Plant Physiol. (1982) 69, 712–716
0032-0889/82/69/0712/05/$00.50/0

Comparative Effects of Substituted Pyrimidines on Growth and Gibberellin Biosynthesis in *Gibberella fujikuroi*[1]

Received for publication March 3, 1981 and in revised form November 2, 1981

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ABSTRACT

The fungicide α-(2,4-dichlorophenyl)-α-phenyl-5-pyrimidine methyl alcohol (triarimol) and four other structural analogs of this substance, in which one or more of the substituents were varied, were tested for their comparative effects on growth and gibberellin biosynthesis in the fungus *Gibberella fujikuroi*. Each of the five analogs tested was capable of inhibiting growth as measured by dry weight in 5-day-old cultures. Three of them [α-(2-chlorophenyl)-α-(4-chlorophenyl)-5-pyrimidine methyl alcohol, fenarimol; α-(2-chlorophenyl)-α-(4-fluorophenyl)-5-pyrimidine methyl alcohol, nuarimol; and triarimol] were effective at appreciably lower concentrations than the other two [α-(4-chlorophenyl)-α-(1-methylethyl)-5-pyrimidine methyl alcohol, experimental compound EL 509; and α-cyclopropyl-α-(4-methoxyphenyl)-5-pyrimidine methyl alcohol, ancymidol].

All five substances also inhibited gibberellin production as measured by gibberellin content of fungus filtrates. The relative effectiveness of the compounds as inhibitors of growth and gibberellin production were similar. These analogs were also shown to inhibit *ent*-kaurene oxidation by microsomal preparations from fungal mycelia. Thus, the site of inhibition of gibberellin biosynthesis may be the same for the fungus as the one affected by this group of substances in higher plant tissues.

The structure-activity relationships between the analogs are opposite to those observed in higher plant tissues. The fungicides fenarimol, nuarimol, and triarimol, which were most effective in inhibiting growth and gibberellin biosynthesis in the fungus, were much less effective than EL 509 and ancymidol in inhibiting growth and gibberellin biosynthesis in higher plants. These results indicate that the *ent*-kaurene oxidase systems from the two sources have somewhat different molecular characteristics, and thus, interact differently with this group of substances.

Ancymidol[3] is a potent inhibitor of growth and GA[4] biosynthesis in higher plants (9–11, 17, 22, 24). It has been shown previously to be ineffective at similar concentrations on the fungus, *Gibberella fujikuroi* (10, 17). Shive and Sisler (22) compared the effects of ancymidol and triarimol on growth and GA contents of beans and *G. fujikuroi*. They reported that these compounds do inhibit GA biosynthesis in the fungus, although triarimol, an experimental fungicide, is more effective than ancymidol. Ali *et al.* (1) also reported on the fungitoxic activity of ancymidol. In the accompanying paper (11), we have compared the effects of a number of ancymidol analogs, including triarimol, on the growth of pea seedlings and the rate of kaurene oxidation in cell-free enzyme extracts of *Marah macrocarpus* liquid endosperm. The results indicate that the analogs with fungicidal activity do inhibit *ent*-kaurene oxidation, but only at relatively high concentrations. Because of the seeming high degree of specificity of ancymidol for three mixed-function oxygenase reactions of the biosynthetic pathway for GAs in the enzyme system from *M. macrocarpus* (10), the apparent differences in the structure-activity relationships of these compounds in higher plants and *G. fujikuroi* (11, 22), and the apparent lack of growth inhibitory activity for the fungus by other GA biosynthesis inhibitors (8, 12, 16, 18), we have compared the effects of five structural analogs, including ancymidol and triarimol, on growth, *ent*-kaurene oxidation, and GA biosynthesis in the fungus *G. fujikuroi*.

MATERIALS AND METHODS

Fungus Culture. The stock culture of *G. fujikuroi* used in these studies was a single-cell isolate from the M 419 strain of American Type Culture Collection No. 917 maintained on potato dextrose agar. The liquid culture medium used for determination of effects of inhibitors on the mycelial growth of *G. fujikuroi* was as described previously (10). Suspensions (1 ml) of 3-d-old cultures (100 ml) of fungus (exponential growth phase) were transferred to fresh media containing appropriate concentrations of potential inhibitors. These flasks were then incubated for 5 d on a shaker under conditions previously described (10).

Determination of Growth. After 5 d incubation, the mycelia were harvested by vacuum filtration on a Buchner funnel. The culture flasks were rinsed twice with distilled H_2O, and the mycelia were also washed gently. The culture filtrates were stored at 3°C for extraction for GAs. The collected mycelia were oven dried on preweighed filter papers for 72 h, and dry weights were determined repeatedly until constant.

GA Extractions. Ten percent of the total volume of each filtrate was withdrawn. The pH of these filtrates was adjusted to 2.5 with dilute HCl. Filtrates were extracted twice with equal volumes of ethyl acetate. Organic extracts were combined and dried over anhydrous sodium sulfate, decanted, and evaporated to dryness under vacuum on a rotary evaporator at 40°C. The dried residue

[1] Supported by Grant PCM 76-19279 from the National Science Foundation and by Grant GM 07065 from the National Institutes of Health.

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[3] Trivial names: Ancymidol (also referred to in the literature as experimental compound EL 531 or the commercial preparation A-REST) is the trivial name for α-cyclopropyl-α-(4-methoxyphenyl)-5-pyrimidine methyl alcohol; triarimol (experimental compound EL 273) is the trivial name for α-(2,4-dichlorophenyl)-α-phenyl-5-pyrimidine methyl alcohol; *ent*-kaurene and *ent*-kaurenoic acid refer to *ent*-kaur-16-ene and *ent*-kaur-16-en-19-oic acid, respectively. Other potential inhibitors used in this study include: fernarimol (EL 222), α-(2-chlorophenyl)-α (4-chlorophenyl)-5-pyrimidine methyl alcohol; nuarimol (EL 228), α-(2-chlorophenyl)-α-(4-fluorophenyl)-5-pyrimidine methyl alcohol; and EL 509, α-(4-chlorophenyl)-α-(1-methylethyl)-5-pyrimidine methyl alcohol. The structures of all five inhibitors are provided in the accompanying paper (11).

[4] Abbreviation: GA, gibberellin.

was then taken up in 1 ml of acetone and stored under refrigeration. The acetone extract was applied to thin layers (0.25 mm) of silica gel 60. The TLC plates were developed to 15 cm with H_2O, (7) and the upper 5 cm of silica gel were removed. The GAs in this portion of the gel were eluted with 10 ml of acetone, and the acetone was evaporated. The extract was taken up in Tris buffer, adjusted to pH 8, and extracted three times with 2 ml benzene to remove nonacidic organic contaminants. The aqueous phases were adjusted to pH 2.5 with HCl and diluted with ethanol to a final concentration of 25% in 0.05% aqueous Tween-20. These preparations were refrigerated for later analysis by the dwarf pea bioassay and GC-MS.

Procedure for GA Bioassays. *Pisum sativum* (Progress No. 9) seeds were planted in sterile vermiculite in plastic pots (15 cm in diameter) 10 to 15 d prior to treatment. These plants were grown in a greenhouse under 16-h photoperiods. The light intensity was supplemented with and day length extended by Gro-Lux fluorescent lamps yielding approximately 800 ft-c. The temperature ranged from 20 to 25°C. Pots were irrigated alternately with water and half-strength Hoagland solution. Pots were thinned to contain 10 uniform seedlings, and each of the 10 plants per pot received a 10 μl application of experimental organic extract on the shoot tip after initial height measurements had been recorded. An additional set of bioassay plants was treated in the same way with a 1:9 dilution of each sample. This was done to insure responses within the linear range of the bioassay and to provide confirming data. Two sets of plants were also used for standard curve determinations. One set was treated with samples of GA_3 ranging in concentration from 0.01 to 100 μg/μl. The other set was treated with the same concentrations of GA_3 which had been previously extracted from buffer and chromatographed under the same conditions as the fungus extracts.

GC-MS and Single Ion Current Monitoring of GAs. Samples prepared for bioassay were also utilized for GC-MS. Aliquots were re-extracted into ethyl acetate at pH 2.5, taken to dryness, dissolved in ether, treated with diazomethane (generated from *N*-methyl-nitroso-*p*-toluene sulfonamide), followed by treatment in closed containers at 60°C for 30 min with *bis*-(trimethylsilyl)-trifluoroacetamide plus 1% trimethylchlorosilane. These derivatized samples were injected into a computerized Varian MAT CH-7 combined gas chromatograh-mass spectrometer fitted with a 4-ft × ⅛-inch column of 7% OV 210 coated on HP Chromsorb W. Chromatography was done isothermal at 230°C with injection and detection chambers at 290°C. Helium was the carrier gas at 30 cc min^{-1}. MS (10 scans min^{-1}) was at 70 ev. The identity of GA_3 was confirmed by comparison of mass spectra and retention times with an authentic standard and with published mass spectra (4). GA_3 content was monitored by single ion current monitoring at m/e = 504.

Incorporation of [2-^{14}C]Mevalonic Acid into GA_3. Aqueous [2-^{14}C]mevalonic acid (0.5 μmol; 10.9 mCi/mmol) was Millipore filtered and added aseptically to growing cultures (50 ml medium buffered at pH 7.5 with 0.05 M potassium phosphate) of *G. fujikuroi* 44 h prior to harvest. The culture filtrates were extracted, first with an equal volume of benzene/acetone (3:1), and then acidified to pH 2.5 and extracted for GAs as described above. The organic extracts were combined, dried over sodium sulfate and chromatographed on thin layers of silica gel to 15 cm in hexane followed by redevelopment to 10 cm with benzene/ethyl acetate/NH_4OH (90:9.5:0.5). This system separates many of the intermediates in the GA biosynthetic pathway from *ent*-kaurene through *ent*-kaurenoic acid while leaving more polar acids at the origin (10). The gel at the origin (0.5 cm) was then removed, extracted with acidic ethyl acetate, and rechromatographed on a new TLC plate with isopropyl ether:acetone:acetic acid (90:30:1.0) with authentic nonradioactive GA_3 as a marker. The gel corresponding to the position of GA_3 in an ancymidol-treated sample was extracted, combined with 100 mg GA_3, and recrystallized to constant specific radioactivity from methanol.

Cell-Free *Ent*-Kaurene Oxidation. Microsomal preparations from mycelia of actively growing *G. fujikuroi* were prepared and assayed as described previously (10) for *ent*-kaurene oxidase activity in the presence and absence of all five inhibitors. The [^{14}C]*ent*-kaurene used as substrate was prepared biosynthetically from [^{14}C]mevalonic acid (13.65 mCi/mmol) in cell-free extracts of *M. macrocarpus* endosperm (10).

Radioactivity Determinations. Radioactive products separated by TLC were located on the plate by means of a Packard radiochromatogram scanner (model 7201) with a 2-mm window. Gel segments were scraped from the TLC plate and counted in a Packard liquid scintillation spectrometer.

Source and Purity of Reagents. *N*-methyl-*N*-nitroso-*p*-toluene sulfonamide, NADPH, flavin adenine dinucleotide, and GA_3 (Grade III) were purchased from Sigma. Regisil RC-2(*bis*-(trimethylsilyl)-trifluoroacetamide + 1% trimethylchlorosilane) was purchased from Regis Chemical, Morton Grove, IL. The potential inhibitors were all gifts from Eli Lilly and Co. They are all presumably racemic mixtures. Ancymidol and EL 509 are experimental plant growth regulators, while fenarimol, nuarimol, and triarimol are experimental fungicides. All other chemicals were reagent grade, and all solvents used in derivatizations were redistilled before use.

RESULTS

The comparative effects of the five substituted pyrimidines on the growth of liquid cultures of *G. fijikuroi* are shown in Figure 1. All five compounds are capable of inhibiting growth. The solid symbols illustrated in Figure 1 represent the mean values obtained from two independent experiments. These results were confirmed in several additional experiments. The maximum SE for these data points was 105, while the mean SE was 25. The concentrations represented by the open symbols (10^{-7} M, 5×10^{-6} M, and 5×10^{-4} M) were tested in only one experiment and contribute some additional clarity to the definition of the curves. It is evident from these data that the analogs can be separated into two families based on their activities as growth inhibitors. There is more than a 10-fold difference in effective concentrations of the recognized

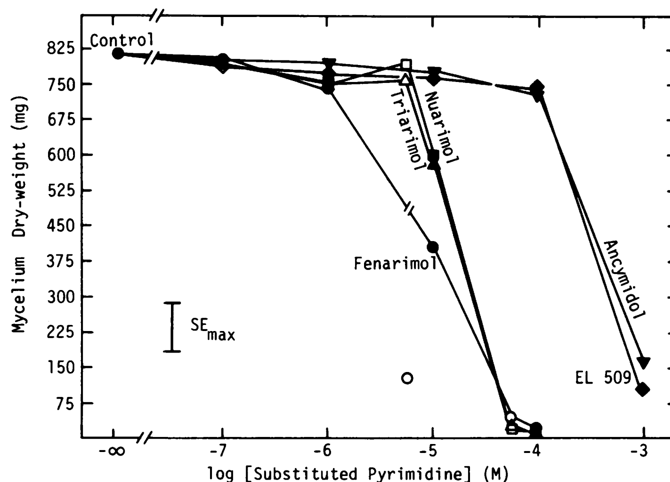

FIG. 1. Effects of substituted pyrimidines on growth of *G. fujikuroi*. Mycelia of 5-d-old cultures grown in the presence of a range of concentrations of five substituted pyrimidines were harvested, dried, and weighed. (○, ●), Fenarimol; (□, ■), nuarimol; (△, ▲), triarimol; (▽, ▼), ancymidol; (◇, ◆), EL 509. Open symbols indicate values from a single experiment; closed symbols represent average values from two or more experiments. The bar indicates the maximum SE for all data points (closed symbols).

fungicides and those analogs commonly known as plant growth regulators.

The GA contents of *G. fujikuroi* culture filtrates were also reduced substantially by all five substituted pyrimidines (Fig. 2A). Data presented in Figure 2 were derived from the same two experiments illustrated in Figure 1. After extraction of the fungus filtrates and partial purification by TLC, the GA contents were estimated using the dwarf pea bioassay. The variation in GA content of the fungus filtrates for any given concentration of inhibitor was quite small in the two experiments. The maximum SE for all data points in Figure 2A was 0.16 in samples averaging 1.17 mg GA_3 equivalents. The standard curves for GA_3 in these bioassays (Fig. 2B) were also reproducible from one experiment to another and were not affected significantly by TLC of the GA_3 samples prior to bioassay. The presence of GA_3 as the primary GA in these extracts was confirmed by GC-MS analysis.

The relationship between GA production and the growth of the mycelium in the presence of different levels on inhibitors was examined by calculating the specific activities (GA_3 equivalents per mg of dry mycelia) from the data of Figures 1 and 2A. The results are plotted in Figure 3. It can be seen that the specific activity of GA production is reduced only in the range of inhibitor concentrations which lead to significant reductions in growth. An interesting, but unexplained, result of these experiments is the apparent stimulation of GA biosynthesis by subinhibitory concentrations of EL 509 and ancymidol (Fig. 2A). Similar apparent stimulation has been observed previously in related experiments. To confirm this result, actively growing cultures of *G. fujikuroi* were incubated with [^{14}C]mevalonic acid, and subsequently extracted and analyzed for GA_3 and some intermediates in the GA biosynthetic pathway. More *ent*-kaurene, GA_3, and another unidentified acidic compound accumulated in the culture filtrates containing 10^{-5} M ancymidol than in its absence (Fig. 4). The identities of *ent*-kaurene and *ent*-kaurenoic acid in these studies were not confirmed, but the GA_3 in the ancymidol-treated sample was identified by recrystallization of constant specific radioactivity in the presence of authentic GA_3. The specific radioactivity declined through three crystallizations to 300 cpm/mg from an initial level of 388 cpm/mg and remained constant through three additional crystallizations.

Tests were performed to determine whether the *ent*-kaurene oxidation system, a known site of inhibition by these substances in higher plants (10), might be involved here as well. The oxidation of *ent*-kaurene in cell-free microsomal preparations from *G. fujikuroi* was examined in the presence of a range of concentrations of four of the inhibitors (Fig. 5). All of them functioned as inhibitors of *ent*-kaurene oxidation in the same general range of concentrations where they inhibited growth and GA production. Although there was some scatter in the data, it appears that the fungicides were effective at a 3- to 10-fold lower concentration than ancymidol, the plant growth regulator.

DISCUSSION

Shive and Sisler (22) compared the effects of ancymidol and triarimol on growth and GA content of bean plants and *G. fujikuroi*. They reported that 2.56 μg/ml (10^{-5} M) triarimol caused a 28% decrease in growth and a 38% decrease in GA-like compounds, while 25.6 μg/ml (10^{-4} M) of ancymidol caused a 7% decrease in growth and a simultaneous 38% decrease in GA-like activity. More recently, Ali *et al.* (1) reported that ancymidol effectively inhibited the growth of five out of nine fungi tested at

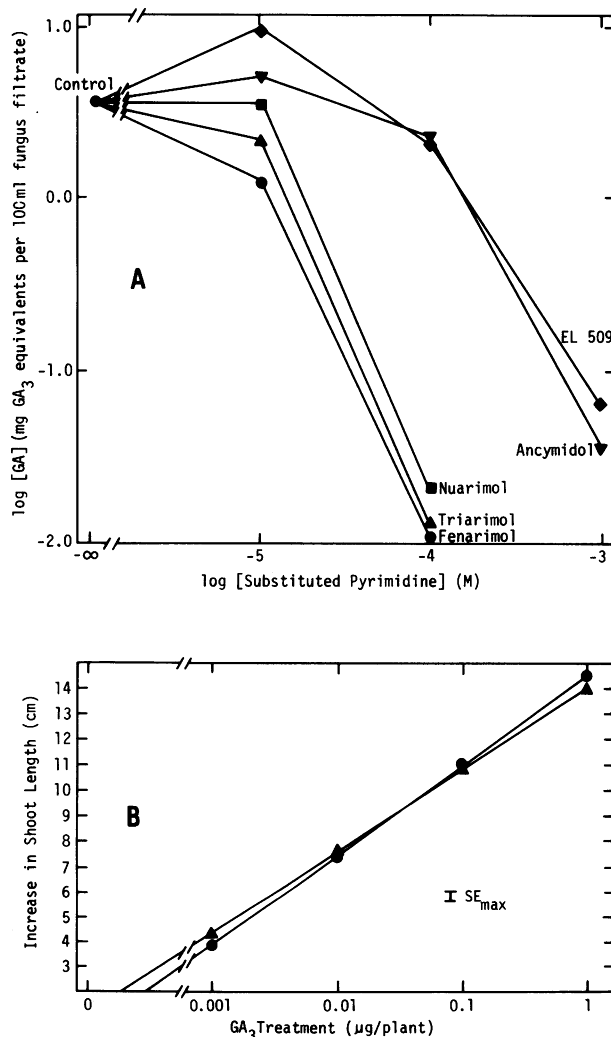

FIG. 2. Effects of substituted pyrimidines on GA biosynthesis of *G. fujikuroi*. The filtrates from cultures as in Fig. 1 were extracted and bioassayed as described in "Materials and Methods." A, GA activity of extracts from fungus filtrates; B, standard curves obtained under two sets of conditions: (▲), standards prepared and used directly in bioassay; (●), standards prepared, chromatographed, and extracted prior to bioassay. Data points in B are mean values from two independent experiments; bar indicates the maximum SE for these points.

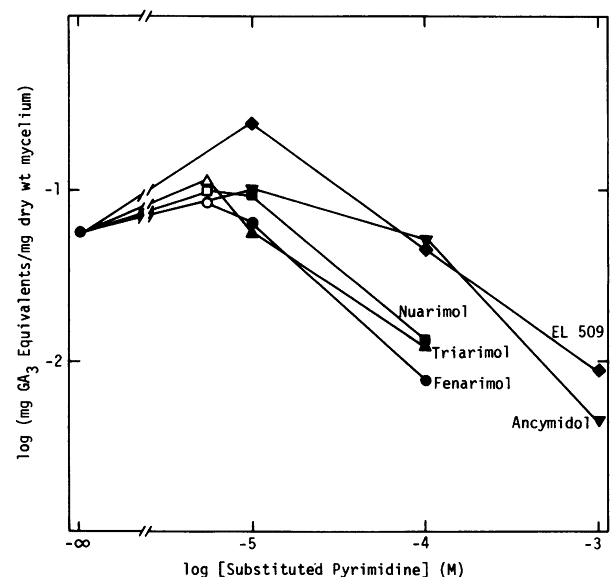

FIG. 3. Effects of substituted pyrimidines on the ratio of GA to dry weight. Data from experiments described in Figs. 1 and 2.

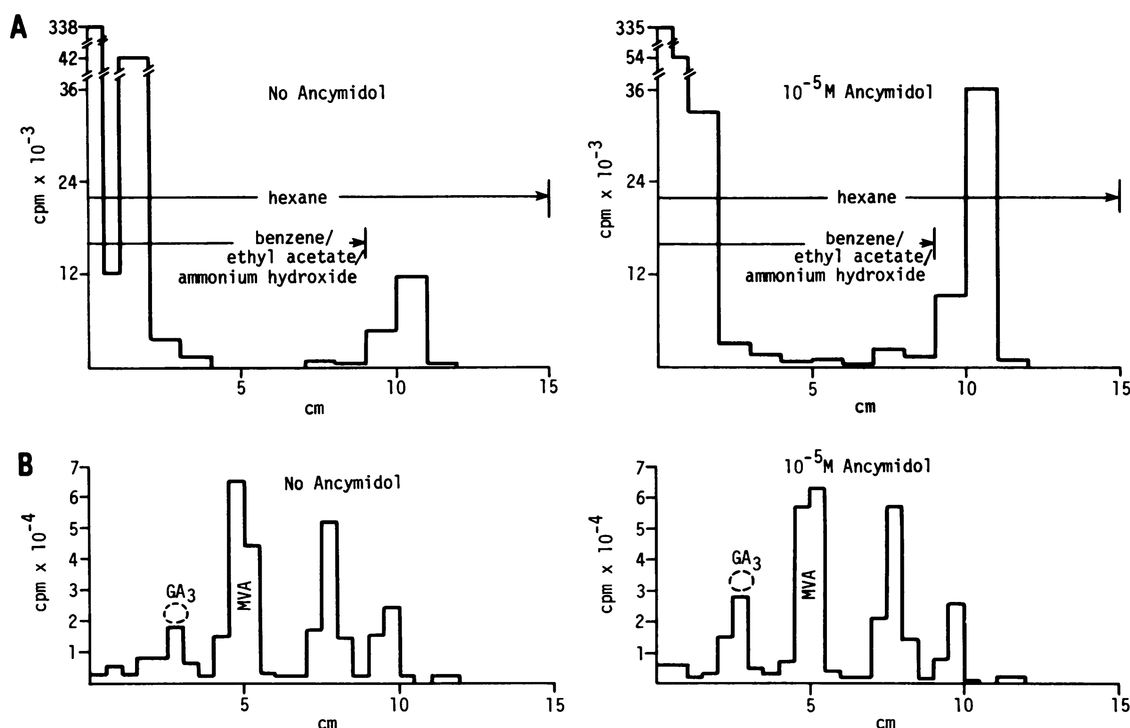

FIG. 4. Histograms of radioactive products of [^{14}C]mevalonate metabolism in cultures of *G. fujikuroi.* Cultures were grown for 44 h in the presence of [^{14}C]mevalonic acid either with or without 10^{-5} M ancymidol. A, Products were extracted and chromatographed 15 cm in hexane and then in benzene: ethyl acetate:NH_4OH (9 cm) to determine early intermediates. *Ent*-kaurene was present at 10 cm; *ent*-kaurenol (5 cm) and *ent*-kaurenal (9 cm) were absent. B, The products from origin (0.5 cm) of chromatograms in A were extracted from gel and rechromatographed in isopropyl ether:acetone:acetic acid (90:30:1) to separate GA_3.

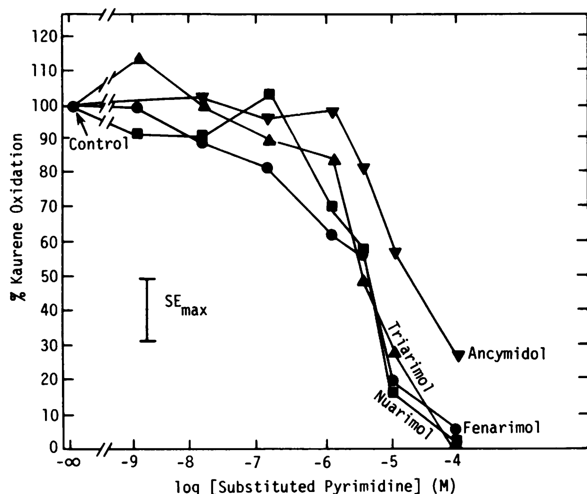

FIG. 5. Effects of substituted pyrimidines on kaurene oxidation in cell-free extracts of *G. fujikuroi.* [^{14}C]*Ent*-kaurene was incubated with microsomes from fungus mycelia in the presence of a range of concentrations of pyrimidines as described under "Materials and Methods." *Ent*-kaurene and its oxidized products were determined by radioassay after TLC separation to calculate the extent of *ent*-kaurene oxidation for each reaction.

concentrations of 20 mg/l (7.8 × 10^{-5} M) or less. Our results confirm and extend the general findings of Shive and Sisler (22). The experimental fungicides were much more effective in inhibiting growth of the fungus than were the compounds recognized as plant growth regulators. As illustrated in Figure 1, the effective concentrations of plant growth regulators were 10 to 20 times greater than those of the fungicides.

The effects of the five substituted pyrimidines on GA biosynthesis were determined in a variety of ways in the present study to avoid possible artifacts due to inhibitor carryover. When the GA content was measured by bioassay, the experimental fungicides reduce the GA-like activity more than the plant growth regulators. Again, the effective concentrations of the two groups of compounds differed by a factor of 10 to 20 (Fig. 2). The results from these bioassays were quite reproducible and independent of the method of GA isolation. The specific activity of GA accumulation (mg recoverable GA_3 equivalents per mg dry weight of mycelium) was slightly higher than the controls at the lowest concentrations of inhibitor tested, but was reduced in comparison with the controls at concentrations of inhibitor which effectively inhibited fungal growth. These results imply that there is an effect of the inhibitors on GA biosynthesis in addition to the general reduction which would be expected from the limitation of fungal growth. The analogs which most effectively inhibited growth of the fungus were also most effective in reducing GA production.

Although the inhibition of GA biosynthesis by the growth retardants is thought to be responsible for the reductions in higher plant growth brought about by these substances, the same explanation is probably not correct for the inhibition of growth of *G. fujikuroi* by the substituted pyrimidines. In the accompanying paper (11), inhibition of growth in peas by 10^{-5} M, but not higher concentrations of inhibitor, was shown to be completely prevented by simultaneous treatment with GA_3. The growth of *G. fujikuroi*, on the other hand, has not been shown to be dependent upon GA. Most other inhibitors of GA biosynthesis either do not significantly affect growth of the fungus (2, 8, 9, 16, 17), or are rapidly metabolized by it (12). In addition, a number of isolates and mutants of *G. fujikuroi* with vastly different capacities for GA production are known, but the rates of growth of these strains appear to be independent of their capacity for GA production. One such example is the mutant strain B1-41a which is blocked

between *ent*-kaurenal and *ent*-kaurenoic acid with less than 3% leakage, but which grows normally (3).

The possibility that triarimol and its analogs might be affecting sterol metabolism in the fungus was not tested in the present study, but it seems likely on the basis of previous reports of this for other fungi (5, 6, 15, 20), barley shoots (6), and rat liver microsomes (13). Staby (23) reported little or no effect of ancymidol on the incorporation of [^{14}C]mevalonic acid into neutral terpenes in cell-free extracts of rat liver, iris, and castor bean, but the ratio of farnesol to cholesterol in rat liver preparations was altered substantially.

Earlier work (10) has shown that ancymidol specifically blocks the oxidation of *ent*-kaurene, *ent*-kaurenol, and *ent*-kaurenal in cell-free extracts of *M. macrocarpus*. More recently Wada has reported the inhibition of GA biosynthesis in *G. fujikuroi* by a group of 17-nor-16-azakaurenes (25), and further demonstrated that 1-*n*-decylimidazole-and 1-geranylimidazole specifically block the oxidation of kaurene and kaurenol in mycelial suspensions of *G. fujikuroi* (26). Conversion of kaurenoic acid to GA was not affected by these substances. The inhibition of kaurene oxidation in cell-free extracts of *G. fujikuroi* by four substituted pyrimidines (Fig. 5) expands the list of substances which can specifically inhibit these oxidative enzymes in the GA biosynthetic pathway of the fungus. It will be of interest to learn if the series of oxadiazolopyrimidines recently described (14) also exert their growth regulatory activity through inhibition of these or similar oxidative reactions.

One of the more interesting aspects of this work is the difference between the structure-activity relationships of these compounds in *G. fujikuroi* and in *M. macrocarpus*. Previous results (10) with ancymidol indicated that its inhibitory action for kaurene oxidative reactions with the microsomes of *M. macrocarpus* endosperm might result from interactions with a Cyt P-450 component. This interaction appeared to be specific in that ancymidol, at concentrations below 10^{-4} M, did not seem to affect the *trans*-cinnamate hydroxylase activity of *Sorghum bicolor* microsomes nor the kaurene oxidative activity of rat liver microsomes. The results presented here suggested the possibility that the experimental fungicides such as triarimol may interact more strongly with the fungal Cyt P-450 and lead to the different specificity of inhibitory action seen in that system. This may also be true for some microsomal mixed function oxygenases of animal tissues. Triarimol inhibits 14-α-demthylase in rat liver microsomes in a manner similar to that shown by CO, suggesting that triarimol may be an effective inhibitor of Cyt P-450 in that tissue (19). Also, dietary concentrations of triarimol in excess of 80 μg/g increase relative liver weights, microsomal protein content, and enzyme activity for metabolism of *p*-nitroanisole in rats (13).

These results and those of others provide evidence that the oxidation of *ent*-kaurene in the fungus occurs via the same route, involving similar enzymes, as in higher plants. The differences in specificity of the two major groups of substituted pyrimidines for the enzymes in higher plants and those of the fungus *G. fujikuroi* suggest, however, that some subtle differences exist in the structural environment of the oxidizing enzymes in higher and lower plants.

The interesting stimulation of GA biosynthesis in the fungus by low inhibitor concentrations (Figs. 2 and 4) remains unexplained. It is not uncommon for a substrate of P-450 to induce the formation of large quantities of the enzyme. One example of such induction by triarimol has already been noted (13). Others were reported recently by Reichhart *et al.* (21). We have observed Cyt P-450 in cell-free extracts of *G. fujikuroi* (R. C. Coolbaugh and C. A. West, unpublished results), but whether or not these inhibitors are inducing the formation of P-450 in the fungus remains to be determined.

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