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·Research report·

Inhibition of Acetolactate Synthase Activity by Chlorsulfuron, Triasulfuron and Their Metabolites in vitro

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Abstract: The metabolism of two sulfonylurea herbicides chlorsulfuron and triasulfuron was investigated by using wheat cytochrome P450 cDNA (CYP71C6v1) which expressed in yeast. The results showed that chlorsulfuron and triasulfuron were hydroxylated at 5-position on the phenyl ring. The difference in IC_{50} of parent compound and 5-hydroxy metabolites to acetolactate synthase (ALS) was small. However, their metabolites injured neither wheat nor bean, when applied as post-emergence treatment. The IC_{50} of chlorsulfuron and its metabolites to wheat ALS were 7.1×10^{-9} and 7.9×10^{-9} mol/L, respectively, and to bean ALS were 3.6×10^{-9} and 4.1×10^{-9} mol/L, respectively. The IC_{50} of triasulfuron and its metabolites to wheat ALS were 4.6×10^{-9} and 5.3×10^{-9} mol/L, respectively, and to bean ALS were 4.7×10^{-9} and 4.9×10^{-9} mol/L, respectively. These results suggested that proper groups at 5-position on phenyl ring of sulfonylurea molecule might possess high herbicidal activity.
Key words: cytochrome P450; chlorsulfuron; triasulfuron; metabolite; acetolactate synthase; inhibition

绿磺隆、醚苯磺隆及其体外代谢物对乙酰乳酸合成酶的离体抑制作用

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摘 要: 利用异源表达于酵母细胞中的小麦细胞色素 P450cDNA (CYP71C6v1) 研究了磺酰脲类除草剂绿磺隆、醚苯磺隆的代谢作用。结果表明, 代谢产物 5羟基绿磺隆和 5羟基醚苯磺隆能够抑制乙酰乳酸合成酶 (ALS酶)活性, 且代谢产物与母体化合物绿磺隆、醚苯磺隆抑制 ALS酶活性的 IC_{50}值差异小, 但是代谢产物在茎叶喷雾小麦和菜豆时, 均未表现出活性。绿磺隆及其代谢产物抑制小麦 ALS酶活性的 IC_{50}值分别为 7.1×10^{-9}和 7.9×10^{-9} mol/L, 抑制菜豆 ALS酶活性的 IC_{50}分别为 3.6×10^{-9}和 4.1×10^{-9} mol/L; 醚苯磺隆及其代谢产物抑制小麦 ALS酶活性的 IC_{50}分别为 4.6×10^{-9}和 5.3×10^{-9} mol/L, 抑制菜豆 ALS酶活性的 IC_{50}分别为 4.7×10^{-9}和 4.9×10^{-9} mol/L。结果表明, 在磺酰脲类分子苯环 5位上进行结构改造, 有可能得到高活性的化合物。
关键词: 细胞色素 P450; 绿磺隆; 醚苯磺隆; 代谢产物; 乙酰乳酸合成酶; 抑制作用
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Introduction

Metabolism of pesticides in plant usually results in deactivation or activation. The activation of pesticide is significant for the discovery of new pesticides. Some organophosphorus insecticides require activation via oxidative desulfuration in the plant to form the oxygen analogue, which are potent anticholinesterases. For example, organophorus systemic insecticide dimethoate, its oxygen analogue, and particularly its N-demethylated derivative by bean cytochrme P450, possess greater insecticidal and mammalian toxicity than the parent compound[1]. Bromoxynil octanoate is a pro-herbicide which is hydrolyzed in the plant to release the photosynthetic inhibitor bromoxynil[2]. The thiocarbamate pre-emergence herbicide EPTC (eptam) is readily converted to its highly active sulfoxide which has been shown in some studies to have greater phytotoxicity. However, the sulfoxide is further metabolized in the plant, and a hypothesis that this is the herbicidally active principle has fallen into doubts[3]. As mentioned previously, insecticides containing oxidizable sulfur can be activated in this way by the plant's metabolism[4,5]. Parent compound of herbicide naproanilide[6] and clomeprop[7] are not active, and acidic compounds formed by hydrolysis in the plant are activated. The activation of specific herbicide occurs only in the controlled weeds, such as the sulfonylurea DPX-L8747 and imazamethabenz-methyl. The intact DPX-L8747 is only weakly active against the target site enzyme, acetolactate synthase (ALS). N-dealkylation in susceptible A. fatua leads to the formation of potent ALS inhibitor[8]. By contrast, resistant wheat and soybean form hydroxylated or glutamylcysteinylglycine derivatives, respectively, of the intact pro-herbicide. The imidazolinone pro-herbicide imazamethabenz-methyl is hydrolyzed to the active ALS inhibitor acid in susceptible weeds, whereas hydroxylation of the intact ester occurs in resistant maize and wheat[9]. The oxadiazolidine ring of methazole requires scission to photosynthetic inhibitor 1-(3, 4-dichlorophenyl) urea in susceptible plants[10]. Selectivity appears to be related to the persistence of this intermediate, governed by the rates of its production and subsequent detoxification by N-demethylation. The pyridazinone pro-herbicide, metflurazon, requires N-demethylation to form the active inhibitor of phytoene desaturase, a target site enzyme of carotenoid biosynthesis[11].

The metabolites from bioactivation reaction could be leading compounds for the discovery of novel active compounds. In this paper, chlorsulfuron and triasulfuron were metabolized by recombinant wheat cytochrome P450 monooxygenase expressed in yeast, and thus 5-hydroxy-chlorsulfuron and 5-hydroxy-triasulfuron were formed. Inhibition of ALS activity by 5-hydroxy-chlorsulfuron and 5-hydroxy-triasulfuron was studied.

1 Materials and Methods

1.1 Chemicals

Chlorsulfuron, triasulfuron, 1-(2-chlorophenylsulfonyl)-3-(4-methyoxy-6-methylh-1, 3, 5-triazin-2-yl) urea, of 98.3% purity, 1-(2-chloroethoxyphenylsulfonyl)-3-(6-methoxy-4-methyl-1, 3, 5-triazin-2-yl) urea, of 98.7% purity, were provided by the Institute of Shenyang Chemical Engineering, China. P450 microsomal protein obtained from wheat cytochrome P450 cDNA (CYP71C6v1) which expressed in yeast[12]. All other chemicals were obtained from Pharmacia Company.

^{1}H NMR was measured with a Bruker DRX-400 spectrometer; The FAB was taken on a Q-TOF mass spectrometer.

1.2 Herbicide metabolism in vitro

Herbicide metabolism and P450 enzyme activity assay in vitro was detected as described by Peter et al[13]. The reaction mixture (25 μL) contained 25～50 μg microsomal protein, 10～150 μmol/L herbicide in 0.1 mol/L MOPS [3-(N-morpholino) propanesulfonic acid] (pH 7.0), 2 mg/mL BSA, 400 μmol/L NADPH (reduced nicotinamide-adenine dinucleotide phosphate), 20 mmol/L glucose-6-phosphate, 0.1 U glucose-6-phosphate dehydrogenase, finally, add 0.1 mol/L MOPS (pH 7.0) to reach a total volume of 25 μL.

The reaction was started by NADPH. Glucose-6-phosphate and dehydrogenase were added to regenerate NADPH for prolonged incubation, although for time of less 30 min they are unnecessary with microsomes. BSA addition increased the activity of microsomes. The incubation was carried out at 27℃

and terminated by adding acetonitrile (75 μL). After that the mixture was centrifuged at 14 000 g for 10 min to remove most of the precipitated proteins, the supernatant was transferred to another microfuge tube containing 150 μL of water and was then analyzed by reverse-phase HPLC with an ODS-18 (4.6 ×250 mm) column, and a mobile phase of acetonitrile + water + phosphoric acid (20 + 80 + 0.2 by volume). All solvents were HPLC grade and filtered and degassed before use. Substrates and products were quantified by a calibration curve using a UV detector (Shimadzu SPD-10AUV-VIS detector, wavelength 225 nm). The peaks were identified by comparing the retention time with analytical standards of herbicides. P450 activity was calculated as pmol product · mg^{-1} microsomal protein · min^{-1}.

1.3 Mass spectral and ^1H NMR analysis

After separation by preparative TLC with benzene-acetone-formic acid (30 ∶10 ∶1 in volume), metabolites of chlorsulfuron or triasulfuron zone were eluted with methanol. The structure of metabolites was analyzed by FAB/MS and ^1H NMR.

1.4 Acetolactate synthase extraction and activity assay

ALS was extracted and enzyme activity were measured in the presence of herbicide or metabolite using the methods outlined by Hart et al [14, 15] with a little modification. All extraction, centrifugation and column separation procedures were conducted on ice or at 4℃. 10-day-seedlings of wheat or bean samples were a composite of newly formed leaves excised from the apex of several plants. Each 10 g of sample was homogenized in a homogenization buffer [0.1 mol/L K_2HPO_4, pH 7.5, 1 mmol/L sodium pyruvate, 0.5 mmol/L $MgCl_2$, 0.5 mmol/L thiamine pyrophosphate, 10 mmol/L FAD (flavin adenine dinucleotide), 10 % (V/V) glycerol] equivalent to 2.5 times the weight of the plant tissue. The homogenate was filtered through eight layers of cheesecloth and then centrifuged at 30 000 ×g for 20 min. The supernatant was brought to 50% saturation with cold $(NH_4)_2SO_4$ and allowed to stand 1 h on ice. The mixture was then centrifuged at 15 000 ×g for 15 min. The supernatant was discarded and the precipitated pellet was dissolved in resuspension buffer (0.1 mol/L K_2HPO_4, 20 mmol/L sodium pyruvate, 0.23 mmol/L $MgCl_2$, pH 7.5). The solution was passed through a Sephadex G-25 column equilibrated with the same buffer. The desalted enzyme preparation was immediately used for enzyme assays.

ALS enzyme assays were carried out in a final volume of 1.5 mL, containing the enzyme preparation, reaction buffer (25 mmol/L K_2HPO_4, 0.625 mmol/L $MgCl_2$, 25 mmol/L sodium pyruvate, 0.625 mmol/L thiamine pyrophosphate, 1.25 μmol/L FAD, pH 7.0), and technical grade herbicide or metabolite at 1.0×10^{-8} to 2.0×10^{-8} mol/L. Reaction tubes were incubated at 35 ℃ for 1 h before the reaction was stopped by the addition of 50 μL of 6 mol/L H_2SO_4, and the solution tubes was heated for 15 min at 60℃. Then 0.5 mL of 0.5 % weight by volume of α-naphthol, which freshly prepared in 2.5 mol/L NaOH, was added consecutively to each tube. The solutions was heated for an additional 15 min at 60 ℃ and the acetoin content measured by the method of Westerfield[16].

There were three replicates for each herbicide concentration. Data presented were the means of the 3 experiments. ALS enzyme activity was presented as a percent of control assays.

1.5 Crop response to metabolites

Wheat and bean at one-leaf stage were dripped with solution consisting of chlorsulfuron, triasulfuron or their metabolites, 20% actetone and 0.25% Tween for experiment. The rates were 1 μg per wheat and 2 μg per wheat. Visual response was taken on the fourteenth day after treatment.

2 Results and discussion

2.1 Analysis of metabolites

A negative FAB mass spectrographic data of the isolated chlorsulfuron metabolites were shown in Table 1. Analysis of chlorsulfuron metabolites demonstrated the presence of a hydroxyl group on the phenyl ring, fragment ions at m/z 127, 191, 206 and 234 corresponding to $C_6H_3Cl(OH)$, $C_6H_3Cl(OH)SO_2$, $C_6H_3Cl(OH)SO_2NH$ and $C_6H_3Cl(OH)SO_2NHCO$ ions, respectively. Furthermore, the ^1H NMR data and the coupling constant of metabolites were consistent with the previous chlorsulfuron metabolism by wheat

cytochrome P450 microsomes and in both wheat and corn, which demonstrated that chlorsulfuron was hydroxylated at the 5-position on the phenyl ring[17].

A negative FAB MS data and ^{1}H NMR data of the isolated triasulfuron metabolites were shown in Table 1. These data provided that hydroxylation of triasulfuron occurred at 5-position on the pheny1 ring.

Table 1 ^{1}H NMR and MS data of the metabolites

Metabolites	MS, m/z	^{1}H NMR, δ(CDCl$_3$)
5-Hydroxy-chlorsulfuron	372 (M-1), 232, 206, 190, 181, 139, 127	7.71 (d, 1H, J=3.7 Hz, Ar-H), 7.16 (d, 1H, J=8.6 Hz, Ar-H), 6,81 (dd, 1H, J=3.7, 8.6 Hz, Ar-H),
5-Hydroxy-triasulfuron	416 (M-1), 276, 234, 181, 170 139, 107	7.67 (d, 1H, J=3.5 Hz, Ar-H), 6.98 (d, 1H, J=8.4 Hz, Ar-H), 6.60 (dd, 1H, J=3.5, 8.4 Hz, Ar-H)

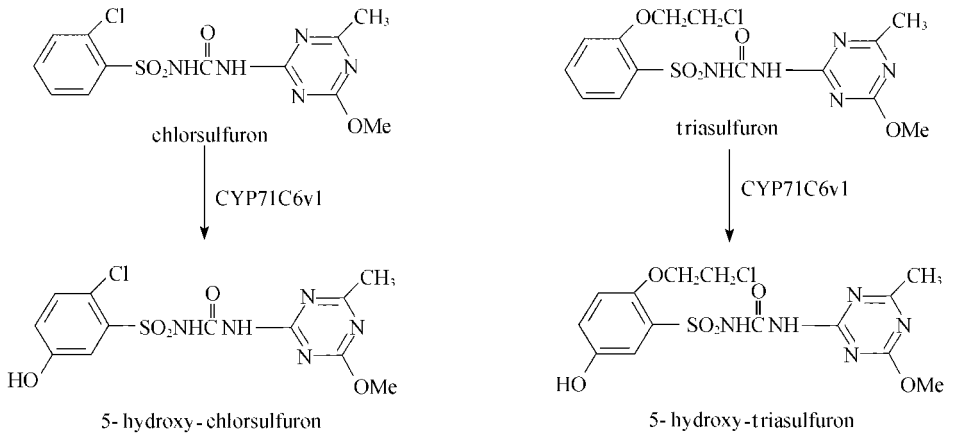

Fig. 1 Metabolism of chlorsulfuron and triasulfuron by CYP71c6v1

2.2 Inhibition of ALS activity by chlorsulfuron and triasulfuron metabolites in vitro

Wheat and bean leaves at one-leaf stage were dripped into solution consisting of chlorsulfuron, triasulfuron or their metabolites, respectively. When the rates were 1 μg per wheat and 2 μg per wheat, there was no any injury of chlorsulfuron, triasulfuron and their metabolites to wheat observed, whereas the injury of chlorsulfuron, triasulfuron and their metabolites to bean was found.

In vitro study showed that chlorsulfuron, triasulfuron and their metabolites inhibited ALS activity. Moreover, the difference in IC_{50} by parent compound and metabolites to ALS activity was very small (Fig. 2). The IC_{50} of chlorsulfuron and its metabolites to wheat ALS were 7.1×10^{-9} and 7.9×10^{-9} mol/L, respectively (Fig. 2a), to bean ALS were 3.6×10^{-9} and 4.1×10^{-9} mol/L, respectively (Fig. 2c). The IC_{50} of triasulfuron and its metabolites to wheat ALS were 4.6×10^{-9} and 5.3×10^{-9} mol/L, respectively (Fig. 2b), and to bean ALS were 4.7×10^{-9} and 4.9×10^{-9} mol/L, respectively (Fig. 2d). The metabolites didn't injury plants but inhibited ALS. Based on the results, we deduced that 5-hydroxy-chlorsulfuron and 5-hydroxy-triasulfuron might be rapidly transformed to glucose conjugate, which had a greatly decreased ability to inhibit ALS[17].

5-Hydroxy chlorsulfuron and 5-hydroxy triasulfuron in vitro could inhibit ALS activity, this result was in contradiction with previous reports (Fig. 3)[18, 19].

Early reports indicated that sulfonylurea could have herbicidal activity only under the case that 5-position on phenyl ring was unsubstituted. However,

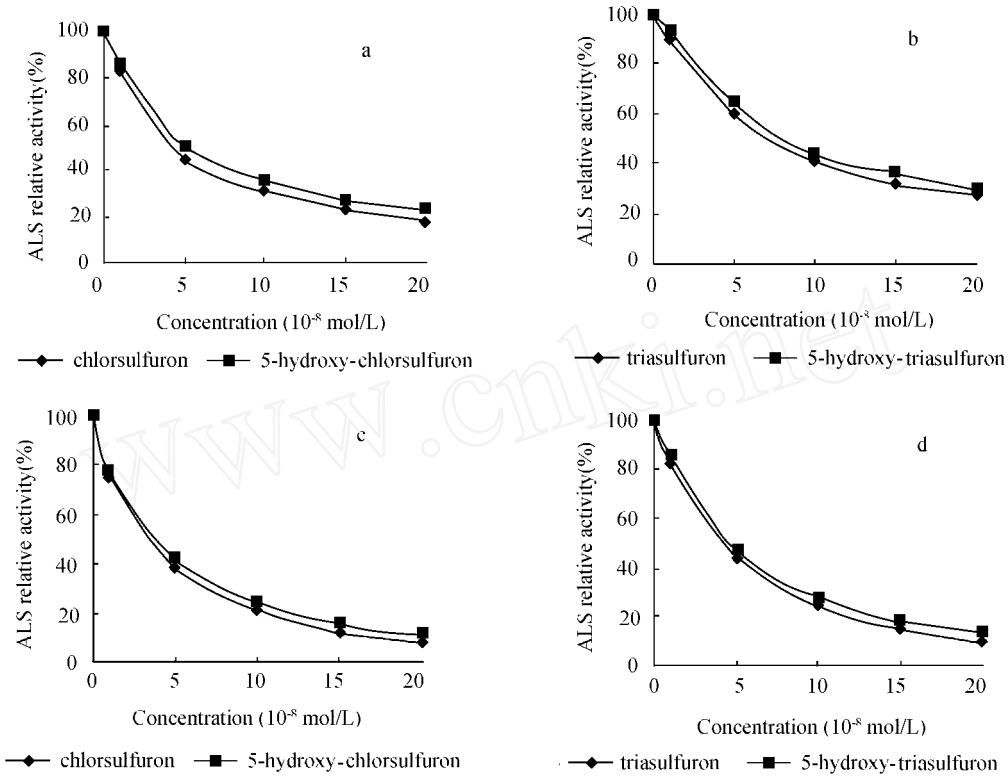

Fig. 2 Inhibition of ALS activity from wheat and bean by chlorsulfuron, triasulfuron and their metabolite

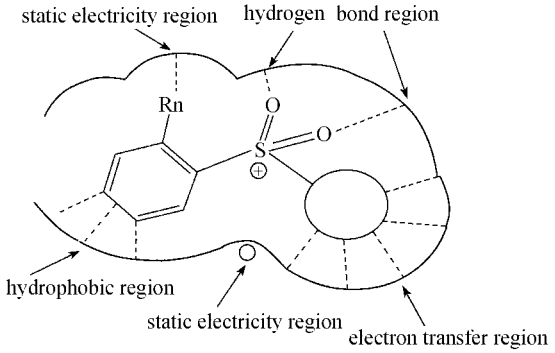

Fig. 3 Elementary model of interaction between sulfonylurea, sulfonamide herbicide molecule and ALS[18, 19]

recent reports[20] demonstrated a series of sulfonylurea compounds with groups at 5-position on phenyl ring had herbicidal activity (Fig. 4). Furthermore, indosulfuron has been commercialized. When the rate was 10 g ai/ha, indosulfuron could control many broadleaf weeds and certain grassy weeds in wheat. Lately Hacker and Bieringer[21] applied for a patent about a series of active sulfonylurea compounds with groups at 5-position on phenyl ring (Fig. 5). These results suggested proper groups at 5-position on phenyl ring of sulfonylurea molecule might possess high herbicidal activity.

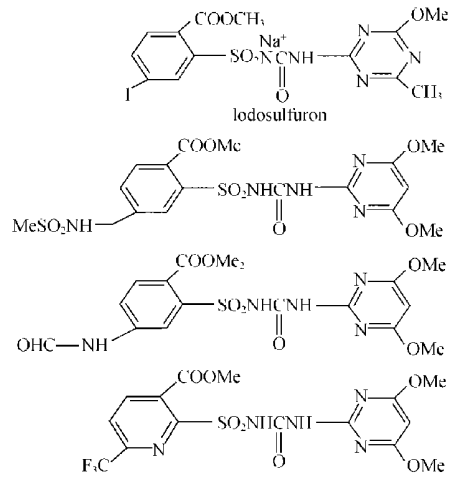

Fig. 4 Structures of sulfonylurea herbicide with groups at 5-position

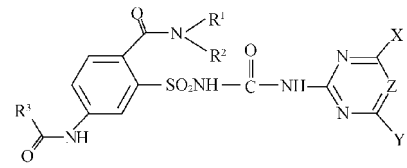

Fig. 5 Herbicide structures of acylated aminophenylsulfonylureas

R^1 and R^2 are hydrogen or alkyl (C_1～C_4), preferably methyl or ethyl, in particular methyl. R^3 is hydrogen or alkyl (C_1～C_4), alkoxy (C_1～C_4), alkenoxy (C_2～C_4), alkynoxy (C_2～C_4), cycloalkyl (C_3～C_6). One of the radicals X and Y is hydrogen or alkyl (C_1～C_4), alkylthio (C_1～C_4), alkylthio (C_1～C_4).

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