the thione function seems to cause a decrease in activity, as suggested by the comparison of the effects exerted by compounds **3d** and **3e**.

On the whole, remarkable is the inhibition brought about by all of the compounds, but mainly by compounds **2d**, **2j**, and **3a**, at the lowest rate tested (10 μg mL^{-1}), one at which tricyclazole is ineffective. To prevent blast, this fungicide is

Scheme 2

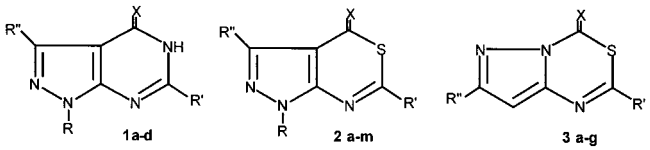


usually applied to rice plants at a concentration of 200 μg mL^{-1}. The possibility of using 20-fold lower rates might thus represent a significant reduction in the environmental fallout of agricultural practice. However, such a prospect relies upon the lack of side effects that may reduce crop productivity. To rule out this possibility, the phytotoxicity of the most powerful compound, **3a**, was evaluated at both the undifferentiated cell and seedlings levels. The rice cultivar employed, cv. Maratelli, was chosen because it is highly sensitive to many strains of *M. grisea*. Results, depicted in **Figure 1**, show that at concentrations >10 μg mL^{-1} the presence of compound **3a** in the culture medium exerted indeed a certain inhibitory effect upon rice growth. This notwithstanding, in the range of 1−10 μg mL^{-1} the compound was able to progressively control fungal proliferation without any significant effect upon plant and cell culture growth.

The ability of several compounds to suppress completely the development of *M. grisea* hyphae is noteworthy. Tricyclazole is commonly used to prevent blast in rice fields. It acts by inhibiting melanin synthesis in appressorial cells, thus weakening fungal walls and avoiding plant colonization by the pathogen (*20*). However, this fungicide is substantially ineffective when applied on infected plants. On the contrary, the most active compounds herein described even at lower concentrations might hinder further pathogen spread in infected plants.

Up to now, no conclusive data have been available about their mode of action. Due to structural similarities with previously characterized azoles, one may suppose that they also may inhibit the synthesis of ergosterol, a main component of fungal membrane (*21*, *22*), or—like tricyclazole—inhibit the production of 1,8-dihydroxynaphthalene-derived melanins (*7*). The latter hypothesis is strengthened by the results obtained in vitro with compound **2j**, which caused a strong depigmentation of fungal mycelium (**Figure 2**). However, this is only indirect evidence. Moreover, a primary effect upon melanin biosynthesis

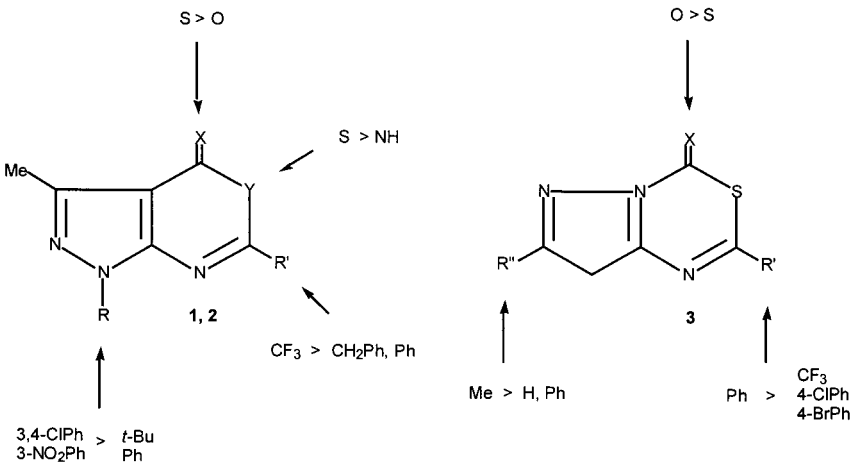
Table 1. Fungicidal Activity of Compounds **1**–**3**[a]



					μg mL^{-1}			
compd	R	R′	R″	X	10	50	100	200
1a	*t*-Bu	CF_3	Me	S	25.0	31.0	35.0	44.0
1b	Ph	CF_3	Me	S	23.0	37.0	55.0	58.0
1c	3,4-ClPh	CF_3	Me	S	52.0	91.0	100	100
1d	3-NO_2Ph	CF_3	Me	S	32.0	61.0	78.0	95.9
2a	*t*-Bu	CF_3	Me	S	nt	nt	42.7	58.3
2b	Ph	CF_3	Me	S	nt	nt	29.6	43.8
2c	3,4-ClPh	CF_3	Me	S	57.7	83.1	90.5	100
2d	3-NO_2Ph	CF_3	Me	S	71.2	89.1	91.7	100
2e	3-NO_2Ph	CF_3	Me	O	21.9	39.6	54.4	81.1
2h	*t*-Bu	Ph	Me	S	14.0	16.0	16.0	17.0
2i	*t*-Bu	Ph	Me	O	14.0	14.0	16.0	19.0
2j	H	CH_2Ph	Me	S	73.0	75.0	76.0	77.0
2k	H	CH_2Ph	Me	O	30.0	57.0	77.0	80.0
2l	H	Ph	Me	S	14.0	15.0	17.0	21.0
2m	H	Ph	Me	O	16.0	24.0	28.0	30.0
3a		Ph	Me	O	92.4	100	100	100
3b		4-BrPh	Me	O	47.0	47.0	58.0	74.0
3c		4-ClPh	Me	O	17.0	62.0	66.0	66.0
3d		CF_3	Me	O	64.5	97.9	99.5	100
3e		CF_3	Me	S	48.4	79.6	79.7	93.2
3f		Ph	H	O	67.0	70.2	73.1	75.1
3g		Ph	Ph	O	15.0	15.0	15.0	15.0
tricyclazole					0	23.5	61.5	94.0

[a] Percentage inhibition of the growth of *M. grisea* evaluated 5 days after treatment; values are means of three trials made in triplicate, with SE never exceeding 5%; nt = not tested.

Scheme 3



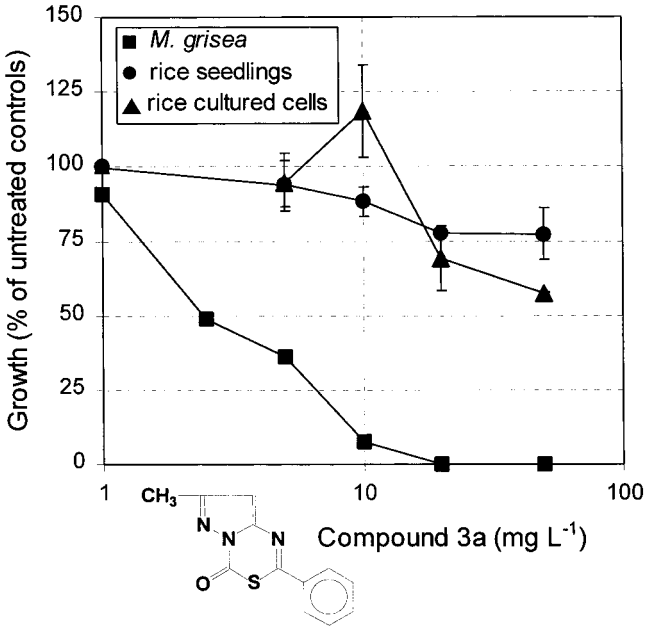


Figure 1. Comparison of the effects of compound **3a** on the growth of *M. grisea* (■) and *O. sativa* suspension cultured cells (▲) or seedlings (●). Mean values ± SD over at least four replications are reported.

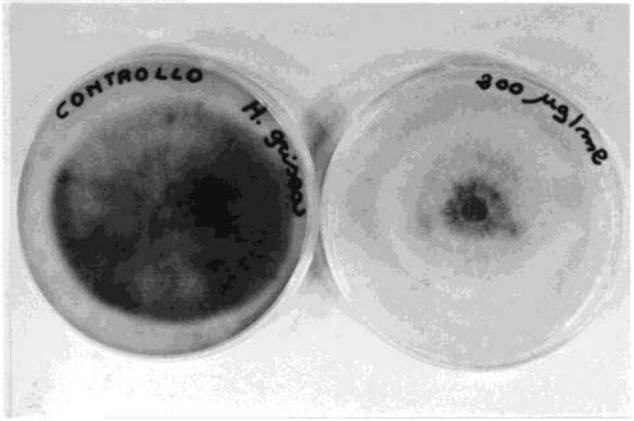


Figure 2. Depigmentation of *M. grisea* mycelium after treatment with **2j** at 200 μg mL^{-1}.

is not consistent with the inhibitory effect on fungal growth. Thus, if any, an interference with melanin metabolism may represent a secondary effect. The sensitivity of both rice plants and cultured cells at high concentrations suggests the possible occurrence of either other targets or side effects involving other pathways in cell metabolism. Experiments are currently in progress to elucidate these aspects.

In any case, several of these compounds proved to possess a remarkable effectiveness against fungal growth. For pyrazolo-[3,4-*d*]pyrimidine and pyrazolo[3,4-*d*]thiazine derivatives (**1**, **2**) the structure−activity relationship is consistent with previous data (*11*, *14*) accounting for a critical role of a trifluoromethyl group and thione function at positions 6 and 4, respectively (**Scheme 3**). Moreover, the current results suggest a 3,4-dichlorophenyl or 3-nitrophenyl at position 1 as a key structural requirement for antifungal activity and can indicate that the substitution of pyrimidine with a thiazine ring acts as amplifier for the mycotoxic activity. In comparison with the series **1** and **2**, the presence of a thiadiazine ring in **3** causes a strong increase of activity. In particular, with respect to pyrazolo[1,5-*c*][1,3,5]-thiadiazine derivatives, compound **3a** (X = O, R′ = phenyl, R″ = methyl) was found to give very good results. Contrary to series **1** and **2**, in this case the phenyl group and the one function seem to cause an increase in activity (**Scheme 3**). The above conclusions may provide researchers with a lead structure to be further explored with the aim to develop new fungicides.

LITERATURE CITED

(1) Oerke, E. C.; Dehne, H. W.; Schonbeck, F.; Weber, A. *Crop Protection and Crop Production*; Elsevier: Amsterdam, The Netherlands, 1994; 808 pp.

(2) Gullino, M. L.; Leroux, P.; Smith, M. S. Uses and challenges of novel compounds for plant disease control. *Crop Prot.* **2000**, *19*, 1−11.

(3) Zeigler, R. S.; Leong, S. A.; Teng, P. S. *Rice Blast Disease*; CAB International: Wallingford, U.K., 1994; 626 pp.

(4) Howard, R. J.; Valent, B. Breaking and entering: host penetration by the fungal rice blast pathogen *Magnaporthe grisea*. *Annu. Rev. Microbiol.* **1996**, *50*, 491−512.

(5) Froyd, J. D.; Paget, C. J.; Guse, L. R.; Dreikorn, B. A.; Pafford, J. L. Tricyclazole: a new systemic fungicide for control of *Pyricularia oryzae* (blast disease) on rice. *Phytopathology* **1976**, *66*, 1135−1139.

(6) Jordan, D. B.; Lessen, T. A.; Wawrzak, Z.; Bisaha, J. J.; Gehret, T. C.; Hansen, S. L.; Schwartz, R. S.; Basarab, G. S. Design of scytalone dehydratase inhibitors as rice blast fungicides: (*N*-phenoxypropyl)-carboxamides. *Bioorg. Med. Chem. Lett.* **1999**, *9*, 1607−1612.

(7) Jordan, D. B.; Basarab, G. S.; Liao, D.-I.; Johnson, W. M. P.; Winzenberg, K. N.; Winkler, D. A. Structure-based design of inhibitors of the rice blast fungal enzyme trihydroxynaphthalene reductase. *J. Mol. Graphics Modell.* **2001**, *19*, 434−447.

(8) Kim, J. C.; Min, J. Y.; Kim, H. T.; Kim, B. S.; Kim, Y. S.; Kim, B. T.; Yu, S. H.; Yamaguchi, I.; Cho, K. Y. Target site of a new antifungal compound KC10017 in the melanin biosynthesis of *Magnaporthe grisea*. *Pestic. Biochem. Physiol.* **1998**, *62*, 102−112.

(9) Giori, P.; Poli, T.; Vicentini, C. B.; Manfrini, M.; Guarneri, M.; Brandolini, V. Synthesis and antifungal activity of pyrazolo[3,4-*d*]pyrimidin-4(5*H*)-thiones. *Farmaco* **1985**, *40*, 795−802.

(10) Vicentini, C. B.; Poli, T.; Guarneri, M.; Brandolini, V.; Manfrini, M.; Giori, P. Derivati del 6-trifluorometil-pirazolo[3,4-*d*]pirimidin-4(5*H*)tioni quali fitofarmaci ad azione fungicida, procedimento per la loro preparazione e composizioni anticrittogamiche che li contengono. Ital. Patent A/87,21121.

(11) Vicentini, C. B.; Poli, T.; Veronese, A. C.; Brandolini, V.; Manfrini, M.; Guarneri, M.; Giori, P. Synthesis and in-vitro antifungal activity of 6-trifluoromethylpyrazolo[3,4-*d*]pyrimidin-4(5*H*)-thiones. *Pestic. Sci.* **1989**, *27*, 77−83.

(12) Poli, T.; Vicentini, C. B.; Brandolini, V.; Veronese, A. C.; Manfrini, M.; Guarneri, M.; Giori, P. Synthesis and in-vitro antifungal activity of 6-trifluoromethylpyrazolo[3,4-*d*]pyrimidines. *Pestic. Sci.* **1989**, *25*, 161−169.

(13) Pavlath, A. E. Theoretical considerations for the application of aromatic and heterocyclic fluorine compounds as pesticides. *Pestic. Sci.* **1986**, *17*, 412−417.

(14) Mares, D.; Romagnoli, C.; Sacchetti, G.; Fabiano, A.; Vicentini, C. B.; Bruni, A. Effectiveness of four new pyrazole-pyrimidines on phytopathogens: ultrastructural evidences on *Pythium ultimum*. *J. Phytopathol.* **2000**, *148*, 395−403.

(15) Vicentini, C. B.; Veronese, A. C.; Guccione, S.; Guarneri, M.; Manfrini, M.; Giori, P. An efficient procedure for the synthesis of pyrazolo[3,4-*d*][1,3]thiazin-4-ones. *Heterocycles* **1993**, *36*, 2291−2301.

(16) Vicentini, C. B.; Veronese, A. C.; Guarneri, M.; Manfrini, M.; Giori, P.; Guccione, S. A new procedure for the synthesis of pyrazolo[1,5-*c*][1,3,5]thiadiazine-4-thiones. *J. Heterocycl. Chem.* **1994**, *31*, 1474−1480.

(17) Murashige, T.; Skoog, F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* **1962**, *15*, 473−497.

(18) Ohira, K.; Ojima, K.; Fujiwara, A. Studies on the nutrition of rice cell culture 1. A simple, defined medium for rapid growth in suspension culture. *Plant Cell Physiol.* **1973**, *14*, 1113−1121.

(19) Forlani, G.; Lejczak, B.; Kafarski, P. The herbicidally active compound *N*-2-(5-chloropyridyl)-aminomethylene-bisphosphonic acid acts by inhibiting both glutamine and aromatic amino acid biosynthesis. *Aust. J. Plant Physiol.* **2000**, *27*, 677−683.

(20) Sisler, H. D. Control of fungal diseases by compounds acting as antipenetrants. *Crop Prot.* **1986**, *5*, 306−313.

(21) Vanden Bossche, H. Importance and role of sterols in fungal membranes. In *Biochemistry of Cell Walls and Membranes in Fungi*; Kuhn, P. J., Trinci, A. P. J., Jung, M. J., Goosey, M. W., Copping, L. G., Eds.; Springer Verlag: Berlin 1990; pp 135−153.

(22) Vanden Bossche, H. Mode of action of pyridine, pyrimidine and azole antifungals. In *Series in Biomedicine: Sterol Biosynthesis Inhibitors: Pharmaceutical and Agrochemical Aspects*; Berg, D., Plempel, M., Eds.; Ellis Horwood: Chichester, U.K., 1988; pp 79−119.

Received for review February 21, 2002. Revised manuscript received May 28, 2002. Accepted May 29, 2002. This work was supported by grants from the Ministero dell'Università e della Ricerca Scientifica e Tecnologica (MURST) of Italy.

JF0202436