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Studies on the Specificity and Site of Action of α-Cyclopropyl-α-[p-methoxyphenyl]-5-pyrimidine Methyl Alcohol (Ancymidol), a Plant Growth Regulator[1]

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ABSTRACT

α-Cyclopropyl-α-[p-methoxyphenyl]-5-pyrimidine methyl alcohol (ancymidol) is an inhibitor of *ent*-kaur-16-ene oxidation in microsomal preparations from the liquid endosperm of immature *Marah macrocarpus* seeds. The K_i for this inhibitor is about 2×10^{-9} M. Ancymidol also blocks *ent*-kaur-16-en-19-ol and *ent*-kaur-16-en-19-al oxidation by the same preparations with a similar efficiency, but does not significantly inhibit *ent*-kaur-16-en-19-oic acid oxidation. Ancymidol appears to be specific for this series of oxidations in higher plant tissues. It does not inhibit the oxidation of kaurene nor kaurenoic acid in rat liver microsomes and has no significant effect on the oxidation of cinnamic acid in microsomal preparations from *Sorghum bicolor* seedlings. Ancymidol also does not inhibit kaurene oxidation *in vitro* nor *in vivo* in cultures of the fungus *Fusarium moniliforme*. The presence of ancymidol did not significantly alter the activities of NADPH-cytochrome *c* reductase, NADH-cytochrome *c* reductase, or NADH-cytochrome b_5 reductase. The addition of ancymidol to suspensions of oxidized *M. macrocarpus* endosperm led to a difference spectrum with an absorption maximum at 427 nm and a minimum at 410 nm.

Ancymidol[4] is a substituted pyrimidine with potent growth regulatory activity in higher plants (1, 10, 19, 22, 23). The inhibition of normal growth by ancymidol can be overcome by applications of gibberellic acid (GA_3) (1, 10, 19, 23). Recently presented evidence (1) indicates that one mode of action of ancymidol is to inhibit the oxidation of kaurene to kaurenol in extracts of *Marah oreganus*. This reaction is the first of a series of oxidations catalyzed by microsomal mixed function oxidases in the biosynthetic pathway of the gibberellins. Although a number of growth regulators have been shown to inhibit reactions in this pathway, ancymidol appears to be unique among them in specifically inhibiting an oxidative reaction. It was therefore of interest to test other oxidative reactions to determine if ancymidol is a general mixed function oxidase inhibitor, or if it has some degree of specificity for the reactions of this pathway.

This paper describes the effects of ancymidol on the oxidation of kaurene and some of its closely related metabolic derivatives in microsomal preparations from immature seed of *Marah macrocarpus*, the fungus *Fusarium moniliforme*, and rat liver. The effects of ancymidol on the hydroxylation of cinnamic acid in microsomal preparations from sorghum seedlings are also reported. In addition, some evidence is presented which indicates that ancymidol interacts directly with Cyt P-450.

MATERIALS AND METHODS

Enzyme Sources. Immature fruits of *M. macrocarpus* (Greene) Greene, wild cucumber, were collected in the spring of 1976 in the Santa Monica Mountains. Immature seeds were removed, rinsed, and stored at −20 C until used. Liquid endosperm was removed from the seeds, ground in a Teflon to glass homogenizer (Thomas), and centrifuged 15 min at 10,000*g*. The resulting supernatant was centrifuged 90 min at 150,000*g* in a Beckman model Ti-60 rotor. The 150,000*g* pellet was then resuspended in 0.1 M Tris-HCl buffer (pH 7.5) containing 25% (v/v) glycerol. The soluble and microsomal preparations were used either directly as an enzyme source or frozen and stored in liquid N_2 for future use.

Sorghum bicolor (Northrup King) seeds were disinfected with 1% (v/v) NaOCl for 1 hr, thoroughly rinsed, and germinated in moist Vermiculite in the dark. The top 2 cm were harvested on the 5th day after planting and used to prepare microsomes. Approximately 20 g of leaf tissue were ground in a chilled mortar and pestle with 2 volumes of 0.15 M K-phosphate (pH 8) containing 0.35 M NaCl and 1 g of insoluble PVP (Polyclar AT). The homogenate was filtered through Miracloth and centrifuged as above for *M. macrocarpus* microsomes. The microsomal pellets were resuspended in 4 ml of 75 mM Tricine (pH 8), frozen, and stored in liquid N_2 until used.

F. moniliforme (ACC 917, M419) stock cultures were maintained on potato dextrose agar (20). The liquid culture medium was that used by Nakata (14) containing glucose (80 g), KH_2PO_4 (2.0 g), $MgSO_4$ (0.5 g), NH_4NO_3 (1.0 g), and trace elements (2.5 ml) made up to a total volume of 1 liter with glass-distilled H_2O. The trace elements solution contained in 100 ml: $FeSO_4 \cdot 7\ H_2O$ (0.1 g), $CuSO_4 \cdot 5\ H_2O$ (0.015 g), $MnSO_4 \cdot H_2O$ (0.01 g), $ZnSO_4 \cdot H_2O$ (0.16 g), and ammonium molybdate (0.01 g). Inocula were prepared by transfer of a small portion of the fungal culture from the slant into 100 ml of sterile culture medium in 250-ml flasks which were incubated on an orbit shaker at 21 C under approximately 500 ft-c of light for 4 days. Ten ml of cell suspension were transferred to 1 liter of fresh medium in a 2-liter flask. Growth was permitted to proceed for 3 to 5 days under the same conditions until the

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[4] Kaurene, kaurenol, kaurenal, kaurenoic acid and 7β-hydroxykaurenoic acid will be used to refer to *ent*-kaur-16-ene, *ent*-kaur-16-en-19-ol, *ent*-kaur-16-en-19-oic acid, and *ent*-kaur-16-en-7α-ol-19-oic acid, respectively. Ancymidol (also referred to in the literature as experimental compound EL 531 or the commercial preparation A-REST) is a trivial name for α-cyclopropyl-α-[p-methoxyphenyl]-5-pyrimidine methyl alcohol; MVA: mevalonic acid.

medium became pink. The mycelia were harvested by filtration, rinsed twice with 500 ml of H_2O and twice with 500 ml of 0.1 M Tricine (pH 8). The washed cells were frozen in liquid N_2 and stored prior to preparation of microsomes. Microsomal fractions were prepared as described previously (25).

The liver from a single Sprague-Dawley rat was minced and homogenized in 40 ml of 0.1 M Tris-HCl buffer (pH 8). The homogenate was centrifuged 15 min at 10,000*g* and the resulting supernatant was recentrifuged at 100,000*g* for 1 hr to yield the microsomal pellet (P_{100}). This was resuspended in 150 ml 0.1 M K-phosphate (pH 7.1) and frozen in liquid N_2 until used.

Preparation of Substrates. [^{14}C]Kaurene was prepared biosynthetically from DL-[2-^{14}C]mevalonic acid (MVA) in large scale incubations with soluble (S_{150}) enzymes from the liquid endosperm of *M. macrocarpus* seeds. Reaction mixtures routinely contained 25 μM [2-^{14}C]MVA (8.05 or 10.9 mCi/mmol), 40 ml of S_{150} (2–3 mg of protein/ml), 2 mM $MgCl_2$, 2 mM $MnCl_2$, 2 mM ATP, and 0.1 M K-phosphate (pH 7.1) in a total volume of 75 ml. Incubations were conducted under a N_2 atmosphere at 30 C for 8 to 10 hr. Reaction mixtures were extracted with benzene-acetone (3:1). [^{14}C]Kaurene was isolated by TLC in hexane on precoated layers (2 mm) of Silica Gel G and further purified by elution from a silicic acid column (1 × 10 cm) with hexane.

[^{3}H]Kaurene was prepared in the same way with [5-^{3}H]MVA (19.92 mCi/mmol) as substrate.

[^{3}H]Kaurenol, [^{3}H]kaurenal, and [^{3}H]kaurenoic acid were prepared biosynthetically by enzymic oxidation of [^{3}H]kaurene (79.68 mCi/mmol) in reaction mixtures containing microsomal preparations of *M. macrocarpus* liquid endosperm. The reaction conditions are described below except that variable incubation times were used for substrate preparations. In these experiments it was noted that seeds stored 8 to 10 months at −20 C routinely gave higher yields of kaurenol and kaurenal than seeds stored for shorter periods of time. The latter gave high yields of more highly oxidized products including kaurenoic acid. Radioactive substrates were extracted from reaction mixtures with benzene-acetone (3:1) and purified by TLC in benzene-ethyl acetate-ammonium hydroxide (90:10:0.05).

In some experiments (noted in the text) a separate sample of [^{14}C]kaurenoic acid (8.91 mCi/mmol) was used. This material was prepared chemically using [^{14}C]methyl iodide and the 17-monomethyl ester of kauran-17,19-dioic acid. The procedures for this partial synthesis of kaurenoic acid will be described elsewhere.

Oxidative Activities in *M. macrocarpus* Microsomes. Kaurene oxidation was assayed in reaction mixtures containing 0.3 μM [^{14}C]kaurene (32.2 mCi/mmol), 5×10^{-5} M FAD, 5×10^{-4} M NADPH, 75 mM Tris-HCl (pH 7.5), and 50 μl of microsomal enzyme suspension (about 100 μg protein) in a total volume of 1 ml. Kaurene oxidation was linear with enzyme concentration through 200 μg/ml. Reactions were incubated 10 min at 30 C in 20-ml scintillation vials on an orbit shaker. Kaurene oxidation was linear with time under these conditions through 20 min. Reactions were stopped by adding 1 ml of acetone and extracted with 2 ml of benzene followed by two extractions (1 ml each) with benzene-acetone (3:1). The organic extract was then concentrated to about 100 μl under a stream of N_2, transferred to the origin of a precoated thin layer plate (2 × 20 cm) with the aid of two additional rinses (100 μl each) of acetone, and chromatographed 15 cm in hexane. The unreacted substrate (10–15 cm) and the oxidized products (origin) were then scraped from the plates and counted by liquid scintillation spectrometry. The per cent oxidation was determined by dividing the cpm in oxidized products by the total number of cpm recovered. Recovery of radioactivity was routinely 90 to 95%.

Kaurenol and kaurenal oxidations were assayed by identical procedures except that the substrate was separated from its products by chromatography in benzene-ethyl acetate-ammonium hydroxide (90:10:0.05).

When kaurenoic acid served as substrate, reaction mixtures were acidified to pH 3 after the benzene-acetone extraction and further extracted twice (1 ml each) with ethyl acetate. In these experiments kaurenoic acid was separated from its products in a solvent system containing benzene-ethanol-7.2 N NH_3 (65:34:1).

Cinnamic Acid Oxidation in Cell-free Extracts of Sorghum Leaves. [^{14}C]Cinnamic acid hydroxylation was measured in microsomal preparations of sorghum leaves essentially by the methods of Potts *et al.* (17). Reaction mixtures contained 50 mM Tricine (pH 8), 1 mM NADPH, 2 mM DL-isocitric acid, 2 mM $MnCl_2$, 0.6 units (μmol min^{-1}) isocitrate dehydrogenase, 40 μM [2-^{14}C]cinnamic acid (4.0 mCi/mmol), and 20 μl of microsomal suspension (110 μg of protein). They were incubated for 10 min at 30 C on an orbit shaker. Reaction rates under these conditions were linear with enzyme content through 100 μl (550 μg of protein). Reactions were stopped by addition of 1 ml of 1% (v/v) HCl in ethanol. *p*-Coumaric acid (50 μg) was added to each reaction and mixtures were extracted twice with 2 ml and once with 1 ml of benzene-acetone (3:1). The organic extract was concentrated under a stream of N_2 to about 100 μl and chromatographed 17 cm in isopropyl ether-benzene-acetic acid (7:2.5:0.5). The coumaric acid was located by radiochromatogram scanning and with iodine vapors and scraped for liquid scintillation counting.

Kaurene and Kaurenoic Acid Oxidation by Rat Liver Enzymes. Kaurene and kaurenoic acid oxidation were assayed in rat liver microsomal preparations essentially by the methods of Murphy and West (13). Reaction mixtures contained 100 μl of microsomal suspension (300 μg of protein), 75 mM Tris-HCl (pH 7.5), 5×10^{-4} M NADPH, 5×10^{-5} M flavin adenine dinucleotide, and either 0.31 μM [^{14}C]kaurene (43.6 mCi/mmol) or 1.0 μM [^{14}C]kaurenoic acid (8.91 mCi/mmol). Reactions were incubated 10 min at 30 C on an orbit shaker and stopped by addition of 1 ml of acetone. Under these conditions reaction rates were linear with enzyme content through at least 200 μl (600 μg of protein). Extractions were the same as for *M. macrocarpus* enzyme extracts. [^{14}C]Kaurene was separated from its products by chromatography in hexane, while products from [^{14}C]kaurenoic acid metabolism were separated by TLC with isopropyl ether-acetic acid (95:5) as the solvent system. The silica gel-containing substrates and products were removed separately and counted by liquid scintillation.

Kaurene and Kaurenoic Acid Oxidation in *Fusarium* Microsomes. Kaurene oxidation was assayed in reaction mixtures containing 20 μl of microsomal suspension (74 μg of protein), 0.31 μM [^{14}C]kaurene (43.6 mCi/mmol), 5×10^{-4} M NADPH, 5×10^{-5} M flavin adenine dinucleotide, and 75 mM Tricine (pH 7.5) in a total volume of 1 ml. Reaction rates were linear with time under these conditions for at least 60 min, but were stopped and extracted as for *M. macrocarpus* enzyme extracts after 45 min. The rates were also linear through 30 μl of enzyme preparation.

Kaurenoic acid oxidation was determined in the same manner as kaurene oxidation except that 10 μl of enzyme extract (43 μg of protein), 1.0 μM [^{14}C]kaurenoic acid (8.91 mCi/mmol), and 75 mM Tricine (pH 8) were used. Product extraction and determination were as described above for *M. macrocarpus* enzyme systems.

Reductase Activities in Microsomes of *M. macrocarpus*. NADPH-Cyt *c* reductase (12), NADH-Cyt *c* reductase (12), and NADH-Cyt b_5 reductase (21) were determined spectrophotometrically as described previously (7). Millimolar extinction coefficients of 21.1 cm^{-1} mM^{-1} at 550 nm for Cyt *c* (24) and 1.02 cm^{-1} mM^{-1} at 420 nm for potassium ferricyanide (18) were used.

Spectral Measurements of Cyt P-450. In contrast to the enzyme assays which were determined with dilute enzyme preparations (10–100 μg of protein/ml), the hemoprotein contents were measured in dense suspensions containing 1 to 2 mg of protein/ml. Cyt P-450 was measured by the methods of Omura and Sato (15) by determining the ΔA between 450 nm and 490 nm in a carbon monoxide difference spectrum. An extinction coefficient of 91 cm^{-1} mM^{-1} was used.

Cyt P-450-binding spectra were obtained with microsomal preparations which were recentrifuged at 10,000*g* for 10 min to remove large particles and the resulting supernatant fraction was diluted with an equal volume of 0.1 M Tris-HCl (pH 7.5) containing 25% (v/v) glycerol. Sample and reference cuvettes were filled with 1 ml each of the microsomes, a base line was established with oxidized minus oxidized enzymes. Ten μl of 10^{-3} M or 10^{-4} M ancymidol was added to the sample cuvette, and the difference spectra were recorded on a Cary model 15 spectrophotometer.

Protein Determinations. The protein contents of enzyme extracts were determined by the method of Lowry *et al.* (11) using BSA as the reference standard. Fifty μl of 0.2% (v/v) Triton X-100 in 0.2 N NaOH was added to each sample (including standards) 2 hr prior to assay.

Radioactivity Determinations. Thin layer chromatograms were routinely scanned on a Packard radiochromatogram scanner, model 7201, to confirm the coincidence of radioactivity with the visualized authentic compounds. The gel corresponding to the position of the visualized standards and/or the peak of radioactivity was then scraped and the samples were counted by liquid scintillation in a Packard liquid scintillation spectrometer.

Reagents. DL-[2-^{14}C]MVA lactone (8.05 and 10.9 mCi/mmol) were purchased from Amersham/Searle and hydrolyzed in 5 mM KOH. DL-[5-^{3}H]MVA (DBED Salt, 6.25 Ci/mmol) was purchased from New England Nuclear and diluted with nonradioactive MVA to 19.92 mCi/mmol. [2-^{14}C]Cinnamic acid (4 mCi/mmol) was purchased from ICN. Omnifluor and [^{14}C]toluene were obtained from New England Nuclear. NADPH, NADH, flavin adenine dinucleotide, and *p*-coumaric acid were purchased from Sigma Chemical. Ancymidol (technical) was a gift of Eli Lilly and Co.

RESULTS

Inhibition of Kaurene Oxidation by Ancymidol. The inhibition of kaurene oxidation in resuspended microsomes from immature *M. macrocarpus* seeds by several concentrations of ancymidol is represented in Figure 1. This result confirms the earlier evidence obtained from the more crude system from *M. oreganus* seeds that ancymidol is active at very low concentrations. The effect of substrate concentration on the rate of kaurene oxidation at several ancymidol concentrations was determined; a double reciprocal Lineweaver-Burk plot of these data is shown in Figure 2A. From this plot it can be estimated that the apparent K_m for kaurene in this system is about 0.5 μM, which agrees well with previous determinations (9). In addition, the decrease in the V_{max} with increasing concentrations of ancymidol seems to rule out a competitive type of inhibition. A replot of the *y* intercepts from Figure 2A as a function of ancymidol concentration (Fig. 2B) gives an estimated K_i for ancymidol of about 2×10^{-9} M.

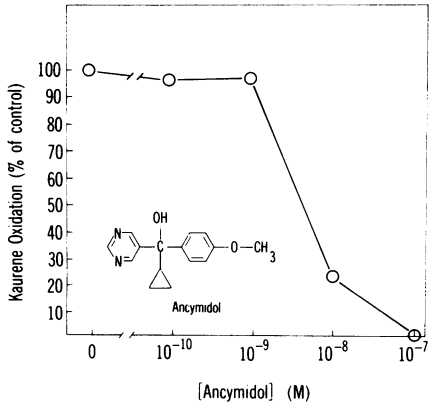

FIG. 1. Effect of ancymidol on kaurene oxidation in extracts of *M. macrocarpus*. Reaction mixtures contained 0.3 μM [^{14}C]kaurene, 5×10^{-5} M FAD, 5×10^{-4} M NADPH, 75 mM Tris-HCl at pH 7.5, and 50 μl of microsomal enzyme suspension (100 μg of protein) in a total volume of 1 ml. The control activity (no ancymidol) corresponds to 21% oxidation of the substrate, or the formation of 63 pmol of oxidized products during the 10-min incubation.

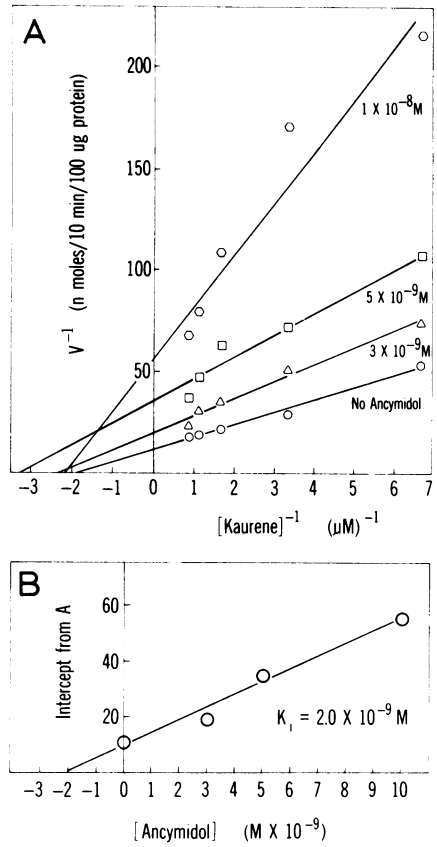

FIG. 2. Effect of kaurene concentration on inhibition of kaurene oxidation by ancymidol. Reaction mixtures were as described in Figure 1 except for the variable substrate concentration. A: Lineweaver-Burk plot of data; B: re-plot of $1/v$ intercepts from (A) against ancymidol concentration.

Specificity of Ancymidol. Kaurene oxidation is only one of several reactions in the gibberellin biosynthetic pathway known to involve Cyt P-450 (13). An initial test of the specificity was to compare the effects of this inhibitor on the oxidations of kaurene, kaurenol, kaurenal, and kaurenoic acid in *M. macrocarpus* microsomes. The results of the extents of oxidation of these substrates over a range of ancymidol concentrations (Fig. 3) indicate that ancymidol has approximately the same inhibitory activity for the oxidations of kaurene, kaurenol, and kaurenal. Kaurenoic acid oxidation exhibited a different inhibition pattern from the others in the presence of ancymidol. Thus, there appears to be some specificity with regard to the reactions in this pathway which ancymidol affects.

It has been previously demonstrated that rat liver microsomes will also oxidize kaurene and kaurenoic acid (13). The oxidation products in this case are not intermediates in gibberellin biosynthesis, but rather degradation products which probably result from general detoxification systems in the liver. Rat liver microsomes seemed to be a good system in which to test the generality of ancymidol action as an inhibitor of transformations of kaurene and its derivatives by mixed function oxidases. When kaurene and kaurenoic acid oxidation were measured in microsomal preparations from rat liver in the presence of a wide range of concentrations of ancymidol, little, if any, effect was observed (Table I).

To test further the specificity of this inhibitor, ancymidol was

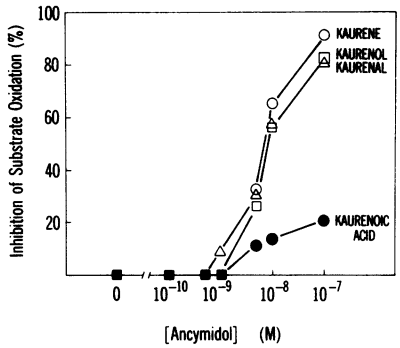

FIG. 3. Comparison of effects of ancymidol on oxidation of [^{14}C]kaurene, [^{3}H]kaurenol, [^{3}H]kaurenal, and [^{3}H]kaurenoic acid. Reaction conditions were the same as in Figure 1. All substrates were present at 0.3 μM. In the absence of inhibitor the following levels of substrate conversions were observed: kaurene, 30%; kaurenol, 71%; kaurenal, 75%; and kaurenoic acid, 30%.

Table I

EFFECT OF ANCYMIDOL ON KAURENE AND KAURENOIC ACID OXIDATION IN MICROSOMAL PREPARATIONS FROM RAT LIVER

[Ancymidol] (M)	Kaurene Oxidation (%)	Kaurenoic Acid Oxidation (%)
0	9.4[a]	20.6[b]
10^{-9}	7.4	23.1
10^{-8}	9.1	22.9
10^{-7}	9.0	22.4
10^{-6}	8.5	26.0
10^{-5}	7.4	23.0
10^{-4}	7.3	17.9

[a]9.4% kaurene oxidation in these reactions is equivalent to the formation of 29.4 pmol of oxidized products during a 10-min incubation.

[b]20.6% kaurenoic acid oxidation corresponds to the formation of 206 pmol of oxidized products during a 10-min incubation.

Table II

EFFECT OF ANCYMIDOL ON CINNAMIC ACID HYDROXYLASE IN MICROSOMAL PREPARATIONS OF SORGHUM SEEDLINGS

[Ancymidol] (M)	p-Coumaric Acid Formed (nmoles)
0	0.71
10^{-9}	0.65
10^{-8}	0.57
10^{-7}	0.69
10^{-6}	0.58
10^{-5}	0.63
10^{-4}	0.56

incubated with microsomal preparations of etiolated sorghum leaves and [^{14}C]cinnamic acid. No inhibition of the conversion of cinnamic acid to coumaric acid was observed (Table II). Thus, ancymidol appears not even to be a general mixed function oxidase inhibitor in plants.

As a final test of the specificity of ancymidol, its effects on gibberellin biosynthesis in *F. moniliforme* were determined. Growing cultures of *F. moniliforme* in buffered nutrient solutions were incubated with [^{14}C]MVA in the presence and absence of 10^{-5} M ancymidol. Gibberellins and intermediates in the gibberellin biosynthetic pathway were then extracted from the culture filtrates and the extracts were chromatographed along with authentic reference standards in a thin layer system. Analysis of the distribution of radioactive products on the chromatograms indicated that ancymidol did not inhibit the production of GA_3, kaurene, or kaurenoic acid. If anything, there was a slight stimulation of the biosynthesis of these products (data not shown).

In order to obtain direct evidence for the effect (or lack of effect) of ancymidol on enzymes from this fungus, microsomal suspensions from the fungus mycelium were prepared and incubated with [^{14}C]kaurene and [^{14}C]kaurenoic acid under conditions which promoted the oxidation of these substrates. The results (Table III) indicate that ancymidol does not inhibit these reactions, in contrast to its effect on the enzymes catalyzing the same reactions from *M. macrocarpus*. The only significant inhibition observed in several experiments was that of kaurene oxidation with 10^{-4} M ancymidol, a concentration 10,000 times greater than that required to cause a similar degree of inhibition in the enzyme system from *M. macrocarpus*.

Studies on Site of Action of Ancymidol. Estabrook *et al.* (4) have presented evidence that the mixed function oxidases for steroids, drugs, and other substances in mammalian hepatic microsomes involve several electron transfer components in addition to Cyt P-450 itself including two flavoproteins. A similar complex appears to be functioning in the microsomes of *M. macrocarpus* (6, 7). To determine if ancymidol acts by inhibiting the transport systems, three reactions were tested using artificial electron acceptors. The results (Table IV) indicate that ancymidol has no significant effect on NADPH-Cyt *c* reductase, NADPH-Cyt b_5 reductase, or NADH-Cyt *c* reductase. It therefore does not exert its effect by acting on those portions of the electron transport systems tested.

Since it is known that many substrates and inhibitors of mixed function oxidase-catalyzed reactions involving Cyt P-450 bind to that component to give a difference spectrum, it was of interest to see if ancymidol would cause such a spectral change. Microsomal suspensions from *M. macrocarpus* seeds were divided in half and

Table III

EFFECT OF ANCYMIDOL ON KAURENE AND KAURENOIC ACID OXIDATION IN MICROSOMAL PREPARATIONS FROM FUSARIUM MONILIFORME

[Ancymidol] (M)	Kaurene Oxidation (%)	Kaurenoic Acid Oxidation (%)
0	29.4[a]	67.3[b]
10^{-9}	28.5	72.1
10^{-8}	20.5	65.4
10^{-7}	25.7	64.2
10^{-6}	26.2	73.1
10^{-5}	24.9	70.5
10^{-4}	11.0	69.2

[a]29.4% oxidation in these reactions corresponds to the formation of 91.1 pmol of oxidized products during a 45-min incubation.

[b]67.3% oxidation in these reactions is equivalent to the formation of 796 pmol of oxidized products during a 10-min incubation.

Table IV

EFFECT OF ANCYMIDOL ON ELECTRON TRANSFER COMPONENTS IN MICROSOMAL PREPARATIONS OF MARAH MACROCARPUS ENDOSPERM

[Ancymidol] (M)	NADPH-Cyt c Reductase (nmoles/min)[a]	NADH-Cyt c Reductase (nmoles/min)[a]	NADH-Cyt b_5 Reductase (nmoles/min)[b]
0	2.27	10.19	45.10
10^{-9}	2.61	11.04	44.12
10^{-8}	2.32	10.24	39.22
10^{-7}	2.80	10.62	44.12
10^{-6}	2.27	9.48	41.18
10^{-5}	2.80	10.20	42.16
10^{-4}	2.61	10.81	44.12

[a]nmoles cytochrome c reduce/min

[b]nmoles ferricyanide reduced/min

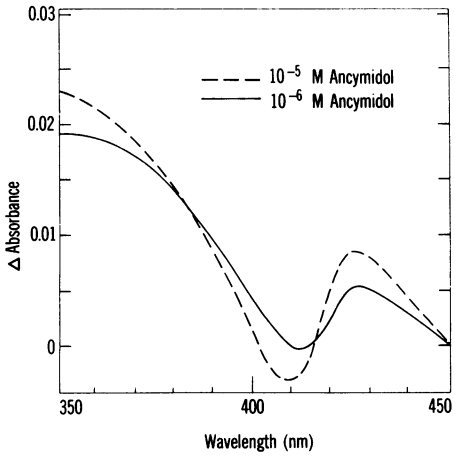

FIG. 4. Interaction of ancymidol with Cyt P-450 in microsomal extracts of *M. macrocarpus*. A base line was established with oxidized enzymes in both sample and reference cuvettes. Then ancymidol was added to the sample cuvette and the difference spectrum was recorded.

placed in two cuvettes in a Cary model 15 dual beam spectrophotometer. Ancymidol was added to the sample cuvette (final concentration of 10^{-6} or 10^{-5} M) and the difference spectrum was recorded. As shown in Figure 4, ancymidol does interact with Cyt P-450 to produce an absorption peak at about 427 nm.

DISCUSSION

Inhibition of Kaurene Oxidation by Ancymidol. The biosynthetic pathway of the gibberellins in *M. macrocarpus* seeds from MVA to GA_{12} can be divided generally into two main parts. The incorporation of MVA into kaurene is catalyzed by soluble enzymes contained in the 150,000*g* supernatant fraction and does not require O_2, while the enzymes involved in the subsequent oxidative steps involved in the conversion of kaurene to GA_{12} are localized in the 150,000*g* pellet, *i.e.* microsomal fraction, and do require O_2 (25). Ancymidol has little or no effect on the biosynthesis of kaurene from MVA at concentrations up to 10^{-4} M in extracts of pea shoot tips and seeds of *M. oreganus* (1). It does, however, strongly inhibit this reaction sequence (68%) at 10^{-3} M.

Previous studies have demonstrated that ancymidol blocks the oxidation of kaurene to kaurenol in cell-free extracts of one species of wild cucumber (*M. oreganus*) (1). In the present study, this result was confirmed with microsomal preparations from *M. macrocarpus*. The lower sensitivity to ancymidol concentration seen in the earlier work is probably due to differences in the type of enzyme preparation and incubation conditions used. In the earlier experiments a crude 10,000*g* supernatant fraction containing microsomes was employed without supplementation other than with substrate. In the present experiments (Figs. 1 and 2) the data were obtained with resuspended 150,000*g* microsomal pellets in the presence of optimal levels of NADPH, flavin adenine dinucleotide, and substrate.

Kinetic data on the inhibition of kaurene oxidation with variable substrate and inhibitor concentrations (Fig. 2) were not consistent with a competitive inhibition. Although some difficulties were experienced in obtaining reliable reaction rates at high substrate concentrations, the data seem to fit a noncompetitive pattern of inhibition most closely.

From the data (Fig. 2) it can also be seen that the K_i of ancymidol for kaurene oxidation is about 2×10^{-9} M. Clearly, this is the most potent inhibitor of this pathway described to date. By comparison, AMO 1618, carvadan, and phosphon, which inhibit kaurene biosynthesis, have K_i values in the range of 10^{-7} to 10^{-6} M (2, 5). A compound which inhibits oxidative reactions in this pathway, SKF 525A, has a K_i value of approximately 10^{-4} M (3, 9).

Specificity of Ancymidol. The reactions subsequent to kaurene oxidation in the gibberellin biosynthetic pathway involve the further oxidation of the alcohol at the 16 position to the aldehyde and the carboxylic acid prior to the oxidation of the carbon at the 7 position. Evidence accumulated to date is consistent with the hypothesis that each of these reactions is catalyzed by a microsomal mixed function oxidase involving Cyt P-450 (3, 14). It was therefore of interest to determine the effects of ancymidol on these reactions. The effect of this inhibitor on the oxidations of kaurene, kaurenol, and kaurenal in microsomal preparations of *M. macrocarpus* were similar (Fig. 3); the K_i values of ancymidol for these oxidations from several determinations were all of the same order of magnitude. The site of kaurenoic acid oxidation, however, was quite distinct in its behavior toward ancymidol. In other studies by Hirano (9) based on the quantitative aspects of the utilization of kaurene, kaurenol, kaurenal, and kaurenoic acid as both substrates, and inhibitors of the utilization of the other metabolites as substrates, it was concluded that there must be separate binding sites for each of these four substrates.

The results with kaurenoic acid seem to be anomalous. In four experiments, 20% inhibition of kaurenoic acid oxidation was the maximum effect observed, even at concentrations of ancymidol up to 10^{-5} M. The effect of kaurene as an inhibitor of kaurenoic acid oxidation was quite similar (9). Since this partial inhibition of kaurenoic acid oxidation by ancymidol was not observed in all experiments, its cause and its significance remain uncertain and will require further investigation. It is clear that kaurenoic acid oxidation behaves differently from the other oxidations tested in the presence of ancymidol. Ancymidol is specific with regard to the reactions it affects in the *M. macrocarpus* system.

Hepatic microsomes have been studied extensively with regard to their drug-metabolizing capabilities (4, 8, 16). The hepatic microsomal Cyt P-450 system is unusual in that it appears to have very broad substrate specificity. Murphy and West (13) have shown that microsomes from rat liver will also oxidize kaurene, kaurenol, and kaurenoic acid. The major product of kaurenoic acid metabolism was 16,17-dihydroxykaurenoic acid. Kaurenol was also dihydroxylated at the exocyclic double bond. Although the products of kaurene oxidation were not carefully characterized, it is expected that it, too, is oxidized in the same positions. In the present study, ancymidol at concentrations up to 10^{-4} M had very little, if any, effect on kaurenoic acid oxidation by rat liver microsomes (Table I). There was a small degree of inhibition at 10^{-4} M for kaurenoic acid oxidation, but no apparent effect on the kaurene oxidation. Ancymidol does not appear to be a general mixed function oxidase inhibitor at low concentrations. Staby (22) has reported that ancymidol affects sterol biosynthesis in rat liver microsomes at 5×10^{-4} M. However, in that study it had the unexpected effect of increasing the ratio of cholesterol to farnesol and the actual levels of cholesterol formed from MVA.

To test whether ancymidol might generally inhibit plant oxidases, the hydroxylation of cinnamic acid in *Sorghum* leaves was selected. This reaction has been studied in some detail (17). It requires O_2 and NADPH and has been shown to involve Cyt P-450. This reaction was unaffected by concentrations of ancymidol up to 10^{-4} M (Table II). This result adds further to the observed specificity of this substituted pyrimidine, indicating that it may be specific for three reactions in a single biosynthetic pathway.

The same gibberellin biosynthetic pathway is thought to exist in the fungus *F. moniliforme*, at least from MVA through the products investigated in this work. It was, therefore, of interest to determine whether ancymidol would have a similar effect in this organism. Ancymidol has been reported not to inhibit gibberellin biosynthesis in the fungus (10). In initial experiments, gibberellin biosynthesis was monitored *in vivo* in actively growing cultures of *F. moniliforme* by adding [^{14}C]MVA and ancymidol simultaneously. [^{14}C]GA_3 was produced in approximately equal amounts in the presence and absence of 10^{-5} M ancymidol.

Further confirmation of the absence of inhibition of gibberellin production in the fungus by ancymidol came from studies on cell-free enzyme preparations. Ancymidol had no significant effect on kaurenoic acid oxidation at the concentrations tested, and blocked kaurene oxidation significantly only at the high concentration of 10^{-4} M. It is noteworthy that the apparent K_i for kaurene oxidase in the fungus is 10,000 times that for the same enzyme in the *M. macrocarpus* microsomes. There appears to be a rather great specificity of ancymidol for three reactions in the higher plant system.

Studies on Site of Action of Ancymidol. A different spectrum was observed between ancymidol mixed with oxidized *M. macrocarpus* microsomes and the oxidized microsomes themselves. The maximum at 427 nm and minimum at 410 nm are reminiscent of the type II difference spectra observed in liver and bacterial Cyt P-450 preparations which have interacted with amines and certain other oxidase substrates and inhibitors (16, 26), although the minima in typical type II spectra are seen at about 395 nm. Orrenius *et al.* (16) have summarized the evidence that such spectral changes reflect the interaction of the supplied substances with Cyt P-450. We conclude that the difference spectrum seen in the present instance is the result of an interaction of ancymidol with the microsomal Cyt P-450.

It is difficult to correlate the quantitative aspects of the ancymidol-binding spectra and the effects of ancymidol as an inhibitor of the oxidase reactions. In order to obtain measurable binding spectra, it was necessary to employ relatively dense microsomal suspensions (about 3 mg of protein/ml), whereas dilute suspensions of microsomes (100–200 μg of protein/ml) were required for linear oxidation rates in assessing inhibition kinetics. Approximately 10^{-6} M ancymidol was required to evoke 50% of the observed spectral changes, whereas the measured K_i for ancymidol in kaurene oxidation under the conditions employed was about 2×10^{-9} M (Fig. 2). Thus, there was a discrepancy in the levels of ancymidol needed in these two phenomena which leads to a question of how closely the two are related.

Hildebrant (8) has discussed the difficulties in correlating the results of binding studies measured by spectral shifts and the characteristics of inhibition of oxidation in the case of the interaction of metyrapone with Cyt P-450 and the inhibition of N-demethylation reactions by this substance in liver microsomes. The conditions under which binding spectra are typically measured with oxidized microsomes in the absence of a physiological reductant and the substrate are quite different from those which pertain during measurement of oxidase activity. Also, it is to be expected that a large fraction of the added inhibitor will be tied up in enzyme-inhibitor complex in cases where the inhibitor is bound strongly to the enzyme and is effective at concentrations where the concentration of added inhibitor molecules is the same range as the concentration of the enzyme to which it is binding. In such cases the amount of inhibitor added to achieve a given level of inhibition becomes proportionately higher as the concentration of enzyme present is increased. This is the case with metyrapone inhibition of N-demethylation in liver microsomes. It can be estimated in the present case that the concentration of added ancymidol required for 50% inhibition of kaurene oxidation in dilute microsomal suspensions is roughly equivalent to the concentration of Cyt P-450 in these suspensions (7). It is difficult to evaluate whether these factors are sufficient to account for the differences seen in the requirement for added ancymidol to realize 50% of the maximal effects on these two processes.

There is a similarity in the behavior of metyrapone as an inhibitor in liver microsomes (8) and ancymidol as an inhibitor in the plant microsomes. The two substances, which have some superficial structural similarities, both bind to Cyt P-450 preparations to give a type II difference spectrum. Both appear to inhibit their substrate oxidations in a noncompetitive manner and are effective at quite low concentrations. From the measurements which have been made to date neither substance appears to act by modifying the rates of electron transport to Cyt P-450.

Ancymidol promises to be a useful tool in further studies on the reactions catalyzed by Cyt P-450 in the gibberellin biosynthetic pathway by virtue of its apparent specificity and its high affinity for the enzymes.

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