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Radiosynthesis and Metabolism of the Insect Antijuvenile Hormone, Precocene II

Tomihisa Ohta, Ronald J. Kuhr,* and William S. Bowers

The antijuvenile hormone precocene II (6,7-dimethoxy-2,2-dimethylchromene) was radiosynthesized with carbon-14 in the 2-*gem*-dimethyl position. Injection of the labeled compound into nine insect species revealed a variation of at least 37-fold in metabolic rate. In vitro studies using cabbage looper and European corn borer gut and fat body tissue homogenates indicated that the metabolic pathway was primarily dependent on catalysis by a mixed-function oxidase system. The principal metabolite in each insect was 6,7-dimethoxy-2,2-dimethylchroman-3,4-diol which was readily conjugated in vivo. Other identified metabolites include 6,7-dimethoxy-2,2-dimethylchroman-3-ol and 3,4-epoxy-6,7-dimethoxy-2,2-dimethylchroman.

A recent bioassay of a lipid extract of *Ageratum houstonianum* revealed that the plant contains chemicals possessing insect antijuvenile hormone activity (Bowers et al., 1976). Isolation and characterization studies showed the active ingredients to be 7-methoxy-2,2-dimethylchromene (precocene I) and 6,7-dimethoxy-2,2-dimethylchromene (precocene II). These compounds are able to induce precocious metamorphosis, cause sterilization, and/or force diapause in certain insects, especially Heteropteran species. Thus, it is possible that such natural products or synthetic analogues could form the basis for development of a fourth generation of insecticide chemicals.

To facilitate structure optimization and mode-of-action studies, we have radiolabeled precocene II with carbon-14 in the 2-*gem*-dimethyl position and have initiated metabolism studies on several insect species. Below are reported synthetic procedures and potential metabolic pathways.

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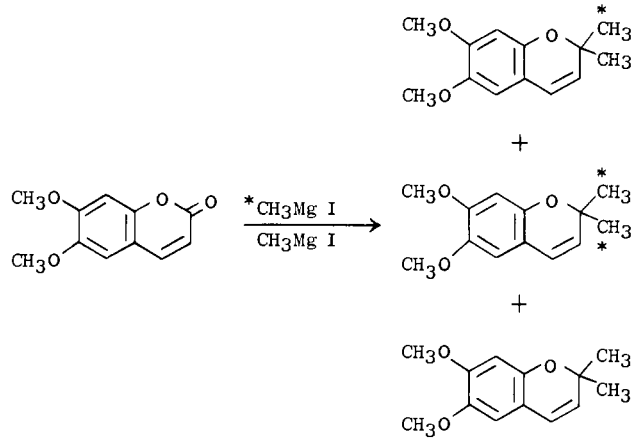
Scheme I



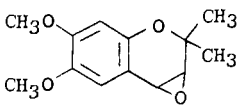
MATERIALS AND METHODS

Radiosynthesis of Precocene II. Unlabeled 6,7-dimethoxycoumarin was prepared from 3,4-dimethoxyphenol and 3-ethoxyacrylyl chloride according to the procedure of Crosby and Berthold (1962). The coumarin was

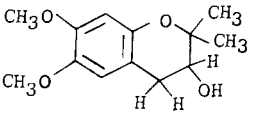
methylated by Grignard reaction with [^{14}C]methyl magnesium iodide in diethyl ether (method similar to Hepworth and Livingstone, 1966) to give radiolabeled precocene II as in Scheme I.

Specifically, 7.5 mg (0.31 mM) of Mg and 40.2 mg (0.283 mM) of methyl iodide were stirred for 15 min in 1.5 mL of diethyl ether. To the mixture was added 250 μL of benzene containing 1.2 mg (0.0085 mM) of [^{14}C]methyl iodide (56 mCi/mmol, Amersham Searle Corp.). After stirring for an additional hour at room temperature, 20.6 mg (0.1 mM) of 6,7-dimethoxycoumarin in 1.5 mL of THF plus 1 mL of benzene was introduced into the flask. One day later, the product was removed by addition of 20% NH_4Cl solution and extraction with ether. Following evaporation of the solvent, the product (20 mg) was sealed in a vial with 2 mL of acetic acid and heated at 110 °C for 1 h. Extraction with ether and purification (>99%) via silica gel thin-layer chromatography (7% ethyl acetate in benzene, R_f 0.5) yielded 6.23 mg of [^{14}C]precocene II (1.23 mCi/mmol). The radioactive precocene II was identical with authentic precocene II synthesized according to Bowers et al. (1976).

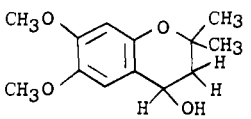
Synthesis of Potential Metabolites. *3,4-Epoxy-6,7-dimethoxy-2,2-dimethylchroman* (epoxy precocene II) was prepared by oxidation of precocene II with *m*-chloroperoxybenzoic acid. The crude product was subjected to Florisil column chromatography, and the fraction which eluted with 15% ether in hexane was purified by gel permeation chromatography (GPC) using a series of two μ-Styragel columns (each 30 cm × 7.6 cm i.d., Waters Assoc.). Elution with benzene gave pure epoxy precocene II: IR (in $CDCl_3$) 1620, 1575, 1505, 1240, 1130, 855 cm^{-1}; mass spectrum *m/e* 237 $(M + 1)^+$.



6,7-Dimethoxy-2,2-dimethylchroman-3-ol was synthesized by $NaBH_4$ reduction of epoxy precocene II. Preliminary clean-up on silica gel thin-layer chromatography (20% ethyl acetate in benzene, R_f 0.3) was followed by GPC as above. Benzene elution yeilded pure product: IR (in $CDCl_3$) 3570, 3450, 1620, 1600, 1510, 1240, 1010, 880, 850 cm^{-1} mass spectrum *m/e* 239 $(M + 1)^+$, 221 $[(M + 1) - H_2O]^+$.



6,7-Dimethoxy-2,2-dimethylchroman-4-ol was prepared by $LiAlH_4$ reduction of 6,7-dimethoxy-2,2-dimethylchroman-4-one or by oxymercuration of precocene II, followed by $NaBH_4$ reduction. Chromatography on Florisil columns and elution with 20% ether in hexane gave pure product: IR (neat) 3450, 3340, 1615, 1595, 1505, 1245, 1020 865, 830 cm^{-1}; mass spectrum *m/e* 239 $(M + 1)^+$, 221 $[(M + 1) - H_2O]^+$ base peak; ^{1}H NMR ($CDCl_3$) δ 1.29 (3 H, s), 1.42 (3 H, s), 2.02 (2 H, m), 3.79 (2 × 3 H, s's), 4.70 (1 H, dd, *J* = 7.7 Hz), 6.30 (1 H, s), 6.90 (1 H, s).



6,7-Dimethoxy-2,2-dimethylchroman-3,4-diol (precocene II diol) was obtained by hydration of epoxy precocene II with 10% H_2SO_4 in 1:1 THF–H_2O. The product was purified by silica gel thin-layer chromatography using ether as the developing solvent (R_f 0.3) and GPC as above: IR (in $CDCl_3$) 3400, 1620, 1600, 1510, 1225, 1010, 850, 835 cm^{-1}; mass spectrum *m/e* 254 (M)+, 236 $(M - H_2O)^+$.

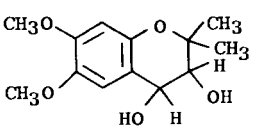


Table I. In Vivo Metabolism of Precocene II in Various Insect Species

Insect	Metabolic rate: Ng mg^{-1} (20 min)$^{a-1}$	Metabolic rate: Ratio	Metabolite distribution, %: Ether-soluble	Metabolite distribution, %: Water-soluble
Milkweed bug	0.39	1.0	<5	>95
Chafer grub	0.62	1.6	20	80
Mealworm	0.79	2.0	18	82
Wax moth	1.02	2.6	18	82
Cotton stainer	1.15	2.9	26[b]	73
Orangeworm	2.11	5.4	19[b]	80
Hide beetle	2.98	7.6	41	59
Cabbage looper	13.1	33.6	35[b]	41
Corn borer	14.3	36.7	38[b]	49

[a] ng of precocene II metabolized (mg of insect body weight)$^{-1}$ (20 min)$^{-1}$; average of four replicate experiments. [b] The ether-soluble and water-soluble percentages do not total 100 because the remainder of the radioactivity was not recovered from the insect.

Metabolism Studies. In vivo metabolic rates were obtained by injecting individual last instar nymphs or larvae with 1 μL of acetone containing 3 μg of [^{14}C]precocene II. The degradation of the administered compound was determined at various time periods after treatment using ether extraction, radioassay, and thin-layer chromatography (TLC) as previously described (Kuhr, 1970, 1971). Water-soluble conjugates were hydrolyzed by incubation with glusulase in pH 4.5 citrate–phosphate buffer for 4 h at 37 °C (Kuhr, 1971). Insect species treated were cabbage looper (*Trichoplusia ni*), European corn borer (*Ostrinia nubilalis*), European chafer grub (*Amphimallon majalis*), milkweed bug (*Oncopeltus fasciatus*), cotton stainer (*Dysdercus cingulatus*), hide beetle (*Dermestes maculatus*), yellow mealworm (*Tenebrio molitor*), navel orangeworm (*Paramyelois transitella*), and wax moth (*Galleria mellonella*).

For in vitro studies, fat body and gut homogenates from last instar cabbage loopers and corn borers were prepared using a described procedure (Kuhr, 1970). Aerobic incubation of 0.5 mL of homogenate with 4 μg [^{14}C]precocene II took place at 25 °C with shaking for 30 min. Included in the 2-mL reaction medium were pH 7.4 phosphate buffer (5 × 10^{-2} M), glucose-6-phosphate (2.4 × 10^{-3} M), glucose-6-phosphate dehydrogenase (1 unit), NADP (1.18 × 10^{-4} M), and KCl (3.86 × 10^{-3} M). Other additions or omissions were tested to gain insight into the enzymes responsible for metabolism. Extraction, radioassay, and chromatography of the ether-soluble products were conducted as before (Kuhr, 1970).

Isolation and Characterization of Metabolities. Radioactive precocene II was diluted with unlabeled precocene II and the above in vitro procedure with cabbage looper fat body was used on a larger scale to biosynthesize sufficient quantities of metabolities for possible identification. The ether-soluble products were separated by preparative TLC on 0.5-mm silica gel G plates developed in 4:1 ether–hexane. Three major radioactive areas located

Table II. Hydrolysis of In Vivo Water-Soluble Metabolites of Precocene II by Glusulase

Insect	% hydrolysis	% of aglycones as precocene II diol[a]
Milkweed bug	47	>95
Chafer grub	42	55
Mealworm	53	41
Wax moth	77	26
Cotton stainer	49	55
Orangeworm	56	71
Hide beetle	52	63
Cabbage looper	62	51
Corn borer	59	53

[a] Tentative identification based on R_f value.

Table III. Specific Activity of Tissue Homogenates from Cabbage Loopers and Corn Borers

	nmol of substrate metabolized/mg of protein/30 min			
	Cabbage looper		Corn borer	
Substrate	Fat body	Gut	Fat body	Gut
Precocene II	12.4	8.28	16.2	12.9
Carbaryl[a]	6.32	4.08	2.19	3.68
Methomyl[b]	0.33	0.23	1.67	0.36

[a] Cabbage looper data from Kuhr (1971); corn borer data from Kuhr and Davis (1975). [b] Cabbage looper data from Davis and Kuhr (1974); corn borer data from Kuhr and Hessney (1977).

by radioautography were extracted from the gel with acetone and rechromatographed using ether or 93:7 benzene–ethyl acetate. This procedure resulted in the isolation of eight metabolites of precocene II.

Each metabolite was further purified by GPC using benzene as the elution solvent, and partial characterization was obtained by comparing retention volumes with those known for synthesized potential metabolites. In addition, tentative identification was afforded by cochromatography on 0.04% Rhodamine 6G TLC plates and comparative retention times on the gas chromatograph (GC). Finally, structural confirmation was accomplished with a Finnigan Model 3100 mass spectrometer connected to a Finnigan Model 9500 gas chromatograph. The 1.5 m × 4 mm i.d. glass column was packed with 3% OV-225 on 100/120 Cromosorb W-HP and the carrier (flow rate 20 mL/min) and reactant gas (source pressure 700 μm) was methane. A source temperature of 30 °C was used together with column temperatures of 170 °C (for epoxy precocene II), 190 °C (for mono-ols), and 220 °C (for diol). The mass spectrometer was interfaced with a Model 6100 computer-controlled data acquisition system. Chemical ionization mass spectra were recorded for all metabolites except precocene II diol. In case of the latter compound, an electron impact mass spectrum was obtained at 70 eV.

RESULTS AND DISCUSSION

The nine insect species showed a 37-fold variation in the rate of precocene II metabolism in vivo (Table I). Most of the metabolites produced were water-soluble in nature, except for those formed by cabbage loopers and corn borers. In case of the latter, there was an equal distribution between ether-soluble and water-soluble products, but about 25% of the injected radioactivity was not recovered from the treated insects (Table I). It is possible that the labeled methyl groups could have been oxidized to $^{14}CO_2$ and expired. Based on R_f values and one dimensional cochromatography on TLC, the major ether-soluble metabolite (50–75% of the total organosoluble products) in each insect was precocene II diol.

Treatment of the water-soluble metabolites with glusulase released from 42–77% of the radioctivity into an ether-soluble form (Table II). TLC of the latter showed that, with the exception of the wax moth, the major aglycone chromatographed in the region of precocene II diol.

Results of in vitro degradation of precocene II by cabbage looper and corn borer tissues are shown in Table III. The findings are compared with previous data obtained from the same insect strains using the insecticides carbaryl and methomyl as substrates. It is apparent that precocene II is much more readily metabolized by fat body and gut enzymes than are the two insecticides. The fact that the toxicity of carbaryl and methomyl closely parallels their metabolic rate in vitro, e.g. methomyl is more toxic than carbaryl to corn borers (Kuhr and Hessney, 1976), suggests that precocene II may be degraded too rapidly to be bioactive in these insects. Indeed, our preliminary studies with loopers have failed to uncover any adverse effects caused by long-term exposure to precocene II, whereas milkweed bugs and cotton stainers, for example, readily succumb to precocene II action (Bowers et al., 1976).

The enzyme(s) responsible for biotransformation of all three substrates appear to be very similar, based on the results shown in Table IV Reduced metabolism in the absence of oxygen or NADPH together with inhibition in the presence of carbon monoxide or piperonyl butoxide

Table IV. Effect of Various Additions or Omissions on the Metabolism of Precocene II, Carbaryl, and Methomyl by Fat Body from Cabbage Loopers and Corn Borers

	% original activity					
	Cabbage looper[b]			Corn borer[c]		
Incubation medium[a]	Precocene II	Carbaryl	Methomyl	Precocene II	Carbaryl	Methomyl
Complete	100	100	100	100	100	100
−Oxygen	77	21	9	53	15	10
−NADP	69	71	61	88	88	82
+CO (80%)	25	7	19	28	17	20
+Piperonyl butoxide	4	3	2	12	3	6
+FMN	71	59	31	63	51	53
+EDTA	101	97	101	83	85	92
+KCN	97	92	98	84	93	102
+GSH	105	100	94	101	101	103
$+Cu^{2+}$	6	3		12	3	4
$+Fe^{2+}$	98	63		97	101	97

[a] All additions present at 10^{-3} M. The complete system consisted of fat body homogenate, substrate, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, NADP, KCl, and water. [b] Carbaryl data from Kuhr (1970); methomyl data from Kuhr (1973). [c] Carbaryl data from Davis and Kuhr (1974); methomyl data from Kuhr and Hessney (1977).

strongly suggest that precocene II metabolism, like that of carbaryl and methomyl, is catalyzed by a mixed-function oxidase system located in the fat body tissue. The reason why precocene II degradation was only mildly affected during incubation in an atmosphere of nitrogen (-oxygen) is not known, but it is possible that more endogenous oxygen was present in the reaction medium for the precocene studies. The response following various additions to the reaction medium was remarkably similar for each substrate.

The largest quantities and greatest diversity of ether-soluble metabolites resulted from incubation of [^{14}C]-precocene II with cabbage looper fat body homogenates. Hence, this system was used as a source for biosynthesis of sufficient amounts of metabolities for potential positive identification. The major metabolite comprised 60.3% of the total organosoluble radioactive products and had an R_f value of 0.12 in 4:1 ether–hexane and 0.31 in ether. The unknown was assigned the structure 6,7-dimethoxy-2,2-dimethylchroman-3,4-diol because it cochromatographed with the chemically synthesized diol in both TLC systems, had the same retention times on GPC and GC, and had an identical mass spectrum as that of synthetic precocene II diol.

The second largest metabolite (17.1% of total) moved on TLC with an R_f of 0.44 in 4:1 ether–hexane and 0.75 in ether. Its retention time on GC and its mass spectrum were the same as that of chemically synthesized 6,7-dimethoxy-2,2-dimethylchroman-3-ol. Interestingly, none of the unknown bands or peaks on TLC or GC corresponded to the dimethylchroman-4-ol.

Finally, one of the minor products (3.9%) was identified as epoxy precocene II based on its R_f values in 4:1 ether–hexane (0.85) and 93:7 benzene–ethyl acetate (0.41), its GC retention time, and its mass spectrum which was identical with 3,4-epoxy-6,7-dimethoxy-2,2-dimethylchroman. At this time, it is not known whether epoxy precocene II serves as an intermediate in the formation of precocene II diol, but it is certainly a possible metabolic pathway.

All of the synthetic metabolites were bioassayed for antijuvenile hormone activity using the milkweed bug as the test organism (Bowers et al., 1976). However, none of the compounds were active.

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Mechanisms by Which Methamidophos and Acephate Circumvent Resistance to Organophosphate Insecticides in the Housefly

Pin Suksayretrup and Frederick W. Plapp, Jr.*

Methamidophos and acephate are nearly as toxic to resistant strains of the housefly as to susceptible strains. Biochemical data indicate these insecticides are poor substrates for microsomal oxidases and glutathione-dependent transferases, enzymes important in detoxifying and conferring resistance to insecticides. The lack of resistance to methamidophos and acephate may be due, therefore, to their lack of reactivity with these enzymes. Both insecticides inhibit housefly cholinesterase and aliesterase in vivo but are poor inhibitors in vitro. Bioassay data show slow knockdown rates for flies exposed to lethal doses of the insecticides. Therefore, the chemicals may have to be metabolically activated in vivo to become toxic.

Methamidophos and its *N*-acetylated derivative, acephate, are relatively new phosphoramidothioate insecticides. Methamidophos shows a broad spectrum of activity against houseflies resistant to various organophosphate (OP), chlorinated hydrocarbon, and carbamate insecticides as well as susceptible housefly strains (Khasawinah, 1970; Quistad et al., 1970). Both insecticides are also toxic to susceptible and OP-resistant *Heliothis* sp. that attack cotton (Plapp, 1972). Acephate is as toxic as methamidophos to the housefly and is much less toxic to mammals (Chevron Chemical Company, 1973a,b).

Flies treated with lethal doses of methamidophos respond slowly (Khasawinah, 1970). Symptoms of poisoning correspond to maximum in vivo thoracic and head cholinesterase (ChE) inhibition. Aliesterase (ali-E) is also inhibited in vivo. No activation or degradation products were found in extracts of houseflies treated with [^{32}P]-methamidophos, but a number of hydrolytic products were detected by in vitro incubation of methamidophos with housefly preparations. It was concluded by Khasawinah (1970) that the relative stability of methamidophos to degradation in vivo is of critical importance in maintaining

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