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Synthesis of Pyrazole Derivatives and Their Evaluation as Photosynthetic Electron Transport Inhibitors

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Two series of new pyrazoles, namely six pyrazolo[1,5-*a*][1,3,5]triazine-2,4-dione and four pyrazolo-[1,5-*c*][1,3,5]thiadiazine-2-one derivatives, were synthesized as potential inhibitors of the photosynthetic electron transport chain at the photosystem II level. The compounds were confirmed by ^{1}H NMR, elemental, and IR analyses. Their biological activity was evaluated in vivo upon both the growth of blue-green algae and the photosynthetic oxygen evolution by eukaryotic algae and in vitro as the ability to interfere with light-driven reduction of ferricyanide by isolated spinach chloroplasts. Some compounds exhibited remarkable inhibitory properties, comparable to those of the reference commercial herbicides lenacil, diuron, and hexazinone. Results suggest that the substitution of triazine with thiadiazine ring may act as amplifier for herbicidal activity.

KEYWORDS: Herbicides; photosynthetic electron transport inhibitors; photosystem II

INTRODUCTION

Control of weeds with herbicides is a main constraint in food production efficiency. Weeds compete with crops for nutrients, water, and physical space, may harbor insect and disease pests, and are thus capable of greatly undermining both crop quality and yield. Several classes of herbicides were developed during the last 50 years that are effective for broad-spectrum weed control. Among them, inhibitors of the photosynthetic electron transport such as triazines and phenylureas, despite the long time from their introduction, still represent a significant fraction of phytochemicals worldwide (*1*). Their molecular target was found to be the plastoquinone-binding site on the D1 protein in the PSII reaction center of the electron transport chain (*2*). A high affinity of these compounds for this domain leads to a block in NADPH production, which is required for CO_2 fixation; moreover, this induces, in turn, the formation of free oxygen radicals, which cause photooxidation of chlorophylls and lipids, thus breaking down thylakoid integrity that is strictly required for chloroplast functionality (*2*).

Several reasons exist today for developing new weed control systems. Modern pesticides should have a favorable combination of properties, including high levels of herbicidal activity, low application rates, crop tolerance, and low levels of toxicity to mammals. Moreover, increasing public concern for the environmental pollution deriving from agricultural practice strictly requires that phytochemicals would be endowed with low recalcitrancy and thus may be rapidly mineralized by the soil microflora. On the other hand, due to an intensive and repeated treatment of fields with the same active principles, starting from 1960 hundreds of weed biotypes have developed herbicide tolerance (*3*). In most cases, resistance is owed to an altered target site: the strong selection exerted by frequent field applications promotes the fixation among weeds of spontaneous mutations in the target protein that confer a reduced affinity for the herbicide (*4*). This has been well documented for herbicides inhibiting the electron transport chain (*5*). Triazine resistance in weeds was first identified in the late 1960s, when the biochemical base of resistance was characterized in a simazine-resistant biotype of *Senecio vulgaris*. Up to now, dozens of photosystem II inhibitor resistant biotypes have been listed in 65 weed species (*3*). Moreover, due to their common mode of action, mutations in the D1 protein are susceptible to conferring a wide pattern of cross-tolerance to both structurally similar herbicides and those from other chemical classes sharing the same target site.

During the last two decades, intensive efforts have thus been undertaken to discover new chemicals with favorable environmental and safety features to selectively control weeds. In several instances, pyrazole derivatives have been found as promising agrichemical products and leads. Among the active principles recently introduced in agricultural practice are the inhibitor of protoporphyrinogen oxidase pyraflufen-ethyl (HRAC group E) (*6*); the bleaching herbicides, pyrazolynate, pyrazoxyfen, and benzofenap, that act through inhibition of 4-hydroxyphenyl-pyruvate-dioxygenase (HRAC group F2); the branched-chain amino-acid-starving sulfonylureas halosulfuron-methyl, pyrazosulfuron-ethyl, and azimsulfuron (HRAC group B); the

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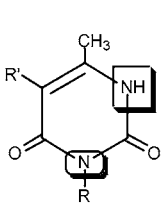
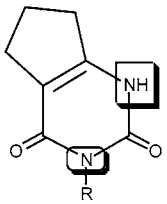
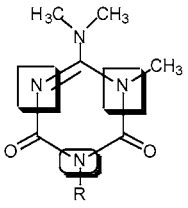
Scheme 1



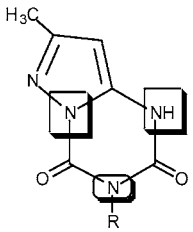
Terbacil: R= *tert*-butyl, R'= Cl
Bromacil: R=*sec*-butyl, R'= Br



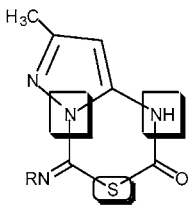
Lenacil: R= cyclohexyl



Hexazinone: R= cyclohexyl



pyrazolo[1,5-*a*][1,3,5]triazine



pyrazolo[1,5-*c*][1,3,5]thiadiazine

inhibitor of cell division, metazachlor (HRAC group K3); and the unknown target herbicide, difenzoquat (HRAC group Z). Moreover, pyrimidine-2,4-dione derivatives, as the uracils lenacil, terbacil, bromacil, and 1,3,5-triazine-2,4-dione derivatives, as the triazolinone hexazinone, have been characterized as photosynthetic electron transport inhibitors (HRAC group C1) (*6*, *7*).

In this view, we undertook the synthesis of some pyrazolo-[1,5-*a*][1,3,5]triazine-2,4-dione derivatives, which may be considered as interesting lead molecules. The substitution of triazine with thiadiazine ring was also considered as a possible amplifier for the activity (**Scheme 1**). An additional purpose was to extend the synthetic and screening program to pyrazolo[1,5-*c*][1,3,5]-thiadiazine-2-one derivatives to explore the potential of this further class of compounds in agriculture.

MATERIALS AND METHODS

Synthesis. *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 $\pm$ 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.

General Procedure for the Synthesis of 5-Amino-3-methyl-pyrazole-1-carboxamides **2** (*X* = *O*). The pertinent isocyanate (0.004 mol) and triethylamine (2 drops) were added to a solution of 5-amino-3-methylpyrazole (0.39 g, 0.004 mol) in dry acetone (20 mL). The mixture was stirred at room temperature for 1−2 h and evaporated to dryness to give a residue, which was purified by column chromatography. By use of this procedure, the following compounds were obtained:

2 (R = Phenyl). Yield, 46%; mp, 95−98 °C (purified by column chromatography; eluent, ethyl acetate/petroleum ether, 2:8). IR (KBr): 3443, 3344, 1700, 1596, 1537 cm^{-1}. ^{1}H NMR (DMSO-d_6): δ 2.11 (s, 3H, Me), 5.23 (s, 1H, CH), 6.43 (s, 2H, NH_2), 7.09−7.69 (m, 5H, Ph), 9.82 (s, 1H, NH).

2 (R = Cyclohexyl). Yield, 75%; oil (purified by column chromatography; eluent, ethyl acetate/petroleum ether, 2:8). IR (neat): 3453, 3348, 2931, 2855, 1694, 1613 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 1.14−1.98 (m, 10H, cyclohexyl), 2.10 (s, 3H, Me), 3.68−3.73 (m, 1H, cyclohexyl), 5.20 (s, 1H, CH), 5.40 (br, 2H, NH_2), 6.92 (d, 1H, NH).

2 (R = Benzyl). Yield, 70%, oil (purified by column chromatography; eluent, ethyl acetate/petroleum ether, 3:7). IR (neat): 3453, 3352, 1697, 1608, 1520 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 2.10 (s, 3H, Me), 4.52 (d, 2H, CH_2), 5.21 (s, 1H, CH), 5.39 (br, 2H, NH_2), 7.29−7.37 (m, 5H, Ph), 9.83 (br, 1H, NH).

2 (R = Ethyl). Yield, 70%; mp, 80−84 °C (purified by column chromatography; eluent, ethyl acetate/petroleum ether, 3:7). IR (KBr): 3386, 2975, 1700, 1617, 1533, cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 1.25 (t, 3H, Me, J = 7.2 Hz), 2.12 (s, 3H, Me), 3.38 (q, 2H, CH_2, J = 7.2 Hz), 5.21 (s, 1H, CH), 5.40 (br, 2H, NH_2), 7.03 (br, 1H, NH).

2 (R = Butyl). Yield, 97%; oil (purified by flash column chromatography; eluent, ethyl acetate/petroleum ether, 2:8). IR (neat): 3453, 3349, 2958, 1702, 1610, cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 0.93 (t, 3H, Me, J = 7.2 Hz), 1.33−1.61 (m, 4H, 2 CH2), 2.11 (s, 3H, Me), 3.33 (q, 2H, CH_2, J = 7.2 Hz), 5,20 (s, 1H, CH), 5.43 (br, 2H, NH_2), 7.06 (br, 1H, NH).

2 (R = Sec-butyl). Yield, 75%; oil (purified by flash column chromatography; eluent, ethyl acetate/petroleum ether, 2:8). IR (neat): 3350, 2968, 1698, 1609, 1520 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 0.96 (t, 3H, Me, J = 7.3 Hz), 1.23 (d, 3H, Me, J = 7.3 Hz), 1.49−1.64 (m, 2H, CH_2), 3.78−3.93 (m, 1H, CH), 5.42 (br, 1H, CH), 6.66 (br, 1H, NH).

General Procedure for the Synthesis of 5-Amino-3-methyl-pyrazole-1-carbothioamides **2** (*X* = *S*). The pertinent isothiocyanate (0.004 mol) and triethylamine (2 drops) were added to a solution of 5-amino-3-methylpyrazole (0.39 g, 0.004 mol) in dry acetone (20 mL). The mixture was stirred at room temperature for 1−2 h, evaporated to dryness to give a residue which was purified by column chromatography. By use of this procedure, the following compounds were obtained.

2 (R = Cyclohexyl). Yield, 63%, oil (purified by column chromatography; eluent, ethyl acetate/petroleum ether, 2:8). IR (neat): 3399, 3291, 2930, 2854, 1607, cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 1.11−2.05 (m, 10H, cyclohexyl), 2.11 (s, 3H, Me), 4.25−4.27 (m, 1H, cyclohexyl), 5.25 (s, 1H, CH), 6.36 (br, 2H, NH_2), 9.04 (d, 1H, NH).

2 (R = Benzyl). Yield, 48%; oil (purified by column chromatography; eluent, ethyl acetate/petroleum ether, 2:8). IR (neat): 3406, 3291, 2922, 1608, 1519 cm^{-1}. ^{1}H NMR ($CDCl_3$): 2.10 (s, 3H, Me); 4.89 (d, 2H, CH_2), 5.28 (s, 1H, CH), 6.37 (br, 2H, NH_2), 7.30−7.41 (m, 5H, Ph), 9.38 (br, 1H, NH).

2 (R = Ethyl). Yield, 31%; oil (purified by column chromatography; eluent, ethyl acetate/petroleum ether, 3:7). IR (neat): 3399, 3285, 2975, 1608, cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 1.30 (t, 3H, Me, J = 7.2 Hz), 2.12 (s, 3H, Me), 3.69 (q, 2H, CH_2, J = 7.2 Hz), 5.27 (s, 1H, CH), 6.37 (br, 2H, NH_2), 9.07 (br, 1H, NH).

2 (R = Butyl). Yield, 40%; mp, 71−73 °C (purified by flash column chromatography; eluent, ethyl acetate/petroleum ether, 2:8). IR (KBr): 3336, 2929, 1604, 1511, 1463 cm^{-1}. ^1H NMR ($CDCl_3$): δ 0.96 (t, 3H, Me, J = 7.2 Hz), 1.34−1.76 (m, 4H, 2 CH_2), 2.12 (s, 3H, Me), 3.64 (q, 2H, CH_2, J = 7.2 Hz), 5.27 (s, 1H, CH), 6.36 (br, 2H, NH_2), 9.12 (br, 1H, NH).

General Procedure for the Synthesis of Pyrazolo[*1,5-a*][*1,3,5*]-*triazine-2,4-dione Derivatives* ***V1*****−*****V6***. Trichloromethyl chloroformate (0.36 mL, 3 mmol) was added to a solution of pertinent amide **2** (X = O) (0.003 mol) in anhydrous tetrahydrofuran (15 mL). After 15 h of stirring at room temperature, the precipitate was filtered off and washed with ethyl ether or purified by column chromatography. By use of this procedure, the following compounds were obtained:

V1 (From **2** R = Phenyl). Yield, 58%; mp, 260−265 °C (purified by column chromatography; eluent, methylene chloride/methanol/toluene, 17:2:1); IR (KBr): 3138, 1765, 1742, 1643, 1553 cm^{-1}. ^1H NMR (DMSO-d_6): δ 2.23 (s, 3H, Me), 5.73 (s, 1H, CH), 7.38−7.49 (m, 5H, Ph), 12.18 (br, 1H, NH).

V2 (From **2** R = Cyclohexyl). Yield, 75; mp, 230−234 °C (purified by column chromatography; eluent, methylene chloride/methanol/toluene, 17:2:1; ethyl acetate/petroleum ether, 8:2). IR (KBr): 3157, 2926, 2855, 1760, 1705, 1643, 1508 cm^{-1}. ^1H NMR (DMSO-d_6): δ 1.36−1.98 (m, 10H, cyclohexyl), 2.18 (s, 3H, Me), 4.52 (m, 1H, cyclohexyl), 5.61 (s, 1H, CH), 11.98 (br, 1H, NH).

V3 (From **2** R = Benzyl). Yield, 89%; mp, 284−6 °C (washed with ethyl ether). IR (KBr): 3347, 2970, 1725, 1636, 1554, 1500 cm^{-1}. ^1H NMR (DMSO-d_6): δ 2.20 (s, 3H, Me), 4.97 (s, 2H, CH2), 5.70 (s, 1H, CH), 7.25−7.36 (m, 5H, Ph), 12.26 (s, 1H, NH).

V4 (From **2** R = Ethyl). Yield, 27%; mp, 270−273 °C (purified by column chromatography; eluent, ethyl acetate/petroleum ether, 8:2). IR (KBr): 3202, 1767, 1706, 1627, 1506 cm^{-1}. ^1H NMR (DMSO-d_6): δ 1.14 (t, 3H, Me, J = 7.1 Hz), 2.19 (s, 3H, Me), 3.83 (q, 2H, CH_2, J = 7.1 Hz), 5.65 (s, 1H, CH), 12.04 (br, 1H, NH).

V5 (From **2** R = Butyl). Yield, 74%; mp, 228−9 °C (purified by column chromatography; eluent, methylene chloride/methanol/toluene, 17:2:1). IR (KBr): 3157, 2957, 1762, 1708, 1639, 1501 cm^{-1}. ^1H NMR (DMSO-d_6): 0.89 (t, 3H, Me, J = 7.2 Hz), 1.20−1.62, (m, 4H, 2 CH_2), 2.19 (s, 3H, Me), 3.78 (t, 2H, CH_2, J = 7.2 Hz), 5.65 (s, 1H, CH), 12.04 (br, 1H, NH).

V6 (From **2** R = Sec-butyl). Yield, 42%; mp, 179−183 °C (purified by column chromatography; eluent, ethyl acetate/petroleum ether, 8:2). IR (KBr): 3433, 2966, 1759, 1702, 1641, 1516 cm^{-1}. ^1H NMR (DMSO-d_6): δ 0.80 (t, 3H, Me, J = 7.2 Hz), 1.38 (d, 3H, Me, J = 7.2 Hz), 1.66−2.08, m, 2H, CH_2), 2.19 (s, 3H, Me), 4.70 (m, 1H, CH), 5.63 (s, 1H, CH), 11.99 (br, 1H, NH).

General Procedure for the Synthesis of Pyrazolo[*1,5-c*][*1,3,5*]-*thiadiazine-2-one derivatives* ***VS2*****−*****VS5***. Trichloromethyl chloroformate (0.36 mL, 0.003 mol) was added to a solution of pertinent amide **2** (X = S) (3 mmol) in anhydrous tetrahydrofuran (15 mL). After 15 h of stirring at room temperature, the precipitate was filtered off and washed with ethyl ether or purified by column chromatography. By use of this procedure, the following compounds were obtained.

VS2 (From **2** R = Cyclohexyl). Yield, 50%; mp, 200 °C (washed with ethyl ether). IR (KBr): 2296, 1727, 1637, 1544 cm^{-1}. ^1H NMR (DMSO-d_6): δ 1.14−1.82 (m, 11H, cyclohexyl), 2.20 (s, 3H, Me), 5.71 (s, 1H, CH), 12.42 (br, 1H, NH).

VS3 (From **2** R = Benzyl). Yield, 27%; mp, 278−280 °C (purified by column chromatography; eluent, methylene chloride/methanol/toluene, 17:2:1; ethyl acetate/petroleum ether, 8:2). IR (KBr): 2951, 1713, 1635 cm^{-1}. ^1H NMR (DMSO-d_6): δ 2.23 (s, 3H, Me), 5.54 (s, 2H, CH_2), 5.80 (s, 1H, CH), 7.24−7.35 (m, 5H, Ph), 12.67 (br, 1H, NH).

VS4 (From **2** R = Ethyl). Yield, 26%; mp, 241−242.5 °C (purified by column chromatography; eluent, methylene chloride/methanol/toluene, 17:2:1; ethyl acetate/petroleum ether, 8:2); IR (KBr): 2939, 1722, 1640, 1499 cm^{-1}. ^1H NMR (DMSO-d_6): δ 1.20 (t, 3H, Me, J = 6.8 Hz), 2.22 (s, 3H, Me), 4.37 (q, 2H, CH_2, J = 6.8 Hz), 5.75 (s, 1H, CH), 12.51 (br, 1H, NH).

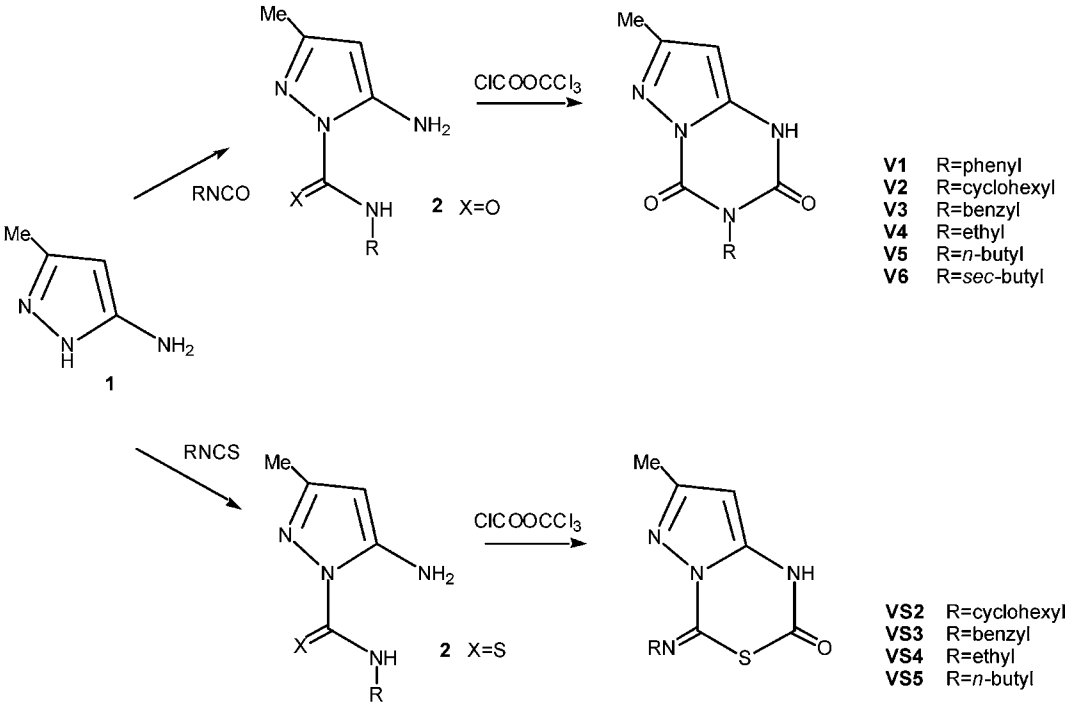
VS5 (From **2** R = Butyl). Yield, 30%; mp, 265−267 °C (purified by column chromatography; eluent, methylene chloride/methanol/toluene, 17:2:1; ethyl acetate,petroleum ether, 8:2). IR (KBr): 2961, 1724, 1642, 1497 cm^{-1}. ^1H NMR (DMSO-d_6): 0.90 (t, 3H, Me, J = 7.2 Hz), 1.22−1.70, m, 4H, 2 CH_2), 2.21 (s, 3H, Me), 4.28 (t, 2H, CH_2, J = 7.2 Hz), 5.75 (s, 1H, CH), 12.52 (br, 1H, NH).

Biological Tests. *In Vivo Activity of Pyrazole Derivatives on Cyanobacterial Cultures.* Cultures of the blue-green alga *Spirulina platensis* Geitler (strain C1) were grown at 24 ± 1 °C under 16-h days (400 μmol m^{-2} s^{-1}) and 8-h nights as described previously (*8*). Late-log-grown cells were used to inoculate 24-well plates, 3.0 mL per well, to a density of 7.5 mg L^{-1} chlorophyll a (Chl-a). Pyrazole derivatives were dissolved in 1 M NaOH, properly diluted, neutralized, and added in a volume of 100 μL to final concentrations of 0.1 and 0.3 mM. In the case of lenacil, a commercial water-dispersible formulation (Venzar, DuPont) was used. A fully randomized design was adopted, and at least six wells in three different plates were used for each treatment. Cultures were grown for 3 weeks. Growth in untreated controls was followed by destructive harvest: 1.2-mL aliquots were withdrawn, and cells were sedimented by centrifugation for 10 min at 20 000*g*. Pellets were dissolved in 1.2 mL methanol; after 30 min in the dark, samples were centrifuged again, and Chl-a content in the supernatant was determined spectrophotometrically on the basis of the Arnon's formula. When controls entered the stationary phase of growth, the increase in Chl-a concentration was determined in all samples. Data were expressed as percent of controls and are means ± SD over replicates. The whole experiment was repeated twice, with very similar results.

Algal Growth Conditions and in Vivo Evaluation of Photosynthetic Activity Rate. Chlorella protothecoides Kruger, ATCC 30411 strain, was grown at 24 ± 1 °C in 1-L Erlenmeyer flasks containing 250 mL of Proteose Peptone medium (1 g L^{-1} Proteose Peptone [Difco], 200 mg L^{-1} KNO_3, 20 mg L^{-1} K_2HPO_4 and 20 mg L^{-1} $MgSO_4 \times 7H_2O$) under 16-h days (200 μmol m^{-2} s^{-1}) and 8-h nights. Mid-log-grown cells (2.9 ± 0.3 mg L^{-1} Chl-a) were harvested by centrifugation for 10 min at 5000*g*, and pellets were resuspended in a proper volume of Bg11 medium (http://www-cyanosite.bio.purdue.edu/media/table/BG11.html) to a cellular density of 2.0 mg L^{-1} Chl-a. The rate of oxygen evolution under saturating light conditions (>2 mmol m^{-2} s^{-1} photosynthetic active radiation) was measured polarographically using a Hansatech (King's Lynn, Norfolk, England) system consisting of a DW2/2 electrode unit, an A1 stirrer, an LS2 light source with A8 fiber optic, and a CB1-D control box. All assays were performed at 24 °C. Reaction was started by switching the light on, and the oxygen concentration (arbitrary units) in the 1.0-mL cell was recorded every 30 s up to 8 min. Then, the light was switched off, and oxygen consumption in the dark was followed for a further 12 min, providing a measure of the cell respiratory activity. The rate of photosynthetic oxygen production was calculated as the sum of oxygen evolution in the light and oxygen consumption in the dark. The inhibitory activity of the pyrazole derivatives was evaluated by adding 10 μL of freshly prepared 10 mM solutions to the stirred electrode cell (final concentration 0.1 mM), 8 min after the reaction had started. The resulting rate was expressed as percent of that in untreated controls. At least three independent measurements were performed for each dose; means ± SE are presented. To improve the solubility of the compounds and increase the rate of traslocation to the chloroplast, assays were carried out also in the presence of 0.001% (v/v) Triton X-100; at this level, well below the critical micelle concentration (0.2 mM, corresponding to about 0.013%), the detergent is though to be unable to interfere with membrane functionality, and was found to be uneffective upon algal photosynthetic activity in vivo (data not shown).

Preparation of Functional Thylakoids from Spinach Leaves and in Vitro Measurement of the Hill Reaction. Thylakoid photosynthetic membranes were isolated from market spinach (*Spinacea oleracea* L.) leaves. Deveined plant material was resuspended in 5 mL g^{-1} of ice-cold 20 mM Tricine [*N*-tris(hydroxymethyl)methylglycine]-NaOH buffer (pH 8.0) containing 10 mM NaCl, 5 mM $MgCl_2$, and 0.4 M sucrose and homogenized for 30 s in a blender at maximal speed. The homogenate was filtered through surgical gauze, and the filtrate was centrifuged at 4 °C for 1 min at 500*g*; the supernatant was further centrifuged for 10 min at 1500*g*. Pelleted chloroplasts were osmotically swollen by resuspension in buffer in which the sucrose had been omitted. The suspension was immediately diluted 1:1 with sucrose-containing buffer, kept on ice and used within a few hours from the

Scheme 2



preparation. The rate of photosynthetic electron transport was measured by following light-driven ferricyanide reduction. Aliquots of membrane preparations corresponding to 20 μg Chl-a were incubated at 24 °C in 1-mL cuvettes containing 20 mM Tricine-NaOH buffer (pH 8.0), 10 mM NaCl, 5 mM $MgCl_2$, 0.2 M sucrose, and 1 mM $K_3Fe(CN)_6$. The assay was initiated by exposure to saturating light (800 μmol m^{-2} s^{-1}), and the rate of ferricyanide reduction was measured at 30-sec intervals for 10 min against an exact blank at 420 nm. Activity was calculated over the linear portion of the curve from a molar extinction coefficient of 1000 M^{-1} cm^{-1}. The effect of pyrazole derivatives upon Hill reaction was evaluated in parallel assays in which the compounds were added to the reaction mixture to concentrations ranging from 10 nM to 1 mM. Each dose was carried out in triplicate, and results were expressed as percentage of untreated controls. Diuron was solubilized in acetone and then diluted with water, as appropriate. Controls received similar amounts of solvent. Under the adopted experimental conditions, value for controls was 46 ± 2 nmol of ferricyanide reduced sec^{-1} (mg Chl-a)$^{-1}$. The concentrations causing 50% inhibition (I_{50}) of in vitro activity were estimated utilizing the linear regression equation of activity values plotted against the logarithm of inhibitor concentration. Confidence limits were computed according to Snedecor and Cochran (*9*).

Under the same conditions but in an uncoupled state (+1 mM NH_4Cl), ferricyanide reduction (161 ± 3 nmol of acceptor reduced sec^{-1} [mg Chl-a]$^{-1}$) was blocked by the addition of the cytochrome b_6f inhibitor 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone, solubilized in ethanol, and water-diluted to a final concentration of 2 μM. Then, an electronic transfer was restored through the addition of 0.1 mM phenylenediamine. The ability of pyrazole derivatives to interfere with this transfer, which excludes photosystem I, was evaluated spectrophotometrically as described on aliquots of thylakoid membranes corresponding to 10 μg Chl-a.

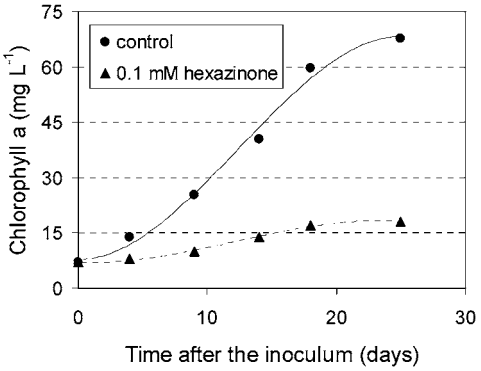
RESULTS AND DISCUSSION

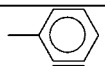
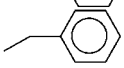
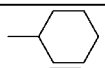
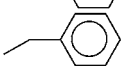
Synthesis. The preparative route to the target products is outlined in **Scheme 2**. 5-Amino-3-methyl-pyrazole-1-carboxamides and 5-amino-3-methyl-pyrazole-1-carbothioamides (**2**) were obtained by reaction of 5-amino-3-methylpyrazole with isocyanate and isothiocyanate, respectively. As expected, the reaction of trichloromethyl chloroformate with (**2**, X = O) proceeded smoothly to afford pyrazolo[1,5-*a*][1,3,5]triazine-2,4-dione derivatives (**V1**−**V6**) and with (**2**, X = S) to give pyrazolo[1,5-*c*][1,3,5]thiadiazine-2-one derivatives (**VS2**−**VS5**), according to a similar procedure previously reported by us for pyrazolo[1,5-*c*][1,3,5]thiadiazine-4-ones (*10*).

Biological Activity. Due to their structural similarity to several commercial herbicides, namely the uracils lenacil, terbacil, and bromacil, and the triazinone hexazinone, which all act by interfering with the photosynthetic electron transport chain, these pyrazole derivatives were susceptible to be endowed with biological activity against photosynthetic organisms. To verify such a hypothesis, their ability to inhibit the obligate photoautotrophic growth of the blue-green alga *Spirulina platensis* was evaluated. Results, summarized in **Table 1**, showed that some of them in the millimolar range were indeed able to significantly reduce cell proliferation. Activity was lower than that of the reference herbicides. However, at 0.3 mM, compounds **V2** and **VS2** caused 90% inhibition of algal growth. Among the pyrazolo[1,5-*a*][1,3,5]triazine-2,4-dione derivatives, the presence of a butyl-(**V4**) and, mainly, of a cyclohexyl-(**V2**) substituent yielded maximal activity. Compounds **VS4** and **VS5** were more effective than their triazine counterparts.

Even if remarkable, growth inhibition is poorly informative with respect to the mode of action. Thus, the effect of millimolar concentrations of the compounds upon the light-driven oxygen production by an eukaryotic alga belonging to the genus *Chlorella* was also investigated. Results (**Table 2**) supported the hypothesis that the pyrazole derivatives may act through interference with the photosynthetic electron transport chain. At 0.1 mM, the same compounds previously found to inhibit growth showed the ability to reduce the rate of oxygen production within a few minutes after the addition to the algal suspension. The structure−activity relationship was similar to that for cyanobacterial growth inhibition. In this case, the effectiveness of compounds **V2** and **VS2** was higher than that of lenacil and similar to that of hexazinone. In several instances, the addition of a detergent to a concentration that had been found to be uneffective upon the rate of oxygen release in controls (data not shown) significantly improved the activity of the

Table 1. In Vivo Effects of Pyrazole Derivatives on the Growth of the Blue-green Alga *Spirulina platensis*[a]



Compound	R	Cell growth (% of controls)	
		0.1 mM	0.3 mM
Hexazinone		7.0 ± 0.3	2.0 ± 0.7
Lenacil		8.2 ± 0.2	1.5 ± 0.5
V1		119.2 ± 4.1	119.6 ± 12.3
V2		31.9 ± 4.2	6.9 ± 1.5
V3		99.5 ± 11.4	92.9 ± 11.7
V4		95.1 ± 4.3	95.3 ± 12.0
V5		88.9 ± 12.3	74.2 ± 14.4
V6		103.8 ± 15.2	87.8 ± 11.1
VS2		33.7 ± 9.6	10.6 ± 10.1
VS3		103.8 ± 6.8	106.8 ± 12.3
VS4		95.1 ± 1.2	80.1 ± 10.1
VS5		96.1 ± 8.0	35.8 ± 3.3

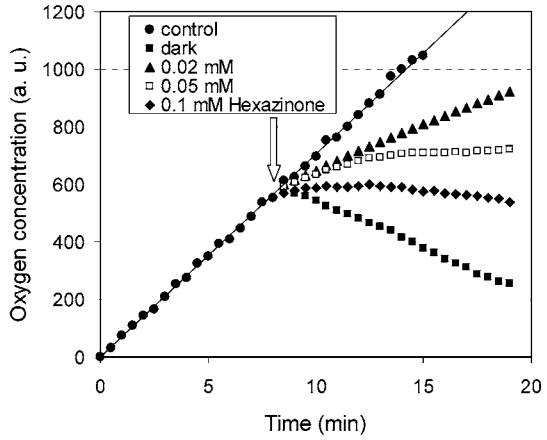
[a] Growth was evaluated as shown in the picture and detailed in Methods on photosynthetically growing cells. Values are expressed as percent of the rate measured in untreated controls and are means ± SD over at least six replications. The whole experiment was repeated a second time, obtaining very similar results.

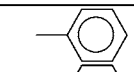
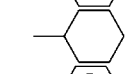
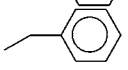
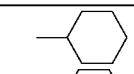
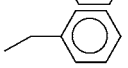
pyrazole derivatives, as well as that of lenacil. This is not unexpected, because small amounts of detergents may significantly improve the solubility in acqueous media for lipophilic compounds and thus increase the rate of herbicide translocation to the active site that in this case should be in the thylakoid membranes inside the chloroplasts.

To strengthen the former evidence supporting the ability of these pyrazole derivatives to act as a photosystem II inhibitors, ferricyanide reduction by isolated spinach thylakoids (the so-called Hill reaction) was measured in the presence of increasing doses of these compounds. Interestingly, all of them showed the ability to inhibit the Hill reaction (**Table 3**). With the only exception of compound **V1**, the concentrations causing 50% inhibition (I_{50}) of in vitro activity ranged from 0.2 to 40 μM, and in most cases it was lower than 10 μM. The effectiveness of the most active compounds is strictly comparable with that of the three commercial herbicides (**Table 3**). In addition, for compounds **V5**, **V6**, **VS4**, and **VS5**, it is higher than that previously found for some herbicidal derivatives of isoxazoledicarboxylic acid (I_{50} 1−30 μM) (*11*). Once again, the pyrazolo-[1,5-*c*][1,3,5]thiadiazine-2-one derivatives showed higher efficacy than the triazine analogues. Because the method employed, which measures the reduction of an electron acceptor after the photosystem I, does not allow to distinguish clearly the site on inhibitor interaction, further experiments were performed to elucidate this point. By addition of both a cytochrome b_6f inhibitor and phenylenediamine, an electron transfer was established from water to ferricyanide that excludes PSI (water ⟹ PSII ⟹ D1 ⟹ phenylenediamine ⟹ ferricyanide). The ability of the most active pyrazole derivatives to also inhibit this transfer at similar rates (**Table 4**) indicated that PSI is not concerned, and that, as for most inhibitors used as herbicides, PSII is the target of their action.

On the whole, the noteworthy high inhibition brought about in vitro with respect to the much lower ability to interfere in vivo with photosynthetic oxygen production most likely depends on the higher accessibility of the target site. The same behavior was evident also for reference herbicides, particularly for lenacil. The relative velocity of in vivo diffusion through both the plasmalemma and the chloroplast envelopes is heavily influ-

Table 2. In Vivo Effects of Pyrazole Derivatives on the Photosynthetic Oxygen Evolution Rate by Intact Cells of *Chlorella prototheocoides*[a]



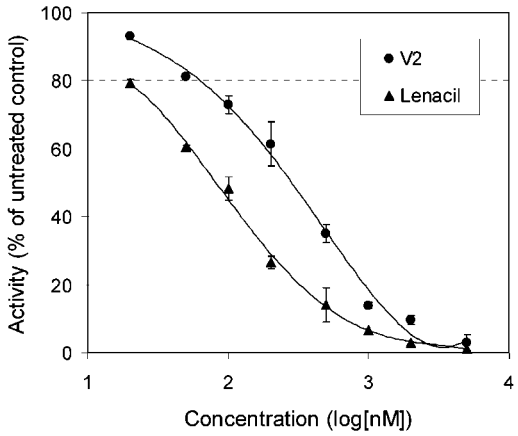
Compound	R	Oxygen evolution (% of controls)	
			+ 0.001% Triton
Control		100.0	99.1 ± 5.0
Hexazinone		24.0 ± 3.8	9.2 ± 1.7
Lenacil		105.0 ± 1.6	52.2 ± 8.3
V1		105.7 ± 0.6	100.1 ± 2.5
V2		60.9 ± 6.9	40.5 ± 2.7
V3		93.8 ± 2.9	96.5 ± 8.3
V4		95.9 ± 0.6	96.9 ± 5.1
V5		107.1 ± 1.2	84.5 ± 6.2
V6		85.9 ± 3.4	81.4 ± 6.6
VS2		39.6 ± 6.3	12.5 ± 3.1
VS3		103.6 ± 1.9	96.9 ± 5.6
VS4		99.0 ± 5.1	105.3 ± 1.9
VS5		92.2 ± 3.6	75.3 ± 5.5

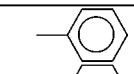
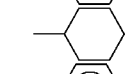
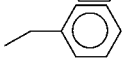
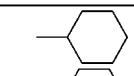
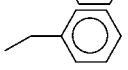
[a] Activity was evaluated as shown in the picture and detailed in Methods on cells harvested during the mid-log phase of growth. The compounds were added to a concentration of 0.1 mM, either in the presence or in the absence of detergent. Values are expressed as percent of the rate measured in untreated controls, and are means ± SE over at least three replications; a.u., arbitrary units.

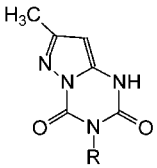
enced by the hydrophobic/hydrophilic properties of a compound. This phenomenon may be amplified in the field, where herbicides have to be translocated from the epidermal cells to the target tissue. On the contrary, the remarkable activity in vitro is less consistent with the substantially poor effect in vivo on algal growth, at least in comparison with the reference compounds. A drastic reduction of activity in planta compared to that in vitro is frequently observed and would be due to poor penetration, poor translocation, or compartimentalization. However, this is unlikely to occur in unicellular algae. As an alternative, such a reduction could rely upon rapid metabolization of pyrazole derivatives. Experiments are in progress to verify this aspect. If confirmed, this property could represent the basis for differential activity against crops and weeds.

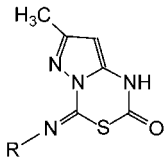
At this initial stage of the research, in which the aim was focused mainly at the analysis of the scaffold effect (thiadiazine vs triazine), a quantitative structure−activity relationship evaluation would be limited by the low number of derivatives available. However, results seem to suggest that the ability to interfere with the photosynthetic electron transport may depend greatly on the steric and conformational properties of the substituent group attached to either ring, with maximal activity achieved, in a decreasing order, with cyclohexyl, sec-butyl and butyl groups. Consistently, both lenacil and hexazinone have a cyclohexyl group, and bromacil a sec-butyl group as substituent. On the contrary, the replacement of aliphatic chains with phenyl or benzyl substituents almost completely abolishes the biological activity, suggesting the conformational freedom as a determinant for the ligand to adapt to the enzyme active site. Almost identical

Table 3. In Vitro Effects of Pyrazole Derivatives on Ferricyanide Reduction by Functionally Intact Cloroplasts Isolated from *Spinacia oleracea* Leaves[a]



Compound	R	I_{50} [b] (μM)
Diuron		0.27 ± 0.02
Hexazinone		0.11 ± 0.02
Lenacil		0.08 ± 0.02
V1		169 ± 86
V2		0.32 ± 0.19
V3		42 ± 8
V4		32 ± 8
V5		6.6 ± 0.8
V6		2.6 ± 0.6
VS2		0.21 ± 0.03
VS3		18 ± 4
VS4		2.9 ± 0.6
VS5		0.57 ± 0.11



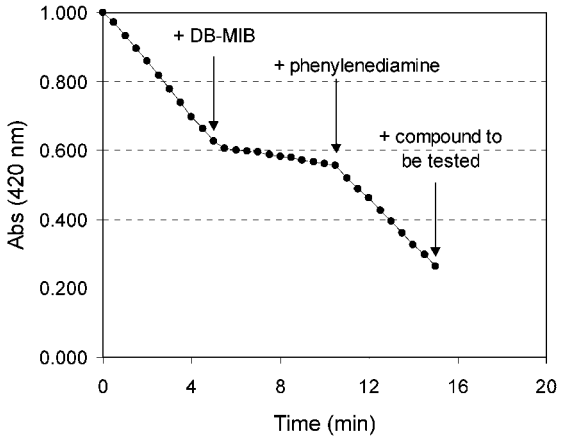


[a] Activity was measured as described in Methods either in the absence or in the presence of pyrazole derivatives at concentrations ranging from 10 nM to 1 mM. Each sample was carried out in triplicate, and values were expressed as percentage of untreated control. [b] The concentrations causing 50% inhibition (I_{50}) of in vitro activity were estimated as shown in the picture utilizing the linear regression equation of the activity values plotted against the logarithm of inhibitor concentration. Confidence limits were computed according to Snedecor and Cochran.

results were reported for herbicidal methyl esters of amino-substituted thiazoles (*12*). In this case also, lipophilicity, as modulated by the length of the hydrocarbon side chain, had a great impact on activity. While compounds with hydrogen, methyl, and ethyl substituents were essentially not active, an increase in the number of carbons resulted in more active derivatives. This was interpreted as a requirement to allow penetration of the thylakoidal membrane to reach the QB binding site; the *n*-propyl side chain provided sufficient lipophilicity, whereas maximum activity was obtained with the isopropyl and *n*-butyl side chains. The effect of cyclohexyl substituent was not tested (*12*).

On the other hand, the comparison between compounds **V2**−**V5** and **VS2**−**VS5**, which have exactly the same substituents, strongly suggests that the thiadiazine ring may act as amplifier of the inhibitory effect. Even though the two rings show isosteric analogies, the substitution of an oxygen with a sulfur results in increased hydrophobicity. Moreover, some conformational variation may derive that could lead to a different positioning of residues, thus modifying ligand interactions with the target active site. Molecular similarity studies clearly show that, even in those cases in which a property is maintained, its quantitative distribution may differ among isosters as a consequence of multiple variations in different fields involved in ligand-biomolecule complementarity. More information with respect to this might come in the future from studies of crystallographic structures, docking experiments, and general computational approaches.

Table 4. In Vitro Effects of Pyrazole Derivatives on Ferricyanide Reduction by Either the Whole Electron Transport Chain or the PSII Alone[a]



	No addition		+ 2 μM DB-MIB + 0.1 mM phenylenediamine	
	nmol s^{-1} [mg Chl-a]$^{-1}$	%	nmol s^{-1} [mg Chl-a]$^{-1}$	%
Control	161.0 ± 2.4	100	144.1 ± 7.3	89.5
+ 2 μM DB-MIB	21.6 ± 1.2	13.4		
+ diuron 1 μM	15.7 ± 1.0	9.8	18.9 ± 4.3	11.7
+ diuron 0.5 μM	34.3 ± 1.4	21.3	41.4 ± 5.2	25.7
+ V2 1 μM	22.2 ± 2.3	13.8	25.3 ± 3.1	15.7
+ V2 0.5 μM	29.8 ± 4.7	18.5	46.2 ± 6.0	28.7
+ V2S 1 μM	17.1 ± 1.4	10.6	23.2 ± 0.7	14.4
+ V2S 0.5 μM	32.2 ± 6.7	20.0	43.7 ± 2.7	27.2

[a] Activity was evaluated spectrophotometrically as described for Table 3 but in an uncoupled state (+1 mM NH_4Cl) on aliquots of thylakoid membranes corresponding to 10 μg Chl-a; ferricyanide reduction was blocked by the addition of the cytochrome b_6f inhibitor 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (DB-MIB). Then, an electronic transfer from water to ferricyanide that excludes photosystem I ($H_2O \rightarrow$ PSII $\rightarrow$ D1 $\rightarrow$ phenylenediamine $\rightarrow$ ferricyanide) was restored through the addition of 0.1 mM phenylenediamine. The ability of the two most active pyrazole derivatives and the reference herbicide diuron to interfere with this transfer was evaluated. Each sample was carried out at least in triplicate, and values were expressed as percentage of untreated control.

Results of laboratory studies may be somehow inconsistent with those obtained in the field, where many factors such as adsorption, translocation, compartimentalization, and possible metabolization might influence herbicide toxicity. Thus, confirmative field trials are now required to assess the real potential of these compounds as agrichemicals. In any case, they proved to possess a remarkable effectiveness in vitro as inhibitors of the photosynthetic electron transport, and some of them showed a significant activity in vivo as well. As the research of new photosystem II inhibitor herbicides is still of great interest for agriculture (*13*), this initial data set strengthens pyrazole triazines and thiadiazines as lead structures to be further explored to develop new active principles.

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