Biological Activity of 6-Pentyl-2*H*-pyran-2-one and Its Analogs

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The *in vitro* biological activities (antifungal, phytotoxic) for a group of fungal metabolites structurally related to 6-pentyl-2*H*-pyran-2-one have been determined. Six compounds were tested and showed a degree of phytotoxicity in one or more of the assays employed. From among the analogs tested, (*R*)-5,6-dihydro-6-pentyl-2*H*-pyran-2-one has been identified as the most active against *Penicillium* species.

Keywords: *Antifungal; phytotoxic; 6-alkyl-2H-pyran-2-one; 5-alkyl-2H-furan-2-one*

INTRODUCTION

The genus *Trichoderma* is a proven source of biologically active natural products. One of these is the metabolite 6-pentyl-2*H*-pyran-2-one (**I**). Previously identified as a component of foodstuffs, and currently employed as a food additive to modify flavor/aroma, the antibiotic activity of **I** was reported following its isolation from fungal cultures (Cutler, 1984a). Activity against a number of phytopathogens has been cited (Claydon *et al.*, 1987), and the activity as biological control agents of certain *Trichoderma* isolates has been attributed, at least in part, to their production *in situ* of **I** (Ghisalberti *et al.*, 1990).

As biodegradable compounds, organic natural products are attractive candidates for development as agrochemicals. Compound **I** is available commercially for use as a food additive. Its high cost hinders its development and, at this time, precludes its economic use as a nontargeted pre- or postharvest application. For this reason, a study was undertaken to determine the spectrum of antifungal and phytotoxic activity of **I** and a group of its commercially available analogs that are naturally occurring in food (Maga, 1976) and used as food additives to modify flavor/aroma; (*R*)-5,6-dihydro-6-pentyl-2*H*-pyran-2-one (**II**), (*RS*)-dihydro-5-hexyl-2*H*-furan-2-one (**III**), (*RS*)-tetrahydro-6-pentyl-2*H*-pyran-2-one (**IV**), (*RS*)-dihydro-5-octyl-2*H*-furan-2-one (**V**) and (*RS*)-tetrahydro-6-heptyl-2*H*-pyran-2-one (**VI**) (Figure 1).

The blue (*Penicillium expansum*, *P. digitatum*), green (*P. digitatum*), and grey molds (*Botrytis cinerea*) of citrus and pome fruit cause substantial postharvest crop losses, and *Sclerotinia* attacks a wide range of vegetable crops. The natural products **I**−**VI** have been assayed in order to assess their potential as part of a sustainable program for the control of these pre- and postharvest phytopathogens.

EXPERIMENTAL PROCEDURES

Maintenance of Test Fungi. The test fungi were field isolates maintained on potato dextrose agar (PDA) as slopes (*P. digitatum*, *P. expansum*, and *P. italicum*) or plates (*B. cinerea*, *Sclerotinia* sp.). Inoculated slopes of the *Penicillium* species were incubated at 20 °C until sporulating and then stored at 4 °C. Inoculated plates of *Sclerotinia* and the two *B. cinerea* isolates were sealed with parafilm and incubated at 20 °C until confluent growth across the agar surface had occurred. Plates were then stored at 4 °C.

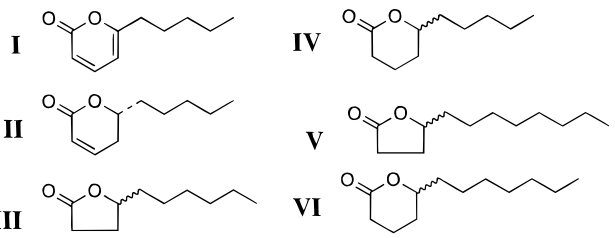

Figure 1. Structures of 6-pentyl-2*H*-pyran-2-one (**I**), (*R*)-5,6-dihydro-6-pentyl-2*H*-pyran-2-one (**II**), (*RS*)-dihydro-5-hexyl-2*H*-furan-2-one (**III**), (*RS*)-tetrahydro-6-pentyl-2*H*-pyran-2-one (**IV**), (*RS*)-dihydro-5-octyl-2*H*-furan-2-one (**V**), and (*RS*)-tetrahydro-6-heptyl-2*H*-pyran-2-one (**VI**).

Agar Diffusion Assay. Suspensions of *Penicillium* spores were prepared by washing PDA slopes with 2 5 mL volumes of aqueous sterile 0.1% (v/v) Tween-80. The spore density of the combined volumes was determined for a 20-fold dilution using an improved Neubauer hemocytometer. The calculated volumes of spore suspension required to yield final spore densities of 10^5, 10^6, and 10^7 spores mL^{-1} were added to 20 mL vol. of molten PDA. For each spore concentration, 3 mL aliquots were transferred to each of the wells of a six-well microtiter plate (Nunc) and allowed to solidify. Solutions of test compounds were prepared in acetone at a concentration of 25 μL mL^{-1}. Twenty microliters of each test compound solution, containing 500 nL (ca. 500 μg) of each test compound, was applied to a 5 mm diameter sterile filter paper (Whatman No. 1) disk. After allowing the solvent to evaporate, an impregnated filter paper disk for each of the test compounds was placed at the center of each well. Plates were incubated for 48 h at 20 °C. The diameters of the resulting zones of total inhibition were measured and recorded (Figure 2).

Agar Dilution Assay. A 2-fold dilution series for each test compound was prepared in sterile 0.1% (v/v) Tween-80 at 4 times final concentration. One volume of the test compound solution was mixed with 3 vol. of molten PDA prepared at four-thirds final strength. For each step of the dilution series, a 3 mL volume of the PDA containing test compound was transferred to individual wells of a six-well microtiter plate (Nunc) and allowed to solidify. Final concentrations were 0.1, 0.05, 0.025, 0.0125, and 0.00625% (v/v) with a control containing 0.025% (v/v) Tween-80 alone. Mycelia bearing agar plugs of 4 mm diameter were aseptically excised from a source plate (*Sclerotinia* sp. or *B. cinerea*) and placed at the center of each well with the colony bearing surface uppermost. Plates were

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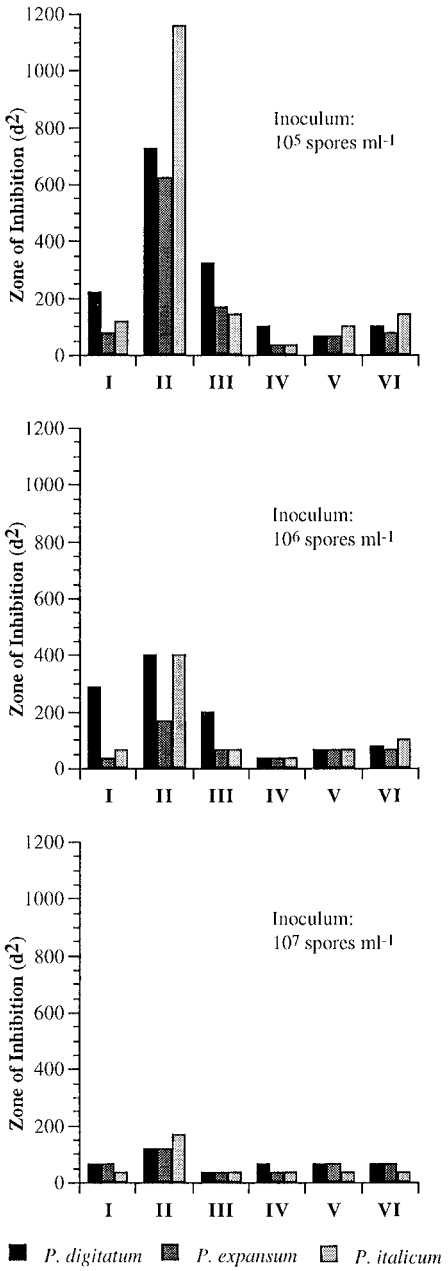

Figure 2. Agar diffusion assay. Sensitivity testing of the three species of *Penicillium* was performed at the three inoculum concentrations indicated and as detailed in the text. Test compounds were applied at the rate of 500 μL per disk; 6-pentyl-2*H*-pyran-2-one (**I**), (*R*)-5,6-dihydro-6-pentyl-2*H*-pyran-2-one (**II**), (*RS*)-dihydro-5-hexyl-2*H*-furan-2-one (**III**), (*RS*)-tetrahydro-6-pentyl-2*H*-pyran-2-one (**IV**), (*RS*)-dihydro-5-octyl-2*H*-furan-2-one (**V**), and (*RS*)-tetrahydro-6-heptyl-2*H*-pyran-2-one (**VI**). Activity is expressed as the square of the diameter/mm^2 (d^2) of the observed total inhibition zone (min, 36; max, 1156) after 2 days of incubation at 20 °C.

incubated at 20 °C for 5 days. Outgrowth from the inoculum plug was recorded (Table 1). Assays were performed in triplicate.

Air Diffusion Assay. A volume of *Penicillium* sp. spore suspension was mixed with 15 mL molten PDA to give a final spore concentration of 10^5 spores mL^{-1} and poured into a 90 mm diameter Petri dish. The agar was allowed to set, and agar plugs of 4 mm diameter excised and placed 20 mm from the center of a water agar plate. A sterile glass coverslip was placed opposite the agar plug along the same diameter and 5 μL of test compound placed at its center. Petri dishes were sealed with parafilm and incubated at 20 °C. Plates were examined for mycelial outgrowth from the agar plugs after 7 days.

Wheat Coleoptile Assay. The assay was performed as previously described (Cutler, 1984b; Cutler *et al.*, 1986).

Table 1. Agar Dilution Assay[a]

	minimum inhibitory concn (%, v/v) of test compd					
test organism	**I**	**II**	**III**	**IV**	**V**	**VI**
B. cinerea isolate 1	0.05	0.025	0.05	0.1	>0.1	0.1
B. cinerea isolate 2	0.05	0.05	0.05	0.1	>0.1	0.1
Sclerotinia sp. Sc4	0.025	0.025	0.025	0.05	0.025	0.025

[a] The minimum concentration required to totally inhibit outgrowth of mycelia from a colony bearing agar plug was determined as detailed in the text for two isolates of *B. cinerea* and one *Sclerotinia* sp. isolate. The inoculum agar plugs were incubated for 5 days at 20 °C on PDA containing the test compound at the concentration indicated; 6-pentyl-2*H*-pyran-2-one (**I**), (*R*)-5,6-dihydro-6-pentyl-2*H*-pyran-2-one (**II**), (*RS*)-dihydro-5-hexyl-2*H*-furan-2-one (**III**), (*RS*)-tetrahydro-6-pentyl-2*H*-pyran-2-one (**IV**), (*RS*)-dihydro-5-octyl-2*H*-furan-2-one (**V**), and (*RS*)-tetrahydro-6-heptyl-2*H*-pyran-2-one (**VI**). The controls for *Botrytis* and *Sclerotinia* demonstrated confluent growth across the agar well surface after 3 and 5 days, respectively.

Table 2. Etiolated Wheat Coleoptile Assay[a]

	wheat coleoptile growth (% control)[b]					
molar concn of test compd	**I**	**II**	**III**	**IV**	**V**	**VI**
10^{-3}	0[c]	0[c]	0[c]	59[c]	0[c]	0[c]
10^{-4}	100	63[c]	100	100	98	100

[a] Inhibition of the growth of wheat coleoptile sections is reported for only two concentrations, for each of the test compounds; 6-pentyl-2*H*-pyran-2-one (**I**), (*R*)-5,6-dihydro-6-pentyl-2*H*-pyran-2-one (**II**), (*RS*)-dihydro-5-hexyl-2*H*-furan-2-one (**III**), (*RS*)-tetrahydro-6-pentyl-2*H*-pyran-2-one (**IV**), (*RS*)-dihydro-5-octyl-2*H*-furan-2-one (**V**), and (*RS*)-tetrahydro-6-heptyl-2*H*-pyran-2-one (**VI**). [b] Duplicate bioassays. [c] Denotes significant inhibition ($P < 0.01$).

Table 3. Lettuce Seed Germination Assay

control	**I**	II	III	IV	V	VI
91	76	84	96	97	94	92

[a] Inhibition of the germination of 100 lettuce seeds cv. Webbs Wonderful was determined after 3 days of incubation in the dark at 20 °C in the presence of test compound vapors; 6-pentyl-2*H*-pyran-2-one (**I**), (*R*)-5,6-dihydro-6-pentyl-2*H*-pyran-2-one (**II**), (*RS*)-dihydro-5-hexyl-2*H*-furan-2-one (**III**), (*RS*)-tetrahydro-6-pentyl-2*H*-pyran-2-one (**IV**), (*RS*)-dihydro-5-octyl-2*H*-furan-2-one (**V**), and (*RS*)-tetrahydro-6-heptyl-2*H*-pyran-2-one (**VI**).

Briefly, wheat seeds (*Triticum aestivum* L. cv. Wakeland) were sown on moist vermiculite and incubated in the dark for 4 days at 22 °C. From individual coleoptiles, 4 mm sections were excised, 2 mm below the tip, and placed in test tubes with 2 mL of a phosphate-citrate buffer (pH 5.6) containing 2% (w/v) sucrose. Test compounds were added in 10 μL of acetone to yield final concentrations of 10^{-3}, 10^{-4}, and 10^{-5} M. Length of the sections was measured after incubation for 18 h (Table 2). Data were statistically analyzed and the 0.01 level of confidence accepted (Kurtz *et al.*, 1965). Assays were performed in duplicate.

Lettuce Seed Germination Assay. The assay was performed as described by Claydon *et al.* (1987). A glass coverslip was placed at the center of a Petri dish lined with moist filter paper and 25 lettuce seed cv. Webbs Wonderful were randomly scattered over the filter paper surface. A volume of 5 μL of test compound was applied to the glass coverslip and the Petri dish lid replaced. Petri dishes, 4 per test compound, were incubated in the dark at 20 °C for 3 days. The number of seeds germinating and the appearance of the emerging radicle was recorded (Table 3).

Phytotoxicity Test on Bean. Seeds of dwarf bean cv. Greencrop were glasshouse grown in a commercial potting mix until the embryo leaves had fully expanded and the trifoliate leaves of the apical tip were in the crook stage. Seedlings at this growth stage were treated in sets of four with an aqueous spray application of test compound in 0.1% (v/v) Tween-80 until thorough wetting of the leaf surface was achieved

(typically 5 mL total volume). Compounds were tested at concentrations of 0.001, 0.01, 0.1, and 1% (v/v) with 0.1% (v/v) Tween-80 alone as a control. Growth and appearance of plants was noted on day 1 and day 7 following application.

RESULTS AND DISCUSSION

6-Pentyl-2*H*-pyran-2-one (**I**) is an antifungal agent (Cutler, 1984a) and has phytotoxic activity (Claydon *et al.*, 1987). Indeed **I** has been isolated from extracts of *Trichoderma* fermentations by bioassay directed fractionation on the basis of its plant growth regulatory activity (Cutler *et al.*, 1986). By contrast Olivier *et al.* (1993) have made claims to the use of the structurally analogous *Trichoderma* metabolites **IV** and **VI** on the basis of their dual function as both antifungal and plant growth stimulating agents.

All six compounds tested demonstrate a concentration-dependent activity as plant growth regulators in the etiolated wheat coleoptile assay (Table 2). Only **II** was inhibitory at a concentration of 10^{-4} M. However, when the inhibitory activity of vapors of the test compounds on the germination of lettuce seeds was determined (Table 3), **II** was less inhibitory than **I.** In addition, the radicle tissue exposed to vapors of **II** showed less of the browning reported for **I** by Claydon *et al.* (1987). None of the saturated lactones tested (**III**, **IV**, **V**, and **VI**) exhibited any inhibitory activity, and radicle appearance was normal. By contrast, the analogous "air diffusion" assay showed no effect of the test compounds' vapors on the germination and mycelial development of *Penicillium* spp. spores. All three species tested were uninhibited and rates of mycelial growth were indistinguishable from those of controls.

In a third phytotoxicity test, bean seedlings were treated with foliar applications of the compounds. No phytotoxicity was observed for any of the test compounds at concentrations of 0.1% (v/v) or less. However, applications of 1% (v/v) of any of the test compounds resulted in an immediate necrosis of leaf tissue (within 24 h), observed as irregular, pale green to golden patches of flaccid leaf tissue. The toxicity was not systemic, and the exposed trifoliate leaves continued to develop normally with further growth of the plant. In contrast, with the other assays employed, the uniformity of these observations for the compounds tested indicates a nonspecific mode of action. Seedlings treated at the lower concentrations remained healthy and grew to maturity.

Inclusion in nutrient agar of **I**, **II**, **III**, **IV**, and **VI** at concentrations of 0.1% (v/v) totally inhibited outgrowth of *B. cinerea* or *Sclerotinia* sp. Sc4 mycelia from an inoculum agar plug. Compound **V** was only active against *Sclerotinia* sp. Sc4 at this concentration. Compounds **I**, **II**, and **III** retained activity against both phytopathogens at lower concentrations (Table 1). At subinhibitory concentrations partial inhibition of mycelial outgrowth was observed.

The *in vitro* antifungal activity of test compounds was anticipated to be both inoculum concentration and time dependent. This is clearly evident in Figure 2 where the areas of zones of inhibition decrease with increasing densities of *Penicillium* spore inoculum. Zones of inhibition were further reduced on prolonged incubation; however, where germination and mycelial growth were inhibited across the whole of the 34 mm diameter well (**II**, *P. italicum* at 10^5 spores mL^{-1}), this inhibition was sustained.

The biodegradability of organic natural products makes them attractive candidates for development as antifungal agents for agricultural use. Alternatively, their production may provide one of many criteria for the selection of microorganisms for use as biological control agents; permitting *in situ* delivery (Cutler and Hill, 1994). The judicious application of the purified metabolites to control both pre- and postharvest phytopathogens requires knowledge of the relative activities (antifungal, phytotoxic) of the compounds. From the data presented here, **II** has been identified as a promising candidate for development as a control agent for blue and green molds. The authors note that the spectrum of activity of **II** includes the opportunistic zoopathogens *Aspergillus fumigatus*, *Candida albicans*, and *Cryptococcus neoformans* (Parker, S. R., *et al.*, unpublished material).

ABBREVIATIONS USED

PDA, potato dextrose agar.

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