J Plant Growth Regul (1985) 4:111–122

Journal of
Plant
Growth
Regulation

Inhibition of Gibberellin Biosynthesis by Paclobutrazol in Cell-free Homogenates of *Cucurbita maxima* Endosperm and *Malus pumila* Embryos

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Received January 23, 1985; accepted April 23, 1985

Abstract. The plant growth retardant paclobutrazol, (PP333) (2*RS*,3*RS*)-1 - (4-chlorophenyl) - 4,4 - dimethyl - 2 - (1,2,4-triazol-1-yl)pentan-3-ol, inhibits specifically the three steps in the oxidation of the gibberellin-precursor *ent*-kaurene to *ent*-kaurenoic acid in a cell-free system from *Cucurbita maxima* endosperm. The KI_{50} for this inhibition is 2×10^{-8} M. The KI_{50} values for the separated 2*S*,3*S*, and 2*R*,3*R* enantiomers of paclobutrazol in this system are 2×10^{-8} M and 7×10^{-7} M, respectively. A cell-free preparation from immature *Malus pumila* embryos converts *ent*-kaurene to gibberellin A_9, whereas no conversion occurs in a similar preparation from *Malus* endosperm. The conversion of *ent*-kaurene by the embryo preparation is inhibited by paclobutrazol with KI_{50} values for the 2*S*,3*S* and 2*R*,3*R* enantiomers of 2×10^{-8} M and 6×10^{-8} M, respectively.

Paclobutrazol (PP333), (2*RS*,3*RS*)-1-(4-chlorophenyl)-4,4-dimethyl-2-(1,2,4-triazol-1-yl)pentan-3-ol, is a potent plant growth retardant, which is active on a broad range of plant species (Lever et al. 1982). It has potential agronomic importance, for example, in controlling the size of fruit trees (Quinlan 1981). Structurally, paclobutrazol is a substituted triazole with two asymetric carbon atoms (Fig. 1), and is produced as a mixture of the 2*R*,3*R* and 2*S*,3*S* enantiomers. Other N-containing heterocyclic growth retardants such as ancymidol

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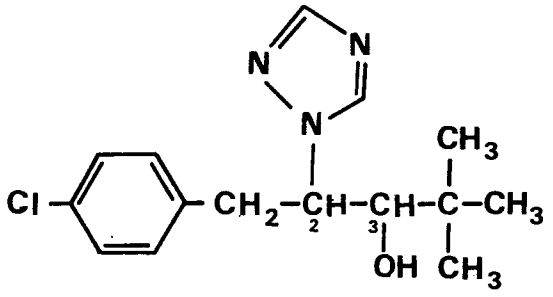

Fig. 1. The structure of paclobutrazol.

and tetcyclacis have been shown to inhibit gibberellin (GA) biosynthesis by blocking specifically the three steps in the oxidation of *ent*-kaurene to *ent*-kaurenoic acid (Coolbaugh et al. 1978, Rademacher et al. 1984). It is likely, therefore, that paclobutrazol has a similar mechanism of action, particularly since it was recently shown to inhibit the oxidation of *ent*-kaurene to unidentified products in a cell-free system from pea apices (Dalziel and Lawrence 1984). In this paper the enzymatic sites of inhibition of paclobutrazol in the GA-biosynthetic pathway are defined in two *in vitro* systems from seed tissues, and the effectiveness of the separated enantiomers is compared.

Materials and Methods

(*R,S*)-[2-^{14}C] mevalonic acid (MVA) (1.96 × 10^{12} Bq mol^{-1}) and (*R*)-[2-^{14}C]-MVA (1.96 × 10^{12} Bq mol^{-1}) were produced from the lactone (Amersham International plc) by hydrolysis with 2 equivalents KOH for 15 min at 30°C. (2*RS*,3*RS*)-Paclobutrazol and the separated 2*R*,3*R* and 2*S*,3*S* enantiomers were kindly provided by Imperial Chemical Industries PLC, Plant Protection Division.

Cell-free extracts were prepared from endosperm of *Cucurbita maxima* L., cv. "Riesenmelone, gelb, vernetzt," essentially as described by Graebe et al. (1974a); however, since slight modifications over the years have brought significant improvements in yield, the modified methods are described fully here. The endosperm was removed from the immature seeds, gently homogenized, filtered through cheese cloth, and centrifuged at 2,000 × *g* for 5 min. The supernatant was dialysed 3× against 0.05 M K-Pi buffer at pH 8.0 containing 2.5 × 10^{-3} M $MgCl_2$ and was then stored in liquid N_2. The ^{14}C-labeled GA-precursors *ent*-kaurene, *ent*-kaurenol, *ent*-kaurenal, *ent*-kaurenoic acid, *ent*-7α-hydroxykaurenoic acid, and GA_{12}-aldehyde were also prepared by a method modified from that used by Graebe et al. (1974a). (*RS*)-[2-^{14}C]MVA (4.7 × 10^{-6} mol, 9.25 MBq) was incubated for 2 h at 30°C with 7 ml endosperm preparation, 5 × 10^{-3} M ATP, 5 × 10^{-3} M PEP, 5 × 10^{-4} M NADPH, 5 × 10^{-3} M $MgCl_2$, and 10^{-3} M $MnCl_2$ in a total volume of 10 ml. The products were extracted and separated by thin layer chromatography (TLC) on silica gel developed in $CHCl_3$-ethyl acetate-acetic acid (75:25:1, v:v). GA_{12}-Aldehyde (Rf 0.60), which was well separated from GA_{12} (Rf 0.39) and *ent*-7α-hydroxy-kaurenoic acid (Rf 0.46), was rechromatographed in the same solvent system.

ent-Kaurenol (Rf 0.76) and *ent*-kaurenoic acid (Rf 0.81) were separated further by TLC on silica gel developed in toluene-ethyl acetate (95:5). *ent*-Kaurene and *ent*-kaurenal (both Rf 0.93) were separated and purified by TLC on silica gel developed first with toluene to 12 cm, and then with petroleum ether (40–60°C) to 17 cm. The identity, purity, and specific activity of the products were determined by combined gas chromatography-mass spectrometry (GC-MS). *ent*-[^{14}C]Kaurene was also prepared from (*R*)-[2-^{14}C]MVA as described above except that NADPH and PEP were omitted from the incubation medium. *ent*-Kaurene, the major product under these conditions, was purified by TLC on silica gel developed in petroleum ether (40–60°C).

Incubations with Paclobutrazol in the C. maxima *System*

(*RS*)-[2-^{14}C]MVA (10^{-8} mol, 1.96×10^5 Bq) was incubated for 1 h at 30°C with the endosperm preparation (75 µl), cofactors as above, and serial dilutions of (2*RS*,3*RS*)-paclobutrazol (10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} M, and control in one experiment and 6×10^{-7}, 4×10^{-7}, 2×10^{-7}, 10^{-7} M, and control in a second experiment) in a total volume of 0.1 ml. ^{14}C-Labeled *ent*-kaurene, *ent*-kaurenol, *ent*-kaurenal, and *ent*-kaurenoic acid (4×10^{-10} mol, 2.35×10^{-3} Bq of *ent*-kaurene, 3.16×10^{-3} Bq of the others) were incubated with the *C. maxima* system and cofactors with and without 10^{-6} M (2*RS*,3*RS*)-paclobutrazol. In all cases the products were separated by TLC on silica gel developed with $CHCl_3$-ethyl acetate-acetic acid (75:25:1). Radioactive bands previously identified by GC-MS (Graebe et al. 1972) were located by scanning and then removed and counted by liquid scintillation spectrometry.

[^{14}C]GA_{12}-aldehyde (4×10^{-10} mol, 3.2×10^{-3} Bq) was incubated with the cell-free system under the same conditions but with 5×10^{-4} M $FeSO_4$ and 5×10^{-3} M ascorbate as cofactors (Hedden and Graebe 1982). Products were separated by TLC on Si gel developed with $CHCl_3$-ethyl acetate-acetic acid (30:70:1).

Effect of Paclobutrazol on ent-*kaurene Oxidation by the* C. maxima *System*

Linear rates of *ent*-kaurene oxidation were ensured by trial time-course incubations with different size aliquots of the cell-free system. The substrate (1,250 Bq; 3.7×10^{-10} mol), added in acetone (4 µl), was incubated with the system at 30°C in the presence of 5×10^{-3} M ATP, 5×10^{-3} M $MgCl_2$, 10^{-3} M $MnCl_2$, 5×10^{-4} M NADPH, and 5×10^{-2} M K-Pi buffer at pH 7.5 in a total volume of 200 µl. Incubations were stopped at 5 min-time intervals by the addition of acetone (1 ml) and, after addition of H_2O (0.8 ml) and a pre-determined volume of 1 N HCl, the products were extracted with ethyl acetate (3×1 ml). *ent*-Kaurene was separated from products by TLC on silica gel developed in petroleum ether (40°C–60°C). Percentage oxidation was determined from the ratio of counts at the origin (total products) to total counts (origin plus *ent*-kaurene). A dilution of 50 µl *C. maxima* preparation (150 µg protein) in 200 µl gave a linear rate of oxidation for 20 min at 30°C. The effect of paclobutrazol

on *ent*-kaurene oxidation was determined from 15-min incubations as described above in the absence (ca 75% oxidation) and presence of (2*RS*,3*RS*)-paclobutrazol or the resolved enantiomers at different concentrations and added in 5 $\times$ 10^{-2} M K-Pi buffer at pH 7.5 (20 µl).

Preparation of Cell-free Systems from Malus

Endosperm and embryos (cotyledons plus embryonic axis) were removed from immature seeds of *Malus pumila* Mill. cv. Cox's Orange Pippin 9–10 weeks after full bloom and frozen separately in liquid N_2. The tissues were macerated in a mortar with 5 $\times$ 10^{-3} M K-Pi buffer at pH 8 containing 2.5 $\times$ 10^{-3} M $MgCl_2$ (1:1, w/v), and then further homogenized in a glass homogenizer. After centrifugation at 5,000 $\times$ *g* for 5 min, the supernatant was frozen in pellets and stored in liquid N_2.

Incubations with the Malus *Cell-free Systems*

Cell-free systems from both endosperm and embryos were tested for the ability to metabolize *ent*-kaurene or GA_{12}-aldehyde under the same incubation conditions as described for the *C. maxima* endosperm system except that 250 µl of the *Malus* preparations were used in 300 µl total volume. Only the embryo preparations contained detectable activity.

1) Preparative-scale incubation with *ent*-[^{14}C]kaurene. The substrate (1.27 $\times$ 10^5 Bq; 3.7 $\times$ 10^{-8} mol) was added in acetone (0.1 ml) to an embryo cell-free preparation (25 ml) supplemented with 5 $\times$ 10^{-3} M $MgCl_2$, 5 $\times$ 10^{-3} M ATP, and 5 $\times$ 10^{-4} NADPH in 25.75 ml total volume. After incubating for 3 h at 30°C the incubation mixture was adjusted to pH 2.5 with 1 N HCl. Acetone (25 ml) was added and the products were extracted with ethyl acetate (3 $\times$ 25 ml). The combined EtOAc phases were washed with water at pH 3 (10 ml) and reduced to dryness *in vacuo*. The products were separated by HPLC (Kontron HPLC System 600) on a 5-µm Hypersil ODS column (25 $\times$ 0.45 cm i.d.) eluted at 1 ml min^{-1} in a linear gradient of 50% MeOH-H_2O to 100% MeOH in 40 min, the solvents containing 50 µl l^{-1} acetic acid. Radioactivity was monitored on stream using a Bertholdt LB 503 Radioactivity monitor. Fractions containing radioactivity were evaporated to dryness *in vacuo*, methylated (CH_2N_2) and trimethylsilylated (MSTFA, 90°C for 30 min), and examined by GC-MS.

2) Preparative-scale incubation with [^{14}C]GA_{12}-aldehyde. The substrate (8.9 $\times$ 10^4 Bq) was added in acetone (50 µl) to an embryo preparation (9.6 ml) diluted with 5 $\times$ 10^{-2} M K-Pi buffer at pH 7.5 (2.4 ml) and supplemented with 10^{-6} M (2*S*,3*S*)-paclobutrazol to suppress conversion of endogenous *ent*-kaurene (total incubation volume 12.12 ml). After incubating for 2 h at 30°C the products were extracted and separated by reverse-phase HPLC as described above.

3) Effect of paclobutrazol on *ent*-kaurene oxidation. *ent*-[^{14}C]Kaurene (10^3 Bq, 2.94 $\times$ 10^{-10} mol) was added in acetone (4 µl) to the incubation mixture

containing the embryo cell-free preparation (0.25 ml), 5×10^{-3} M $MgCl_2$, 5×10^{-3} M ATP, 5×10^{-4} M NADPH, and different concentrations of (2*R*,3*R*)- or (2*S*,3*S*)-paclobutrazol dissolved in 5×10^{-2} M K-Pi at pH 7.5 (30 µl) in a total volume of 0.3 ml. After incubating for 2 h at 30° the reaction was stopped by the addition of acetone (1 ml), and the products were extracted as described above. The percent oxidation was determined as described for the incubations with *C. maxima*. Percent inhibition at each paclobutrazol concentration was calculated relative to the control incubation in the absence of paclobutrazol.

GC-MS

GC-MS was carried out using a VG 1212 MS coupled to a Dani 3800 HR GC and a VG 2015 data system. Derivatized samples were injected in 1 µl MSTFA onto a BP-1 WCOT quartz capillary column (25 m × 0.25 mm i.d.) at 50°C without splitting. After 0.5 min the split (50:1) was opened and after 1 min the column was heated ballistically to 200°C and then at 4°C min^{-1} to 300°C. Mass scanning from 700-20 amu in 1-sec cycles was started at 200°C. The injector temp was 200°C and the carrier gas (He) inlet pressure was 0.8 bar. The column was coupled directly to the ion source which was maintained at 250°C. An electron energy of 70 eV and an emission current of 100 uA were used. Products were identified by comparison of their mass spectra with those of authentic standards. Specific radioactivities were estimated from the proportions of isotope peaks by the method of Bowen et al. (1972).

Reproducibility

The inhibition by paclobutrazol of *ent*-kaurene oxidation in the *C. maxima* system is well documented by the experiments described. The inhibition curves were repeated at least once with no significant differences in the KI_{50} values. Confidence limits for individual points on these curves were not determined. The agreement between small scale and large scale incubations assures reproducibility of the results reported for the apple seed system. KI_{50} values were obtained from single experiments in this case.

Results

Cell-free homogenates of *Cucurbita maxima* endosperm convert MVA via *ent*-kaurene to *ent*-kaurenoid metabolites and GAs when incubated in the presence of NADPH and $MgCl_2$ as cofactors (Graebe et al. 1972, 1974a,b). In the presence of $MnCl_2$, later steps in the GA-biosynthetic pathway are inhibited so that GA_{12} is the final GA product (Fig. 2). In order to determine the general concentration range at which paclobutrazol might be inhibitory and to obtain preliminary information about the site of inhibition, a preparation was incubated with (*RS*)-[2-^{14}C]MVA (10^{-4} M), cofactors, and a series of (2*RS*,3*RS*)-paclobutrazol concentrations (10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7} M) as described in

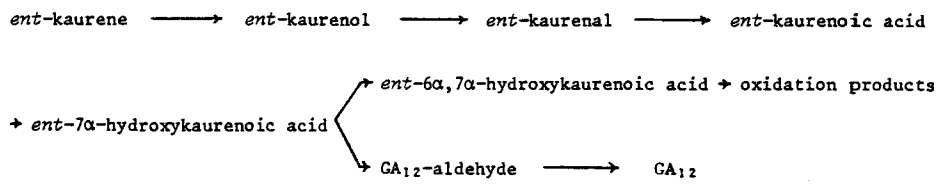

Fig. 2. Steps catalyzed by microsomal oxidases in the GA biosynthetic pathway in *C. maxima*.

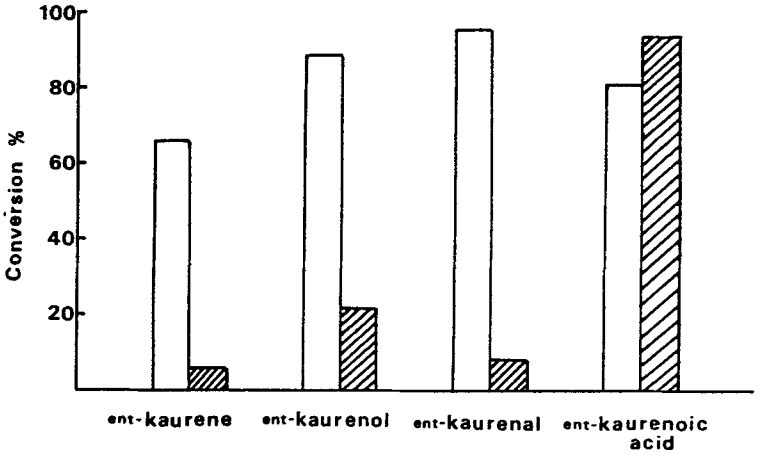

Fig. 3. The percentage conversion of [^{14}C]-labeled *ent*-kaurenoid GA-precursors into later intermediates in the absence (open columns) and presence (hatched columns) of 10^{-6} M paclobutrazol by a cell-free system from *C. maxima* endosperm.

Materials and Methods. Separation of the products by TLC showed that the incubation with 10^{-7} M paclobutrazol gave the same products as the control incubation. The other three concentrations yielded a single fraction of radioactivity migrating on TLC like *ent*-kaurene or *ent*-kaurenal, which are not resolved by the chromatographic system used. Thus it was concluded that paclobutrazol at concentrations of 10^{-6} M and higher inhibits the oxidation of *ent*-kaurene or a step shortly thereafter. When the experiments were repeated with a narrower range of paclobutrazol concentrations, 10^{-7} M again had no effect, whereas 2×10^{-7} M strongly inhibited the formation of products as before. To locate precisely the step(s) that are inhibited ^{14}C-labeled *ent*-kaurene, *ent*-kaurenol, *ent*-kaurenal, and *ent*-kaurenoic acid (4×10^{-6} M) were incubated with the system in the presence or absence of (2*RS*,3*RS*)-paclobutrazol (10^{-6} M). The results, presented in Table 1 and illustrated in Fig. 3, show that paclobutrazol inhibits the conversion of *ent*-kaurene, *ent*-kaurenol and *ent*-kaurenal, but not of *ent*-kaurenoic acid. Conversion of *ent*-[^{14}C]kaurenoic acid was somewhat higher in the presence of paclobutrazol, probably because there was reduced competition from endogenous *ent*-kaurenoic acid that is normally produced from endogenous *ent*-kaurene during the incubation (Hedden and Graebe 1981).

In a separate experiment GA_{12}-aldehyde (4×10^{-6} M) was incubated with the cell-free preparation in the presence of ascorbate and $FeSO_4$ (Hedden and Graebe 1982) and in the absence or presence of (2*RS*,3*RS*)-paclobutrazol (10^{-7},

Table 1. Incorporation of *ent*-[^{14}C]kaurenoid precursors of GA biosynthesis into later intermediates in the absence (−) and presence (+) of 10^{-6} M (*2RS,3RS*)-paclobutrazol. Products were separated by TLC and 7 discrete bands of radioactivity known to correspond to the products given below were removed and counted.

		Products						
Substrate		*ent*-kaurene + *ent*-kaurenal	*ent*-kaurenol + *ent*-kaurenoic acid	*ent*-7α-OH kaurenoic acid	GA_{12}-aldehyde (Bq)	GA_{12}	*ent*-6α,7α-diOH kaurenoic acid	Unidentified material at origin
ent-kaurene	−	714	43	0	135	274	285	175
	+	1,232	26	0	0	0	0	53
ent-kaurenol	−	47	190	0	150	435	387	433
	+	132	1,337	29	0	0	43	164
ent-kaurenal	−	76	84	0	177	484	472	498
	+	1,572	30	15	0	0	35	57
ent-kaurenoic	−	0	315	0	172	398	395	464
acid	+	0	88	0	31	207	361	967

10^{-6}, and 10^{-5} M). At all concentrations of paclobutrazol there was no significant difference over the control in the incorporation of GA_{12}-aldehyde into fractions previously identified (Graebe et al. 1974a,b) as GA_{43} (85%), GA_{13} (6%), GA_4 (2%), and GA_{12}/GA_{12}-aldehyde (4%).

In order to determine the KI_{50} of paclobutrazol for the inhibition of *ent*-kaurene oxidase in *C. maxima* endosperm, the 2,000 × *g* supernatant was diluted so that a linear rate of oxidation was obtained over 20 min. This was achieved for incubations with 1.8×10^{-6} M *ent*-[^{14}C]kaurene at 30°C by using 50 µl (150 µg Lowry protein) of the preparation, $MgCl_2$ (5×10^{-3} M), ATP (5×10^{-3} M), NADPH (5×10^{-4} M), and potassium phosphate buffer (5×10^{-2} M) in a total volume of 200 µl. The extent of oxidation (ca 75% after 15 min) was measured after separation of *ent*-kaurene from total products by TLC as described in Materials and Methods. The effect of paclobutrazol at different concentrations on the amount of *ent*-kaurene oxidized in 15 min (expressed as % control) is shown in Fig. 4. The concentration of paclobutrazol required to inhibit the oxidation by 50% (KI_{50}) is estimated from this plot as 2×10^{-8} M.

Figure 4 also contains the inhibition curves for the separated 2*R*,3*R* (+) and 2*S*,3*S* (−) enantiomers. The KI_{50} values for the enantiomers are 7×10^{-7} M and 2×10^{-8} M, respectively.

It was decided to test the efficiency of paclobutrazol as an inhibitor of *ent*-kaurene oxidase in a second system and for this purpose the apple was chosen as a species for which the compound is known to be effective as a growth retardant. Cell-free homogenates were prepared from apple endosperm and from immature apple embryos and tested in the presence of Mg^{2+}, NADPH, and ATP for *ent*-kaurene oxidase activity. No activity could be detected in the preparations from endosperm, but the preparations from immature embryos converted *ent*-[^{14}C]kaurene to several products. After a large-scale (25 ml) incubation the products were separated by reversed-phase HPLC using a gradient of methanol-water containing 50 µl l^{-1} acetic acid. The radiochromatogram profile shown in Fig. 5 indicated seven peaks of radioactivity. The three earliest eluting products were examined by GC-MS after methylation and trimethylsilylation. Peak 1 (the major product) contained [^{14}C]GA_9 (identified as its methyl ester). The ^{14}C label was apparent from the presence of isotope peaks associated with the major fragment ions. The relative intensities of these ions indicated that the product was diluted considerably by endogenous metabolite. The fragment ion at m/z 298 (M^+-32) was accompanied by an ion at m/z 306 due to the presence of four ^{14}C atoms in some molecules, and the specific radioactivity of the GA_9 was estimated from the relative intensities of these ions (Bowen et al. 1972) as 1.1×10^{12} Bq mol^{-1}. Compared with a specific radioactivity of 3.4×10^{12} Bq mol^{-1} for the substrate this represents a 2:1 dilution by endogenous material.

The derivatized material from peaks 2 and 3 (Fig. 5) contained *ent*-7α-hydroxykaurenoic acid MeTMSi and GA_{12}-aldehyde Me, respectively, both containing ^{14}C. However, the mass spectra were too weak to estimate their specific radioactivities. There were insufficiently labeled compounds in peaks 4–6 to make an identification. The last peak (peak 7) corresponded to the substrate, *ent*-kaurene.

The intermediacy of GA_{12}-aldehyde between *ent*-kaurene and GA_9 was con-

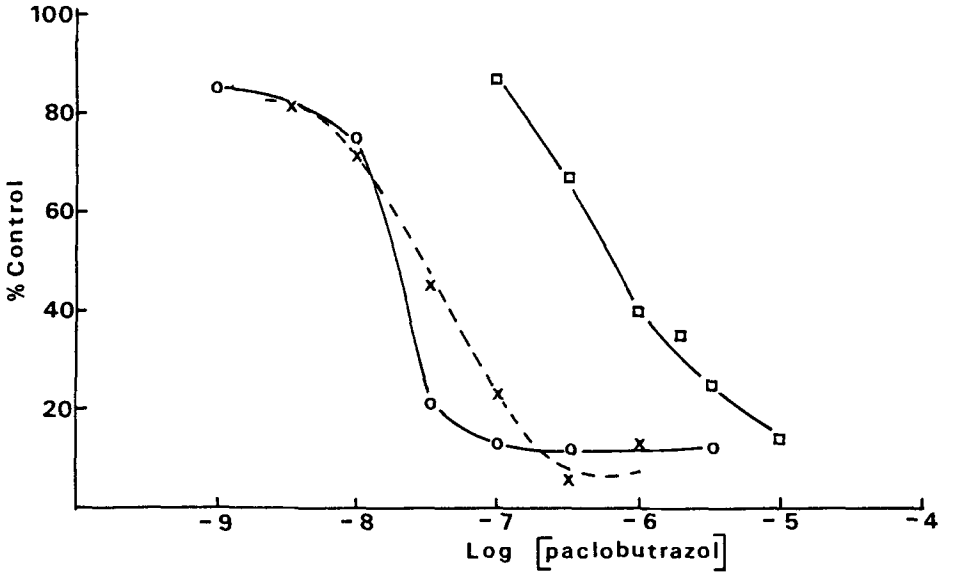

Fig. 4. The effect of (2*RS*,3*RS*)-paclobutrazol (--x--) and the 2*R*,3*R* (-□-) and 2*S*,3*S* (-O-) enantiomers on the oxidation of *ent*-[^{14}C]-kaurene by the *C. maxima* endosperm preparation. The conversion in the presence of paclobutrazol, expressed as percent of the conversion in the absence of paclobutrazol (control), is plotted against $\log_{10}$[paclobutrazol] in M.

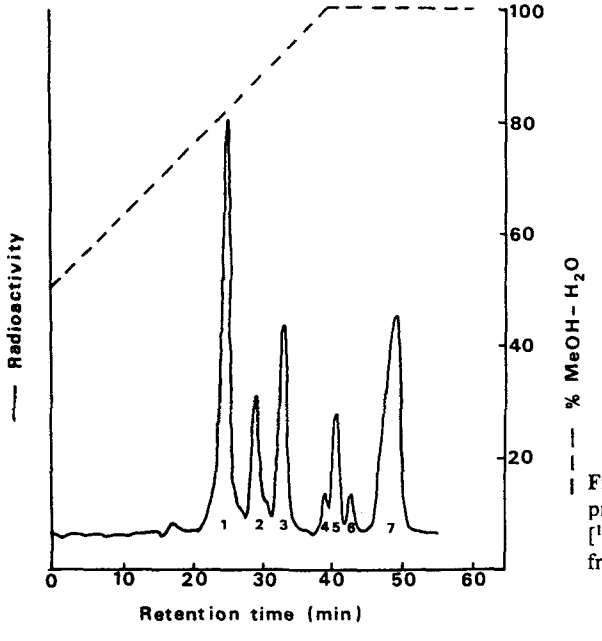

Fig. 5. HPLC separation of products from an incubation of *ent*-[^{14}C]kaurene with a cell-free system from immature apple embryos.

firmed by incubating the apple embryo preparation with [^{14}C]GA_{12}-aldehyde. Trial incubations had shown that no exogenous co-factors were required to obtain efficient conversion of GA_{12}-aldehyde to essentially a single product.

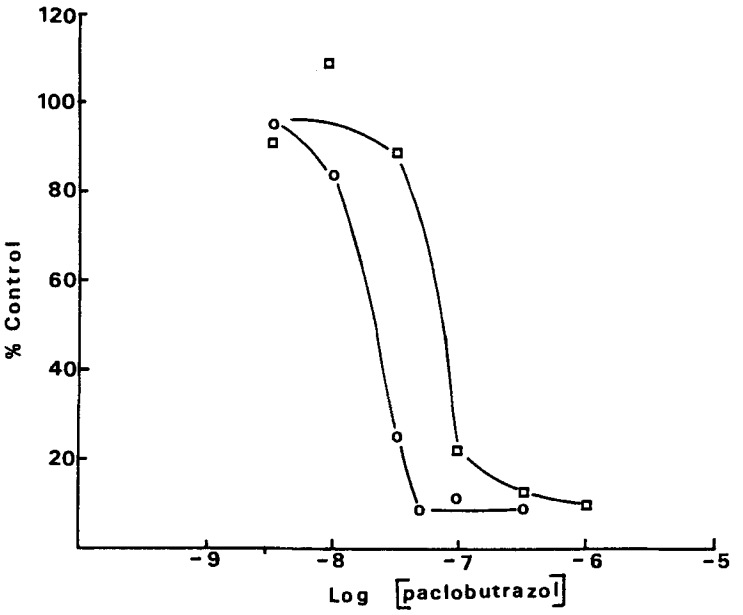

Fig. 6. The effect of (2*R*,3*R*)- (-□-) and (2*S*,3*S*)-paclobutrazol (-○-) on the oxidation of *ent*-[^{14}C]kaurene by a cell-free system from immature apple embryos. The axes are as in Fig. 4.

After a large-scale incubation in the presence of 10^{-6} M (2*S*,3*S*)-paclobutrazol to inhibit the conversion of the endogenous pool of *ent*-kaurene and so reduce dilution of the ^{14}C-label, this product was identified by GC-MS of the Me ester as GA_9. Its specific radioactivity was determined from the mass spectrum as 2.5×10^{12} Bq mol^{-1}.

The effect of the resolved enantiomers of paclobutrazol on the oxidation of *ent*-kaurene by the apple embryo preparation was examined in a similar manner to the experiments with *C. maxima* endosperm, although, because of the relatively slow rates of conversion, end point analysis was used instead of true reaction rates. *ent*-[^{14}C]Kaurene (2.9×10^{-10} mol; 9.9×10^2 Bq) was incubated for 2 h at 30°C with the apple preparation (250 µl, 250 µg Lowry protein), cofactors as for the *C. maxima* system and (2*R*,3*R*) or (2*S*,3*S*)-paclobutrazol in a total volume of 300 µl. In the absence of paclobutrazol 50% conversion occurred in 2 h. The results are shown in Fig. 6. The KI_{50} values for the (2*S*,3*S*) and 2*R*,3*R* enantiomers of paclobutrazol were 2×10^{-8} M and 6×10^{-8} M, respectively.

Discussion

Paclobutrazol is shown to be an extremely effective inhibitor of GA biosynthesis *in vitro*. It inhibits the three successive oxidative steps between *ent*-kaurene and *ent*-kaurenoic acid, but not the conversion of *ent*-kaurenoic acid to *ent*-7α-hydroxykaurenoic acid (Fig. 2). Steps after GA_{12}-aldehyde are also not affected. Thus it has the same enzymatic sites of action as ancymidol and

the other nitrogen-containing heterocyclic growth retardants that have been investigated, and exhibits similar specificity and efficiency (Coolbaugh et al. 1978; Rademacher et al. 1984). The 2*S*,3*S* (−) enantiomer is an equally effective inhibitor of *ent*-kaurene oxidase in both the *C. maxima* and *Malus* cell-free systems. Dalziel and Lawrence (1984) reported a much higher KI_{50} value for the racemic mixture in a preparation from *Pisum* shoot apices (10^{-6} M as opposed to 2×10^{-8} M). The reason for the discrepancy in the two values is unclear, but may be due to differences in the enzymes in vegetative and seed tissue.

There is an apparent difference in the sensitivity of the cell-free system to paclobutrazol depending on whether MVA or *ent*-kaurene is used as the substrate. When MVA was the substrate it was evident from the pattern of products obtained that little inhibition occurred until the paclobutrazol concentration exceeded 10^{-7} M. However, Fig. 4 shows that 10^{-7} M paclobutrazol reduces the conversion of exogenous *ent*-kaurene to about 30% that of the control. This discrepancy may be due to the longer incubation time employed with MVA. Alternatively, it could indicate that oxidation of *ent*-kaurene is more sensitive to inhibition when this intermediate is supplied than when it is formed *in situ*. *ent*-Kaurene produced *in situ* may have preferential access to the enzyme active site.

The KI_{50} values for the enantiomers correlate positively with their respective potencies as growth retardants. The 2*S*,3*S* enantiomer is more efficient at reducing the growth of apple seedlings than is the 2*R*,3*R* enantiomer. In comparison, the latter is the better fungicide (Sugavanam 1984). By analogy with the closely related compound diclobutrazol, as well as other triazole fungicides, the fungicidal activity of the 2*R*,3*R* enantiomer is due presumably to interference with ergosterol biosynthesis via inhibition of lanosterol 14-demethylation (Baldwin and Wiggins 1984). Sugavanam (1984) has presented evidence from computer modelling that the 2*S*,3*S* and 2*R*,3*R* enantiomers of paclobutrazol can be superimposed on *ent*-kaurene and lanosterol, respectively, i.e., on the respective substrates for the inhibited enzymes in the GA and fungal sterol biosynthetic pathways. It has been shown for other triazole fungicides, such as triadimefon, that they may also affect sterol metabolism in higher plants (Buchenauer and Rohner 1981). Thus, at least at high concentrations, inhibition of sterol biosynthesis may be a factor in reduced plant growth by the racemic mixture. The effect of the enantiomers on the GA and sterol content of elongating plant tissues remains to be determined.

Although it was less active, the 2*R*,3*R* enantiomer inhibited *ent*-kaurene oxidation appreciably in both cell-free systems and considerably more so in the apple embryo preparation. The 2*R*,3*R* enantiomer contained less than 2% of the 2*S*,3*S* enantiomer as impurity, which could account for some, but by no means all, of the activity. It is possible that conversion of the 2*R*,3*R* enantiomer to a more active form occurred during the incubation. Thus after oxidation at C-3 to the ketone, racemization at C-2 would occur readily, giving rise to a potentially active product. Such a conversion would be more likely with the apple embryo preparation because of the longer incubation times.

In the cell-free system from immature *Malus* embryos *ent*-[^{14}C]kaurene was converted to [^{14}C]GA_9 with ^{14}C-labeled *ent*-7α-hydroxykaurenoic acid and

GA_{12}-aldehyde accumulating as intermediates. No intermediates between GA_{12}-aldehyde and GA_9 were apparent nor were any of the other C_{19}-GAs known to be native to immature apple seeds (Hoad 1978). It is also surprising that neither *ent*-kaurene nor GA_{12}-aldehyde were metabolized by endosperm preparations even though this tissue contains high concentrations of GAs (Hedden, unpublished results). It remains to be determined whether this lack of extractable enzyme activity in the endosperm actually reflects the situation *in vivo* or is an artifact of extraction.

Acknowledgments. We thank Mr. M. Bolton (East Malling) and Mrs. G. Bodtke-Owusu Boakye (Göttingen) for able technical assistance, and the Deutsche Forschungsgemeinschaft for support to J.E.G.

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