

# The herbicidally active experimental compound Hoe 704 is a potent inhibitor of the enzyme acetolactate reductoisomerase

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Growth inhibition of plants and bacteria by the experimental herbicide Hoe 704 (2-methylphosphinoyl-2-hydroxyacetic acid) was alleviated by the addition of the branched-chain amino acids to growth media. Hoe 704 caused a massive accumulation of acetoin and acetolactate, indicating its direct interference with the branched-chain amino acid biosynthetic pathway. The second enzyme of this pathway, acetolactate reductoisomerase (EC 1.1.1.86), was found to be subject to strong inhibition by Hoe 704. The inhibition was time-dependent and competitive with the enzyme's substrate, acetolactate. This report establishes acetolactate reductoisomerase as a new target for a herbicidal compound.

Acetolactate reductoisomerase; Enzyme inhibition; Herbicide; Hoe 704

## 1. INTRODUCTION

Several enzymes of amino acid biosynthetic pathways have been identified as the targets of recently developed herbicides [1]. The pathway leading to biosynthesis of the branched-chain amino acids, valine, leucine and isoleucine (fig.1), is of particular interest because its first enzyme, acetolactate synthase (EC 4.1.3.18), was found to be the target of three different new classes of herbicides [2]. These findings raise the question of whether an inhibitor of another enzyme of the same pathway might also have herbicidal properties. We report here that the herbicidally active experimental compound Hoe 704 (2-methylphosphinoyl-2-hydroxyacetic acid) is a potent inhibitor of the second enzyme in the branched-chain amino acid biosynthetic pathway, acetolactate reductoisomerase (EC 1.1.1.86).

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*Abbreviations:* AHB, 2-aceto-2-hydroxybutyrate; AL, 2-acetolactate; PMSF, phenylmethanesulfonyl fluoride

## 2. MATERIALS AND METHODS

2-Acetolactate, 2-aceto-2-hydroxybutyrate and Hoe 704 (all racemic mixtures) were synthesized at Hoechst AG. Maize (*Zea mays* L.) was cultivated in pots in a greenhouse at 24°C/16°C (day/night). Sterile cultures of duckweed (*Lemna gibba* L.) were maintained at room temperature in 200-ml Erlenmeyer flasks on nutrient solution [3]. *Escherichia coli* DH-1 was grown in the minimal medium of Vogel and Bonner [4], supplemented with 0.4 µg/l thiamine pyrophosphate and 0.5% glucose. Media were autoclaved prior to the addition of branched-chain amino acids which were added following filter sterilization. Acetoin and acetolactate were extracted from plant material with three changes of 80% methanol at 80°C for 20 min each. Acetolactate was determined after its conversion to acetoin by incubation with 0.5 M H<sub>2</sub>SO<sub>4</sub> for 15 min at 60°C. Acetoin was determined according to Westerfeld [5].

Acetolactate synthase was assayed as described by Ray [6]. Acetolactate reductoisomerase was purified from *E. coli* and a *Daucus carota* cell culture by essentially identical procedures: the cells were disrupted in 50 mM Tris (pH 7.5), 10 mM MgSO<sub>4</sub>, 5 mM mercaptoethanol, 1 mM EDTA and 1 mM PMSF, and the proteins fractionated by ammonium sulfate precipitation. The reductoisomerase precipitated between 40 and 60% ammonium sulfate saturation and was further purified by subsequent chromatography on Q-Sepharose, phenyl-Sepharose and a Mono-Q column (details of the purification will be published elsewhere). The purified enzymes from both sources were stable for at least 2 months at –20°C. Acetolactate reductoisomerase was determined as in [7] with the following modifications: the reaction mixture contained 30 mM

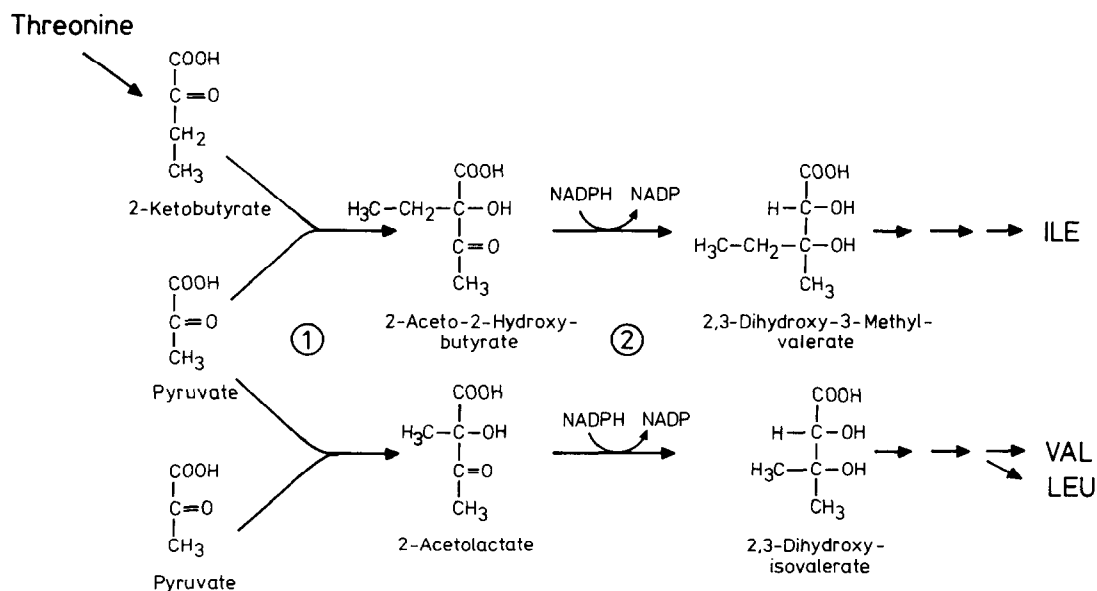


Fig.1. Biosynthesis of branched-chain amino acids. Numbered steps are those catalysed by the enzymes mentioned in the text: (1) Acetolactate synthase; (2) acetolactate reductoisomerase.

phosphate buffer (pH 7.5), 16 mM 2-acetolactate (or 2-aceto-2-hydroxybutyrate), 0.2 mM NADPH and enzyme solution in a total volume of 1 ml. After 5 min incubation at 30°C, the reaction was initiated by the addition of the enzyme. The oxidation of NADPH was followed at 340 nm with a Beckman DU-50 spectrophotometer.

### 3. RESULTS

The compound Hoe 704 (2-methylphosphinoyl-2-hydroxyacetic acid, fig.2) was discovered as a nonselective herbicide active against both monocotyledonous and dicotyledonous species [8]. The first response of plants to treatment with Hoe 704 is a rapid and complete arrest of growth, while toxic symptoms develop only after several days. These symptoms bear striking similarities to those observed after exposure of plants to herbicides of

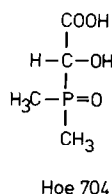


Fig.2. Structure of Hoe 704.

either the sulfonylurea or imidazolinone type [9,10]. These latter herbicides are known to act by inhibiting the first enzyme of the branched-chain amino acid biosynthetic pathway, acetolactate synthase (EC 4.1.3.18; alternative name: aceto-hydroxyacid synthase) [1]. Interference of Hoe 704 with branched-chain amino acid biosynthesis was indicated by the initial observation that inhibition of growth by Hoe 704 of duckweed (*L. gibba*) a higher plant (table 1), as well as of the bacterium

Table 1

Growth of *L. gibba* in the presence of Hoe 704 ( $2 \times 10^{-5}$  M) and different combinations of branched-chain amino acids ( $10^{-4}$  M each) in the culture medium

Supplement	Plant fresh wt (% control)	
	Without	With Hoe 704
None	100 (control)	12
Val, Leu, Ile	91	111
Val, Leu	9	9
Val, Ile	91	76
Leu, Ile	10	8
Val	4	4
Leu	4	4
Ile	14	4

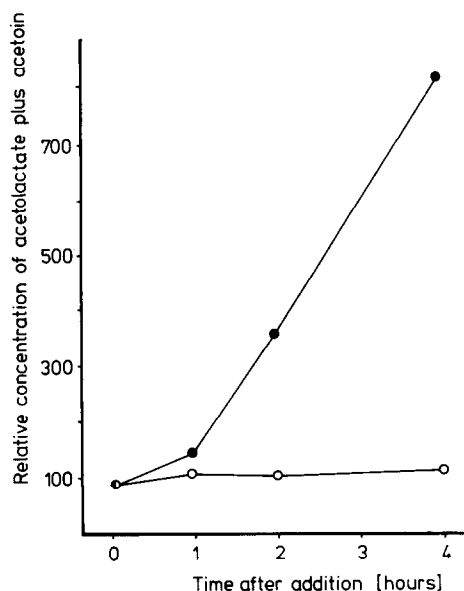


Fig.3. Relative concentration of acetoin plus acetolactate in *L. gibba* (●) and culture medium (○). At time zero,  $2 \times 10^{-5}$  M Hoe 704 was added to the growth medium. (Concentration of acetoin and acetolactate in control plants and corresponding media = 100.)

*Klebsiella pneumoniae* (not shown), was alleviated by the addition of the three branched-chain amino acids to the growth media. While no effect of Hoe 704 on the activity of acetolactate synthase was observed in vitro, it was found that plants treated with Hoe 704 accumulate massive amounts of acetolactate and acetoin, the decarboxylation product of acetolactate (fig.3, table 2).

This observation strongly suggested that the second enzyme in the pathway, acetolactate reductoisomerase (EC 1.1.1.86), which converts either 2-acetolactate or 2-aceto-2-hydroxybutyrate to

Table 2

Accumulation of acetolactate and acetoin in corn plants treated with Hoe 704

	Acetoin	Acetolactate
Control plant	1.17	n.d.
Treated plant	1150	350

6-week-old corn plants were sprayed with the herbicide (in 0.025% Tween 80) at a dosage equivalent to 1 kg/ha, and leaves were analysed 2 weeks later. Results given as nmol/100 mg dry wt. n.d., not detectable

Table 3

Inhibition ( $IC_{50}$  values) of purified acetolactate reductoisomerase from *E. coli* *D. carota* by Hoe 704

Substrate:	Enzyme source			
	<i>D. carota</i>		<i>E. coli</i>	
	AHB	AL	AHB	AL
	19.4	8.2	14.9	8.0

2-Aceto-2-hydroxybutyrate (AHB) and 2-acetolactate (AL) were used as substrate.  $IC_{50}$  values expressed in  $\mu$ M

2,3-dihydroxyisovalerate or 2,3-dihydroxy-2-methylvalerate, respectively, by an internal side chain shift and concomitant reduction (see fig.1), is subject to inhibition by Hoe 704. This enzyme was therefore extracted and purified from *E. coli* and a carrot cell suspension culture. Both enzymes were found to be strongly inhibited by the compound (table 3). The extent of inhibition increased with time until a steady state had been attained (fig.4), and a Lineweaver-Burk plot constructed from the velocities of NADPH oxidation in the steady state clearly indicated competitive inhibition. For the *E. coli* enzyme, with acetolactate as

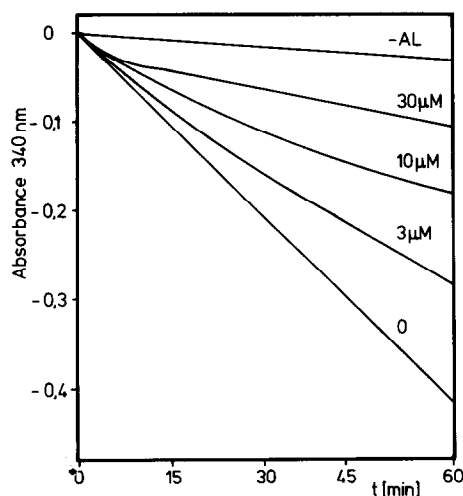


Fig.4. Time course of the reaction catalysed by the *D. carota* acetolactate reductoisomerase with 2-acetolactate as substrate in the absence (0) and presence of increasing concentrations of Hoe 704. The time course of a control reaction without acetolactate is also shown (-AL). Enzyme concentration in assays was 0.9  $\mu$ g/ml. Oxidation of NADPH was followed by measuring the decrease in absorbance at 340 nm.

substrate, a  $K_i$  value of 0.82  $\mu$ M was determined from a Dixon plot.

#### 4. DISCUSSION

The results of the physiological and biochemical experiments described here strongly indicate that the herbicidal activity of Hoe 704 is due to its direct interference with acetolactate reductoisomerase, the second enzyme in the branched-chain amino acid biosynthetic pathway (fig.1). Further evidence that acetolactate reductoisomerase is the target of Hoe 704 is provided by the observation that a carrot cell culture adapted to growth in increasing concentrations of the compound overaccumulates this enzyme (Donn, G. and Schulz, A., in preparation). Fig.4 demonstrates that Hoe 704 is a slowly equilibrating inhibitor of this enzyme, and further work will be required in order to investigate in detail the nature of the inhibition. Preliminary evidence suggests that only one of the enantiomers of Hoe 704 is active as an inhibitor of acetolactate reductoisomerase. While the properties of this enzyme from microorganisms have been thoroughly studied [7,11–13], and the nucleotide sequences of the corresponding genes are known for *E. coli* and *Saccharomyces cerevisiae* [14,15], there is little information available on the plant enzyme [16,17]. The availability of the acetolactate reductoisomerase overproducing carrot cell culture has allowed us to purify the plant enzyme to homogeneity. The purification protocol and properties of this enzyme will be published elsewhere. The novel inhibitor of this enzyme will be a valuable tool in the elucidation of its complex reaction mechanism.

Previous attention had focussed on acetolactate

synthase as the target of new herbicides [2]. Our work has clearly shown that inhibition of another enzyme of the same pathway leads to plant death. This observation is of relevance to future herbicide design.

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