

The Mechanism of Action of Bensulfuron-Methyl on Hydrilla

M. R. RATTRAY, G. MACDONALD, D. SHILLING AND G. BOWES¹

ABSTRACT

Bensulfuron-methyl has been reported to specifically inhibit acetohydroxyacid synthase (AHAS, E.C. 4.1.3.18) in susceptible terrestrial plants. AHAS is the first enzyme unique to the synthesis of the branched-chain amino acids leucine, isoleucine and valine. Extraction of AHAS from the submersed aquatic plant hydrilla (*Hydrilla verticillata* (L.f.) Royle) and the assay conditions used are described. *In vitro* inhibition of AHAS by bensulfuron-methyl was significant. The addition of 1 nM of herbicide resulted in a 25% inhibition of enzyme activity. Increases in herbicide concentration caused significant increases in inhibition (1 mM = 93% inhibition). The I_{50} was calculated to be 22 nM. The level of

inhibition was also shown to be time-dependent. *In vivo* inhibition of hydrilla by bensulfuron-methyl was also investigated and assessed as growth inhibition ($I_{50} = 110$ nM). These results suggest that the inhibition of hydrilla growth by bensulfuron-methyl is due to the inhibition of AHAS.

Key words: acetohydroxyacid synthase, AHAS, sulfonylurea.

INTRODUCTION

The aquatic herbicide bensulfuron-methyl (methyl 2-[[[[[(4,6 dimethoxypyrimidin-2-yl)amino]carbonyl]amino]sulfonyl] methyl]benzoate): Mariner®) is currently being tested for its effectiveness in controlling nuisance aquatic plants. Bensulfuron-methyl is a member of the class of herbicides known as the sulfonylureas which includes such herbicides as chlorsulfuron (Glean®) and chlorimuron-ethyl

¹Departments of Botany and Agronomy, University of Florida, Gainesville, FL 32611, USA.

(Classic®). Sulfonylureas exhibit extremely high levels of herbicidal activity (as low as 1 g ha^{-1}) and very low mammalian toxicity. As herbicides, they are very potent inhibitors of plant growth with visual symptoms often occurring within 1 or 2 days of treatment in some broadleaf species. A wide range of secondary plant responses often develop depending on the species being treated and the environmental conditions.

The mechanism-of-action of the sulfonylurea herbicides involves the inhibition of the enzyme acetohydroxyacid synthase (AHAS, EC4.1.3.18; also known as acetolactate synthase, ALS). AHAS is the first enzyme unique to the biosynthesis of the branched chain amino acids leucine, valine and isoleucine. AHAS catalyzes (a) the condensation of two pyruvate molecules to form CO_2 and α -acetolactate which leads to the production of valine and leucine and (b) the condensation of one molecule of pyruvate with α -ketobutyrate to form CO_2 and α -aceto- α -hydroxybutyrate which leads to isoleucine biosynthesis. Several publications have recently discussed the methodologies involved in assaying AHAS and the effects of inhibitors such as the sulfonylurea herbicides on the activity of this enzyme (Singh *et al.* 1988, Ray 1984, Schloss 1990, Landstein *et al.* 1990, Durner *et al.* 1991 and Shaner and Little 1989).

In this paper, the effect of bensulfuron-methyl on the growth of hydrilla and the activity of AHAS extracted from hydrilla will be discussed.

METHODS AND MATERIALS.

In vivo study. Hydrilla was established from 10-cm apical stem segments into 40-L aquaria under greenhouse conditions (16:8 h light/dark, $1000 \mu\text{mol m}^{-2} \text{s}^{-1}$ PAR, 30/20C day/night). Plants were grown for 18 days prior to the onset of the treatment. Bensulfuron-methyl was added as a one-time addition to bring the initial water concentrations to 0, 0.12, 1.2, 2.4, 12, 24, 122 and 244 nM. Five plants from each treatment were then removed for growth analysis after 3 days and 1, 2, 3, 4 and 6 weeks. At each harvest, shoot length, shoot number and fresh weight were recorded. Shoot tissue was dried for 3 days at 70C and the dry weights were then determined. The amount of growth or change (increase) in growth was also determined by subtracting the growth at each harvest time from the initial growth determined at the time of treatment. Regression analysis ($P < 0.10$) was used to determine the effect of rate and time for each growth parameter. These equations were then used to derive I_{50} values for the change in shoot length, number of shoots, and fresh and dry weights. I_{50} values will only be reported for the final harvest.

In vitro study. AHAS was extracted from hydrilla following the procedure of Ray (1984). The extraction solution consisted of 100 mM potassium phosphate (pH 7.5), 1 mM

sodium pyruvate, 1 mM MgCl_2 , 0.5 mM thiamine pyrophosphate (TPP), 10 μM flavin adenine dinucleotide (FAD), and 10% (w/v) glycerol. Plant material (10 to 20 g of 1- to 2-cm apical shoots) was ground in a mortar with a pestle and the homogenate filtered through 8 layers of cheesecloth. The filtrate was then centrifuged at 25000 g for 20 min. $(\text{NH}_4)_2\text{SO}_4$ (50% w/v) was then added to the supernatant. This mixture was then centrifuged at 25000 g for 20 min and the supernatant discarded. The pellet was dissolved in 50 mM potassium phosphate (pH 7.0), 20 mM sodium pyruvate, and 0.5 mM MgCl_2 . This was then desalted through a Sephadex G25 column prior to assaying for enzyme activity.

The assay method used for AHAS was based on the procedure of Singh *et al.* (1988). AHAS activity was measured by estimating the amount of acetolactate produced, after conversion by decarboxylation in the presence of acid to acetoin. Standard reaction mixture (500 μl total volume) contained the enzyme in 25 mM potassium phosphate buffer (pH 7.0), 200 mM sodium pyruvate, 0.5 mM TPP, 20 mM MgCl_2 , and 25 μM FAD. Various concentrations of bensulfuron-methyl were also added where appropriate. The reaction mixture was incubated at 37C for 60 min after which the reaction was stopped with the addition of 50 μl 6N H_2SO_4 . The reaction product was then decarboxylated at 60C for 15 min. The acetoin formed was determined by incubating with creatine (0.17%) and 1- α naphthol (1.7%) by the method of Westerfield (1945). Maximum color formation was achieved by incubation at 60C for 15 min and a further 15 min at room temperature. The absorption of the color complex was measured at 530 nm. Protein concentrations were determined following the procedure of Bradford (1976).

RESULTS AND DISCUSSION

Bensulfuron-methyl has been shown to inhibit the growth of hydrilla (Van and Vandiver 1992, Haller *et al.* 1992) and other aquatic plants (Anderson and Dechoretz 1988). However, to date, there has been little research into the actual mechanism-of-action of bensulfuron-methyl on aquatic plants. In this study, we have investigated both the inhibition of vegetative growth in hydrilla and the inhibition of AHAS, a key enzyme in branched chain amino acid biosynthesis.

Based on shoot length, the effect of bensulfuron-methyl at 12 nM or higher significantly reduced growth (Figure 1). Growth was inhibited at 12 and 24 nM by approximately 40 and 50% of the control, respectively. At the two highest concentrations no increases in shoot length were recorded. Six weeks after initial treatment I_{50} values based on shoot length, number of shoots and fresh and dry weights ranged from 85 to 183 nM (Table 1). In several previous studies (Anderson and Dechoretz 1988, Haller *et al.* 1992, Van and

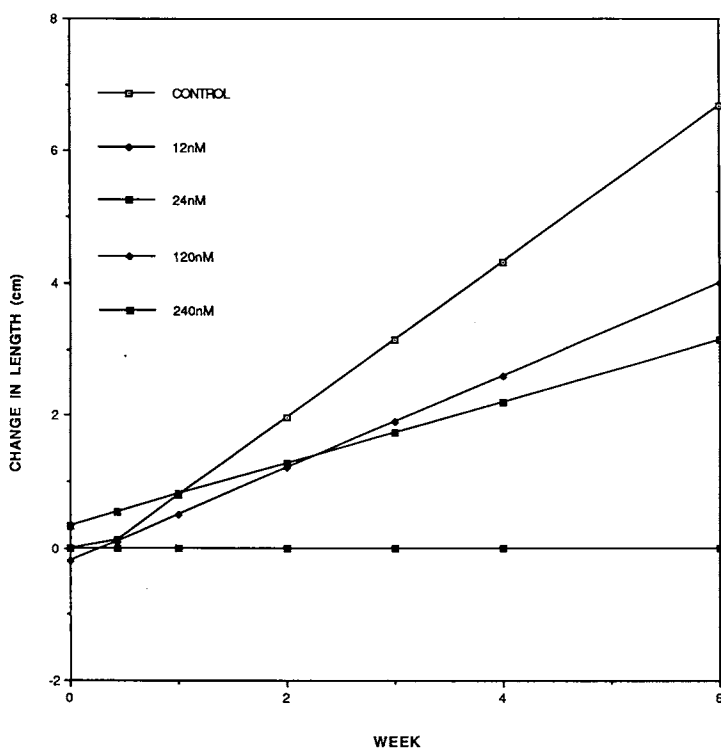


Figure 1. Effect of increasing concentrations of bensulfuron-methyl on the shoot growth of hydrilla.

TABLE 1. COMPARISON OF INHIBITION (50%) VALUES CALCULATED FOR HYDRILLA BASED ON GROWTH AND AHAS ACTIVITY, AND FOR SEVERAL TERRESTRIAL SPECIES BASED ON AHAS ACTIVITY (FROM RAY 1984)

Species	Treatment	I ₅₀ (nM)
Hydrilla		
(a) Growth		
-Shoot length	Bensulfuron-methyl	95.3
-Shoot number	Bensulfuron-methyl	101.6
-Fresh weight	Bensulfuron-methyl	85.3
-Dry weight	Bensulfuron-methyl	182.7
(b) AHAS activity	Bensulfuron-methyl	22.0
Pea	Chlorsulfuron	21.0
Wheat	Chlorsulfuron	18.5
Soybean	Chlorsulfuron	23.0
Tobacco	Chlorsulfuron	28.3
Green Foxtail	Chlorsulfuron	25.8
Johnsongrass	Chlorsulfuron	35.9
Morning Glory	Chlorsulfuron	24.4

Vandiver 1992), vegetative growth of hydrilla as well as other aquatic plants was shown to be inhibited by a minimum of 24 nM. However, to this date no I₅₀ values for bensulfuron-methyl have been published. However, it is important to point out that the I₅₀ values calculated for this herbicide will be very dependent upon the rate of growth of the treated plant and the

duration of exposure. I₅₀ values determined at the shorter time intervals were higher than those presented (data not shown). The susceptibility of plants to sulfonylurea herbicides also varies greatly depending on various factors, including growth stage (Beyer *et al.* 1988). In hydrilla, Haller *et al.* (1992) noted that newly sprouted hydrilla plants were more susceptible to bensulfuron-methyl than mature plants. This would result in variable I₅₀ values depending on the growth phase of the plants.

One interesting symptom observed during this experiment was the apparent "hardening" of tissue when hydrilla is treated with this compound. Initial measurements suggest that there is a significant increase in the percent dry matter content in treated hydrilla relative to control plants (data not shown). The cause of such an increase is unclear but will be investigated as part of this ongoing project. Another important point related to this "hardening" is the need to be careful when selecting which growth parameter is suitable to use as an estimate of growth for the calculation of I₅₀ values. We concluded from the experiments described in this paper that by only using dry weight data, the activity of bensulfuron-methyl on hydrilla growth would be underestimated. In this instance, shoot length provided the most consistent measurement and appeared to provide the best estimate of herbicidal efficacy.

Secondly, we investigated the *in vitro* effect of bensulfuron-methyl on the activity of AHAS extracted from hydrilla. Bensulfuron-methyl inhibited AHAS activity with the addition of as little as 1 nM and the inhibition was dose dependent (Figure 2). At 1 μM, enzyme activity was 93% inhibited after a 60-min incubation period. An I₅₀ of 22 nM was calculated from these data and it is similar to values previously published for this class of herbicides with terrestrial plants (Ray 1984; Table 1). However, a comparison of the I₅₀ values calculated for hydrilla growth and hydrilla AHAS activity indicated that the value is five-fold higher in the whole plant system (Table 1). This difference was not unexpected and the relative difference, as stated earlier, will be dependent upon the growth stage of the plants treated. In addition, the I₅₀ values for growth parameters reflect virtually all factors influencing herbicidal activity. Factors such as microbial decomposition, plant metabolism, and adsorption to soil colloids would tend to decrease the concentration of the herbicide at the active site thus increasing the I₅₀.

The inhibition of AHAS activity was also shown to be time dependent; inhibition increased with increasing time in the presence of herbicide (Figure 3). This suggests that this enzyme may be even more sensitive to inhibition since even very low concentrations of bensulfuron-methyl might eventually cause some level of inhibition. The results obtained in this part of the study are consistent with the known mode-of-

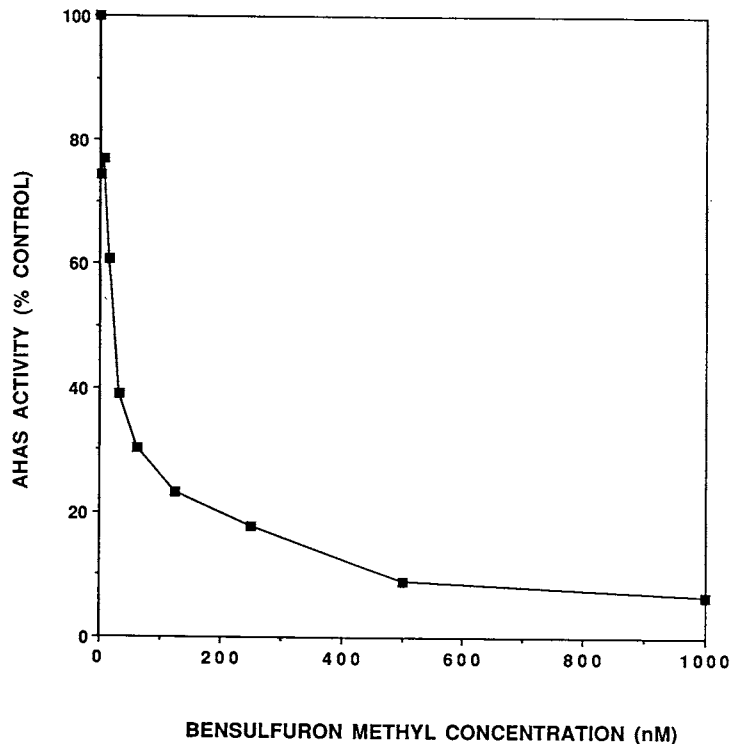


Figure 2. Effect of increasing concentrations of bensulfuron-methyl on AHAS activity following a 60-min incubation period. AHAS was extracted from hydrilla.

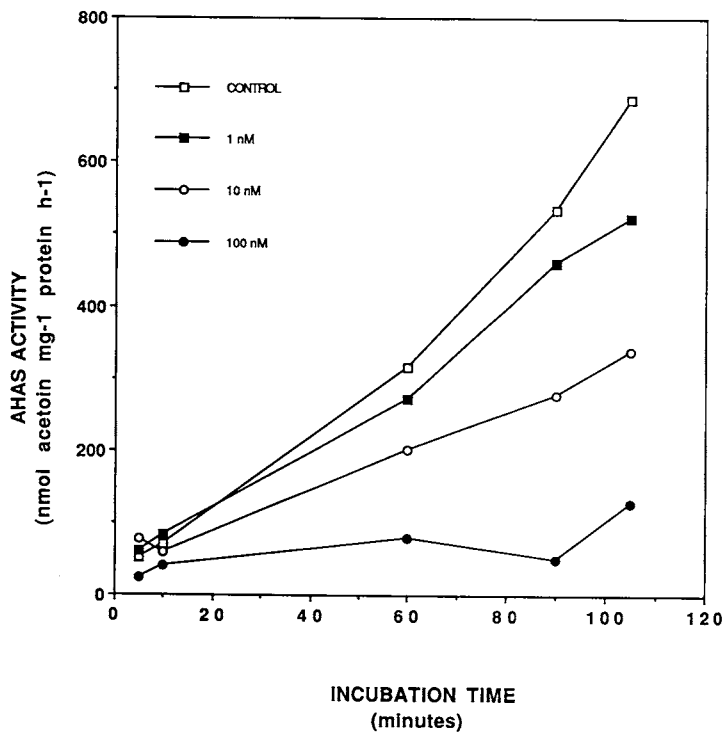


Figure 3. Effect of time on the inhibition of AHAS activity in hydrilla shoot tips by bensulfuron-methyl

action of the sulfonylurea herbicides, namely the inhibition of AHAS (Ray 1984, Singh *et al.* 1988, Schloss 1990, Durner *et al.* 1991). In conclusion, the results presented in this paper demonstrate that the herbicidal effect of bensulfuron-methyl on hydrilla growth appears to be due to the inhibition of AHAS, a key enzyme in branched-chain amino acid biosynthesis.

ACKNOWLEDGMENTS

The authors would like to thank Dr. Joseph Joyce for supporting this project and Dr. William Haller for useful discussions. This work was funded by USDA Science and Education Grant Number 5B-43YK-9-0001.

LITERATURE CITED

- Anderson, L.W.J. and N. Dechoretz. 1988. Bensulfuron methyl: A new aquatic herbicide. Proc. Aquat. Plant Control Res. Prog. Misc. Paper A-88-5. US Army Engineer Waterways Experiment Station, Vicksburg, MS. pp. 224-235.
- Beyer, E.M., M.J. Duffy, J.V. Hay and D.D. Schlueter. 1988. Sulfonylurea herbicides. *In* Herbicides: Chemistry, Degradation, and Mode of Action. Marcel Dekker, Inc. NY.
- Bradford M.M. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248-254.
- Durner, J., V. Gailus and P. Boger. 1991. New aspects on inhibition of plant acetolactate synthase by chlorsulfuron and imazaquin. *Plant Physiology* 95:1144-1149.
- Haller, W.T., A.M. Fox and C.A. Hanlon. 1992. Inhibition of hydrilla tuber formation by bensulfuron methyl. *J. Aquat. Plant Manage.* 30:48-49.
- Landstein, D., D.M. Chipman, S. Arad and Z. Barak. 1990. Acetohydroxy acid synthase activity in *Chlorella emersonii* under auto- and heterotrophic growth conditions. *Plant Physiol.* 94:614-620.
- Ray, T.B. 1984. Site of action of chlorsulfuron. *Plant Physiol.* 75:827-831.
- Schloss, J.V. 1990. Acetolactate synthase, mechanism of action and its herbicide binding site. *Pesticide Sci.* 29:283-292.
- Shaner, D.L. and D. Little. 1989. Effects of imazaquin and sulfometuron methyl on extractable acetohydroxyacid synthase activity in maize and soybeans. BCPC Mono No. 42:197-198.
- Singh, B.K., M.A. Stidham and D.L. Shaner. 1988. Assay of acetohydroxyacid synthase. *Anal. Biochem.* 171:173-179.
- Van, T.K. and V.V. Vandiver, Jr. 1992. Response of monoecious and dioecious hydrilla to bensulfuron methyl. *J. Aquat. Plant Manage.* 30:41-44.
- Westerfield, W.W. 1945. A colorimetric determination of blood acetoin. *J. Biol. Chem.* 161:495-502.