Phytochemistry, Vol. 29, No. 9, pp. 2793–2796, 1990.
Printed in Great Britain.

0031-9422/90 $3.00+0.00

RING-METHYL HYDROXYLATION OF CHLORTOLURON BY AN INDUCIBLE CYTOCHROME P450-DEPENDENT ENZYME FROM MAIZE

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(*Received in revised form* 9 *February* 1990)

Key Word Index—*Zea mays*; Gramineae; pesticide metabolism; chlortoluron; cytochrome P450 monooxygenase; hydroxylation.

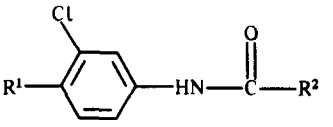
Abstract—An enzyme catalysing ring-methyl hydroxylation of the substituted phenylurea herbicide chlortoluron was detected in isolated microsomes from germinating maize. Chlortoluron hydroxylation was absolutely dependent on NADPH and molecular oxygen. The enzyme was inhibited by carbon monoxide in the presence of oxygen and this inhibition could be reversed by light. Several known inhibitors of cytochrome P450 enzymes inhibited chlortoluron hydroxylation to varying degrees. It is concluded that the newly detected enzyme is a cytochrome P450-dependent mixed function oxidase. Enzyme activity was stimulated 15-fold by treatment of maize seeds with the herbicide antidote CGA 154281 [4-(dichloroacetyl)-3,4-dihydro-3-methyl-2H-1,4-benzoxazine].

INTRODUCTION

Oxidative metabolism of herbicides in plants has been reported in a number of cases to be mediated by microsomal mixed function oxidases, though involvement of cytochrome P450 has not always been definitely proven [1]. Chlortoluron [*N'*-(3-chloro-4-methylphenyl)-*N*,*N*-dimethylurea] is a substituted phenylurea herbicide used for selective weed control in winter wheat (*Triticum aestivum* L.) and barley (*Hordeum vulgare* L.) [2]. This herbicide is metabolized in plants by a combination of oxidative *N*-demethylations and hydroxylation of the ring-methyl group [3, 4]. The benzyl alcohol derivatives may slowly undergo further oxidation to benzoic acids. All oxidized metabolites of chlortoluron are available for glycosyl conjugations. The relative tolerance of wheat and barley to chlortoluron has been attributed largely to the high capacity of these species to perform ring-methyl hydroxylation [4]. On the other hand, efficient *N*-didemethylation may afford herbicide tolerance as observed in cotton (*Gossypium hirsutum* L.) [4]. Only recently *N*-demethylation of chlortoluron has been demonstrated in microsomes from Jerusalem artichoke (*Helianthus tuberosus* L.) tubers, and this activity could be ascribed to a previously unknown cytochrome P450 isozyme [5]. *N*-Demethylation of the related phenylurea herbicide monuron has been demonstrated in microsomes from cotton seedlings [6]. Based on inhibitor studies *in vivo* cytochrome P450 has been suggested to be involved in ring-methyl hydroxylation of chlortoluron [7, 8]. In the present report, we describe the ring-methyl hydroxylation of chlortoluron *in vitro* by an inducible cytochrome P450-dependent mixed function oxidase from germinating maize (*Zea mays* L. cv Blizzard).

RESULTS AND DISCUSSION

Incubation of ^{14}C-labelled chlortoluron (1) in the presence of microsomes isolated from germinating maize and NADPH led to the formation of several more polar products (Table 1). The two major metabolites from [^{14}C]chlortoluron were identified as ring-methyl hydroxylated derivatives, namely *N'*-(3-chloro-4-hydroxymethylphenyl)-*N*,*N*-dimethylurea (I) and, *N'*-(3-chloro-4-hydroxymethylphenyl)-*N*-methylurea (II). These together accounted for over 80% of the radiolabelled metabolites. The ratio between ring-methyl hydroxylated chlortoluron (I) and its *N*-monodemethylated analogue (II) remained constant during an incubation period from 0.5 to 6 hr. Minor compounds resulted from further oxidation of the benzyl alcohol derivative (I) to the corresponding benzoic acid (V) and from *N*-monodemethylation of [^{14}C]chlortoluron, forming *N'*-(3-chloro-4-methylphenyl)-*N*-methylurea (III). *N*-didemethylated [^{14}C]chlortoluron (IV) as well as some unidentified metabolites were found in trace amounts (Table 1). No ring-methyl hydroxylated *N*-didemethylated chlortoluron was detected using either 1D-TLC or 2D-TLC. Neither the ethyl acetate extract nor the initial



1 R^1 = Me, R^2 = $N(Me)_2$

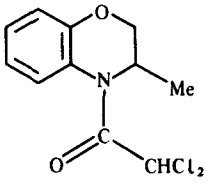


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Table 1. Radiolabelled metabolites formed from ^{14}C-labelled chlortoluron in isolated maize microsomes

Compounds	R^1	R^2	Radioactivity (cpm)*
I	CH_2OH	$N(Me)_2$	17340±2040
II	CH_2OH	NHMe	9960±1680
III	Me	NHMe	1560±480
IV	Me	NH_2	490±60
V	COOH	$N(Me)_2$	2100±80
Unidentified			1260±170

Isolated microsomes were incubated with [phenyl-U-^{14}C]-chlortoluron (10 kBq) in the presence of an NADPH generating system for 1 hr at 30°. Products were analysed by 2D-TLC.

Position of R^1 and R^2 groups were presented in the chemical structure of **1**.

*Data represent mean values of three experiments ±s.d.

assay mixture contained any detectable amount of polar conjugates. Thus, in the maize microsomal system hydroxylation of the ring-methyl group significantly predominated over *N*-demethylation. This is in agreement with a previous report on *in vivo* metabolism of chlortoluron in a maize cell suspension culture [8].

Formation of ring-methyl hydroxylated [^{14}C]chlortoluron required the presence of NADPH and molecular oxygen. Complete loss of ring-methyl hydroxylation was observed upon removal of either NADPH or oxygen from the reaction mixture. This indicates that a mixed function oxidase is involved in chlortoluron ring-methyl hydroxylation. Strong evidence for the participation of cytochrome P450 in this reaction is provided by CO-inhibition experiments (Table 2). Ring-methyl hydroxylation of [^{14}C]chlortoluron was significantly diminished by CO in the presence of oxygen and this inhibition could be partially reversed by illumination of the reaction mixture with white light. The effects of known inhibitors of plant cytochrome P450 enzymes [9–11] on [^{14}C]chlortoluron hydroxylation in the microsomal system are shown in Table 3. Strong interference with the microsomal enzyme was observed upon addition of the plant growth regulators and kaurene oxidase inhibitors [12], tetcyclacis and paclobutrazol. The former had previously been shown to inhibit both *N*-demethylation and ring-methyl hydroxylation of the herbicide in maize and cotton cells *in vivo* [8]. Considerable inhibition of chlortoluron ring-methyl hydroxylation by tetcyclacis was detected at a concentration of 1 μM. The fungicide propiconazole, also known to inhibit plant obtusifoliol 14α-methyl demethylase [13], was effective only at very high concentrations (Table 3). From the results presented above it is concluded that ring-methyl hydroxylation of chlortoluron in the isolated maize microsomes is catalysed by a cytochrome P450-dependent mixed function oxidase.

A variety of foreign compounds including phenobarbital, several herbicides, and divalent manganese ions have been reported to increase cytochrome P450 concentration in ageing Jerusalem artichoke (*Helianthus tuberosus* L.) tuber tissue and to stimulate cytochrome P450-linked in-chain lauric acid hydroxylase and cinnamic acid 4-hydroxylase [14, 15]. Increasing evidence indicates that herbicide-degrading enzymes in plants can be activated or induced by certain chemicals referred to as herbicide antidotes or 'safeners' [16]. In this study, we used maize tissue derived from seed that has been treated with a recently developed safener for maize, CGA 154281 [4-(dichloroacetyl)-3,4-dihydro-3-methyl-2H-1,4-benzoxazine] [17]. This safener treatment increased the activity of the microsomal chlortoluron ring-methyl hydroxylating enzyme up to 15-fold as compared to untreated control plants (Fig. 1). No effect was observed on the ratio of the two main metabolites (compounds I and II) formed following treatment with CGA 154281 up to 5 g active ingredient per kg of seed. The total cytochrome P450 concentration was also significantly (2-fold) elevated in microsomes isolated from CGA 154281-treated plants (Fig. 1). Several explanations would account for the disproportionality observed between the increase in cytochrome P450 content and the stimulation of ring-methyl hydroxylation following CGA 154281-treatment: (i) if chlortoluron ring-methyl hydroxylation is supported by the same cytochrome P450 isozyme in control as well as in treated plant microsomes, one must assume that this isozyme contributes only marginally to the total cytochrome P450 content in control maize. (ii) Another possibility would be that in CGA 154281-treated plant microsomes an additional, not constitutive cytochrome P450 isozyme contributes to the enhancement of the ring-methyl hydroxylating activity. Chlortoluron ring-methyl hydroxylase activity was rather low even in induced seedlings. It might be speculated that this microsomal enzyme activity would be higher in more tolerant species or after treatment with other safeners.

Nevertheless, the present report provides evidence for the involvement of a cytochrome P450-dependent enzyme in the ring-methyl hydroxylation of chlortoluron in maize. We

Table 2. Inhibition of [^{14}C]chlortoluron ring-methyl hydroxylation by carbon monoxide

	Chlortoluron ring-methyl hydroxylation		
Composition of gas atmosphere	Relative activity (%)	Inhibition (%)	Reversion (%)
Air + light*	100	—	—
CO–O_2–N_2 1:2:7	43	57	—
CO–O_2–N_2 1:2:7 + light*	68	32	44

*White light was provided by a slide projector. 100% activity corresponded to 802±13 pmol hr^{-1} mg^{-1} (mean ±s.d. of two experiments).

Table 3. Effects of inhibitors of cytochrome P450 enzymes on [^{14}C]chlortoluron ring-methyl hydroxylation in maize microsomes

Inhibitor		Inhibition of chlortoluron ring-methyl hydroxylation (%)
None	—	0*
Tetcyclacis	100	92
(μM)	10	79
	1	60
Paclobutrazol	100	91
(μM)	10	41
Propiconazole	200	59
(μM)	100	14

*0% Inhibition corresponded to a specific activity of 345 ± 12 pmol hr^{-1} mg^{-1}. All data are means of two experiments.

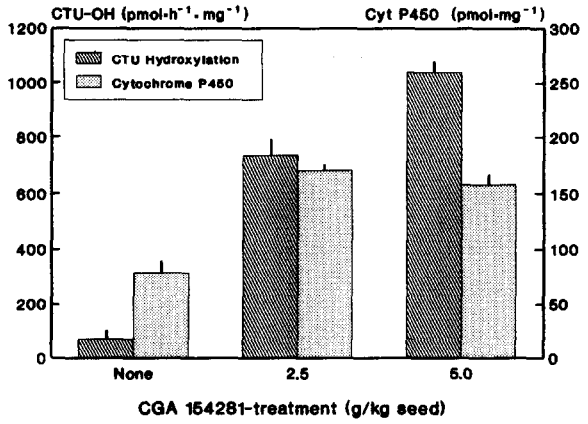

Fig. 1. Effect of the herbicide antidote CGA 154281 on chlortoluron ring-methyl hydroxylation and cytochrome P450 concentration in maize microsomes. Maize seed was treated, where indicated, with CGA 154281 (2.5 g kg^{-1} or 5 g kg^{-1}) and allowed to germinate for 48 hr. Microsomes were prepared from the seedling tissues and assayed for chlortoluron ring-methyl hydroxylase activity and cytochrome P450 concentration (see Experimental). CTU, chlortoluron; CTU-OH, ring-methyl hydroxylated chlortoluron. Bars represent means $\pm$s.d. of three experiments.

have also shown that this enzyme activity is strongly stimulated by treatment of plants with a structurally unrelated, non-herbicidal compound that acts as a herbicide antidote *in vivo* [17]. The mechanism of the observed cytochrome P450 induction, however, remains to be elucidated. It is the aim of our current work to characterize other enzymes involved in oxidative herbicide metabolism and to investigate their regulation by endogenous and exogenous factors.

EXPERIMENTAL

Chemicals. [phenyl-U-^{14}C]Chlortoluron (sp. act. 1.95 MBq mg^{-1}), non-labelled reference derivatives of chlortoluron, and CGA 154281 [4-(dichloroacetyl)-3,4-dihydro-3-methyl-2H-1,4-benzoxazine] were synthesized in the laboratories of Ciba-Geigy Ltd, Basel, Switzerland.

Plant material. Seeds of maize (*Zea mays* L. cv Blizzard) were coated with the safener CGA 154281: 1 kg of seed was treated with a dry powder containing 2.5 or 5 g of CGA 154281 active ingredient in a 25% (w/w) wettable powder formulation by rotation in a flask for 30 min. Coated seeds were allowed to germinate in moist vermiculite for 48 hr at 25°, in the dark. Seedling tissues including shoots and radicles were then excised from the caryopses and stored on ice prior to the isolation of microsomes.

Preparation of microsomes. All operations were performed at 4°. Maize seedling tissue was homogenized using an Ultra-Turrax homogenizer in 3 vol of 0.1 M Tris–HCl pH 7.5, 0.5 M sucrose, 10 mM $MgCl_2$, 1 mM EDTA and 5 mM GSH. The homogenate was filtrated through two layers of Miracloth and centrifuged at 6000 *g* for 10 min. The resulting supernatant was further centrifuged at 105000 *g* for 90 min. The microsomal pellets were resuspended in 0.1 M K-Pi buffer, pH 7.5, containing 4 mM $MgCl_2$, 1 mM EDTA, 1 mM GSH and 20% (v/v) glycerol. These membrane preparations were then stored at −80° without detectable loss of activity over a period of 6 months.

Enzyme assay. Standard assays for chlortoluron ring-methyl hydroxylation contained in a final vol of 0.5 ml: 0.1 M Na-Pi buffer, pH 7.4, 1 mM NADPH, 2 mM G-6-P, 1 IU G-6-P dehydrogenase, [phenyl-U-^{14}C]chlortoluron (10 kBq) and non-labelled chlortoluron to a final concn of 200 μM, and 2 mg microsomal protein. Incubations were carried out at 30° for 1 hr and terminated by the addition of 0.5 ml 4 M HCl. Under the assay conditions, product formation was found to be linear with respect to time and protein concentration. For some experiments, O_2-depleted incubation mixtures were obtained by the addition of 50 nmol Glc, 2.5 IU Glc oxidase and 10 IU catalase.

Analytical procedures. Chlortoluron and its products were extracted ($\times 3$) with 5 ml EtOAc. Extraction efficiency exceeded 98% in all experiments. The extracts were concd *in vacuo* and initially analysed by 2D-TLC on silica gel 60 F_{254} plates (Merck) first in $CHCl_3$–EtOH–conc NH_3 (36:8:1), followed by $CHCl_3$–EtOH–HOAc (9:1:1) and thereafter routinely by 1D-TLC in the latter solvent system. Radioactive zones were localized and quantified using a TLC linear analyzer. Metabolites were identified by co-chromatography with nonlabelled reference compounds. Cytochrome P450 concentrations were measured spectrophotometrically [18]. Protein was determined using the Bio-Rad protein assay.

Acknowledgements—We wish to thank Martine Boehler and Fritz Karlin for their skillful technical assistance, and Dr F. Waechter for valuable discussions and critical reading of the manuscript.

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