Acetohydroxyacid Synthase Inhibitors: N-Phthalyl-L-valine Anilide and Related Compounds

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The potency of L-valine as an inhibitor of *Zea mays* acetohydroxyacid synthase (AHAS) is increased more than 8000-fold on conversion to its N-phthalyl anilide derivative which is active at 2 μM. The D-valine, α-aminobutyric acid, isoleucine and phenylalanine analogs are 11- to 43-fold less potent, and similar N-phthalyl anilide derivatives of other branched-chain amino acids are essentially inactive. Full potency is retained on replacing the phthalimide moiety of the valine anilide with cyclohexane-1,2-dicarboximide or 1-cyclohexene-1,2-dicarboximide groups and partial activity with 4-cyclohexene-1,2-dicarboximide and methyl- or dimethylmaleimide groups. Inhibition of the enzyme and of root growth by the valine derivatives may result from binding at or near the site involved in feedback control of AHAS by L-valine.

Introduction

Acetohydroxyacid synthase (AHAS) (EC 4.1.3.18) is the first common enzyme of the biosynthetic pathways leading to the branched-chain amino acids valine, leucine and isoleucine. It is inhibited *in vitro* by millimolar levels of valine and leucine [1] or valine and isoleucine [2], depending on the AHAS source, probably by acting at a regulatory site distinct from the active site [3]. The most potent AHAS inhibitors are imidazolinone herbicides such as Scepter® [4] and sulfonylurea herbicides such as chlorsulfuron [5, 6], acting *in vitro* at micromolar and nanomolar levels, respectively. Since the imidazolinone herbicides may be considered derivatives of valine [7], the enzyme inhibition responsible for their herbicidal action might involve the allosteric site normally occupied by valine as part of the feedback mechanism regulating AHAS activity. On this basis, other derivatives of branched-chain amino acids may also show specific AHAS inhibition. This study identifies such compounds and considers their structure-activity relationships.

Materials and Methods

Synthesis of compounds

Compounds **1**–**18** (Tables I and II), synthesized as candidate AHAS inhibitors, gave suitable proton magnetic resonance (PMR) spectra [Bruker WM-300 spectrometer, tetramethylsilane as internal standard, deuterochloroform as solvent, coupling constants *(J)* in Hz] for the assigned structures. The identity of key compounds was confirmed by mass spectrometry (Hewlett Packard 5985 instrument, chemical ionization at 230 eV with methane).

Compounds **1**–**10** were prepared from the appropriate N-phthalyl-amino acids [10] *via* the acid chlorides. For example, N-phthalyl-DL-valine (2.5 g) in chloroform (20 ml) was treated with thionyl chloride (1 ml) and the mixture refluxed 1 h. The solvent and excess reagent were removed *in vacuo* and the crude acid chloride treated with a solution of aniline (1.3 g) in pyridine (10 ml). After several hours at room temperature, the mixture was poured into dilute hydrochloric acid (5%, 100 ml) and the product (**5** DL) filtered and crystallized from ethanol giving colourless needles (2.7 g, 84%), m.p. 170–172 °C. $[M + 1]^+$ 322. PMR δ 0.93(d), $J = 6.5$ ($C\underline{H}_3$); 1.18(d), $J = 6.7$ ($C\underline{H}_3$); 2.93(m), $[(CH_3)_2C\underline{H}]$; 4.53(d), $J = 11.6$ $[(CH_3)_2CH-C\underline{H}]$; 7.05–7.65(m), (Ar$\underline{H}$); 7.75–7.95(m) (phthalyl $\underline{H}$); 9.12(br s), (N$\underline{H}$). This general procedure was also used to prepare the reduced phthalyl derivatives (**12**–**14**) and the succinyl derivative (**15**).

An alternative procedure *via* valine anilide (**11**) was used to prepare maleyl derivatives **16**–**18** from the corresponding maleic anhydride by refluxing in glacial acetic acid [11]. Compound **11,** a colourless oil, was obtained in 90% yield on deprotection of **5** DL by treatment with hydrazine hydrate in ethanol

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[12]. $[M + 1]^+$ 192. PMR δ 0.87(d), J = 6.9 ($C\underline{H}_3$); 1.04(d), J = 6.9 ($C\underline{H}_3$); 1.52(br s), ($N\underline{H}_2$); 2.45(m), [$(CH_3)_2C\underline{H}$]; 3.37(d), J = 3.7 [$(CH_3)_2CH–C\underline{H}$]; 7.06–7.62(m), (Ar$\underline{H}$); 9.50(br s), ($N\underline{H}$). Typically, **11** (1.3 g) above and citraconic anhydride (0.8 g) in glacial acetic acid (5 ml) was boiled under reflux for 6 h. The reaction mixture was poured into 10% sodium chloride solution and the product extracted with chloroform (3 × 20 ml). The combined extracts were washed with water, dried and evaporated. The crude product was purified by chromatography on a short silica column using chloroform as eluent. After crystallization from ethyl acetate-hexane, compound **17** (0.6 g, 32%) was obtained as colourless needles, m.p. 92–93 °C. $[M + 1]^+$ 286. PMR δ 0.86(d), J = 6.6 ($C\underline{H}_3$); 1.11(d), J = 6.6 ($C\underline{H}_3$); 2.12(d), J = 1.7 ($C\underline{H}_3$); 2.77(m), [$(CH_3)_2C\underline{H}$]; 4.29(d), J = 11.6 [$(CH_3)_2CH–C\underline{H}$]; 6.40 (q), J = 1.8 (maleyl $\underline{H}$); 7.05–7.59(m), (Ar$\underline{H}$); 8.86(br s), ($N\underline{H}$).

AHAS Assay

Literature procedures [5, 13] were modified for routine inhibitor studies. The combined shoot and root tissue from etiolated 5-day-old maize *(Zea mays)* seedlings was stored at −80 °C for 1–20 days in aliquots of 7.5 g fresh weight. Each portion of stored material was ground in a mortar and pestle with 15 ml of 0.1 M potassium phosphate pH 7.0 buffer containing 1 mM sodium pyruvate, 0.5 mM thiamine pyrophosphate, 0.5 mM magnesium chloride, 10 μM flavin adenine dinucleotide and 10% v/v glycerol. The homogenate was filtered through 8 layers of cheese cloth and centrifuged at 27,000 × *g* for 20 min. Ammonium sulfate was added to the supernatant to give 50% saturation. After stirring for 1 h below 5 °C the precipitate was collected by centrifugation at 27,000 × *g* for 30 min. The pellet was dissolved in 0.1 M potassium phosphate pH 7.5 buffer containing 20 mM sodium pyruvate and 0.5 mM magnesium chloride and desalted on a small column (Pharmacia K9) of Sephadex G-25 equilibrated with the same buffer. The enzyme preparation was used immediately and was sufficient for 44 assays, *i.e.* about 170 mg fresh weight equivalent per assay.

Enzyme reaction mixtures consisted of 20 mM sodium pyruvate, 0.5 mM thiamine pyrophosphate, 0.5 mM magnesium chloride, 10 μM flavin adenine dinucleotide and the test compound in 0.8 ml of 0.02 M potassium phosphate pH 7.0 buffer. Assays initiated by adding the enzyme (0.2 ml) involved incubation for 1 h at 37 °C, addition of 6 N sulfuric acid (0.1 ml), and heating for 20 min at 55 °C. Acetoin was determined by colorimetric assay [14] involving addition of creatine (0.5% w/v, 1 ml) and then a freshly prepared solution of α-naphthol (4% w/v in 2.5 N sodium hydroxide, 1 ml) with heating for an additional 15 min at 55 °C before determining the absorbance at 530 nm. Under these conditions acetolactate formation is essentially proportional to the protein concentration and the specific AHAS activity is about 400 nmol acetolactate · mg protein^{-1} · hr^{-1} [13].

The new compounds from synthesis were assayed at 0.3, 1, 3, 10, 30 and 100 μM and other chemicals were tested at appropriate concentrations. Tabulated data are the means of at least two separate experiments with triplicate analyses and are reproducible within ±3%. Compounds inhibiting less than 5% are considered inactive. The anilides and herbicides were dissolved in dimethyl sulfoxide (DMSO) which was added to the assay mixture at a final concentration of up to 1%; this amount of DMSO gives less than 5% inhibition. Alternative cosolvents, such as ethanol, tetrahydrofuran and acetonitrile, are more inhibitory and dimethylformamide is not suitable, giving 50% inhibition at 0.1%. About 70% of the AHAS activity as assayed is sensitive to the inhibitors examined (Fig. 1). Accordingly, the inhibitor concentrations for 50% inhibition (I_{50}s) are normalized for the 70% sensitive portion, *i.e.*, 35% inhibition is the corrected I_{50}. Chlorsulfuron and Scepter as comparison compounds give I_{50} values of 8 nM and 0.8 μM, respectively (Fig. 1). Inhibition by Scepter as an internal standard at its IC_{50} value varied by ±3% among the assays considered here.

Root growth assay

Seeds of wild mustard *(Brassica kaber)* and barnyardgrass *(Echinochloa crusgalli)* were allowed to germinate on agar (0.65%) in a petri dish until the roots were 5–7 mm long [15]. The seedlings were then transferred to a second petri dish containing the test substance in agar. The dish was positioned with the roots pointing down and the root lengths were measured after 16 hrs in the dark at 25 °C relative to untreated controls.

Results and Discussion

AHAS Inhibition by amino acids (Table I)

Valine and leucine are the most effective inhibitors with enhanced potency by a cooperative effect between these amino acids but with no further increase on addition of isoleucine. The inhibitory activity of DL-valine is due to the L isomer; D-valine is inactive. These findings with maize enzyme follow reported patterns [16, 17] for barley enzyme, although the sensitivity of the maize enzyme is generally lower.

AHAS Inhibition by N-phthalyl derivatives of amino acid anilides (Table I)

Protection of the L-valine amino group with a phthalyl residue and conversion of the carboxylic function to an anilide (**5** L) increases the inhibitory potency by more than 8000-fold, *i.e.*, from an I_{50} of 20 mM to 2.3 μM (Fig. 1). The derivative from L-valine (**5** L) is more than 11-fold more potent than from D-valine (**5** D). Other inhibitory amino acid derivatives with up to one-tenth the potency of the valine derivative **5** DL are those from α-aminobutyric acid (**3**), isoleucine (**8**) and phenylalanine (**10**); surprisingly, leucine derivative **7** is inactive.

The remarkable isomeric and steric specificity of group R, showing highest potency for the valine derivatives (**5**) and discriminating the L- and D-isomers, suggests an action at or near the site of feedback inhibition by L-valine. The known patterns for cooperative effects with combinations of amino acids indicate that there are at least two inhibitor binding sites with different specificities, *i.e.*, norvaline, norleucine and leucine are cooperative with valine while isoleucine, valine and α-aminobutyric acid cooperate with leucine [16]. It may be significant that effective inhibitors are obtained on derivatization of the "valine group" (*i.e.*, **3**, **5** and **8**) but not the "leucine group" (*i.e.*, **4**, **6** and **7**), supporting the suggestion [16] of separate inhibition sites for valine (and its analogues) and leucine (and its analogues). However, enhanced inhibition between DL-leucine and N-

Table I. Physical data and I_{50} values for inhibition of AHAS activity by amino acids and their derivatives of general structure **I**.

R	Amino acid	N-Phthalyl anilide No.	N-Phthalyl anilide m.p. [°C]	I_{50}, [μM] (or % inhib) NH_2CHCOH	I_{50}, [μM] (or % inhib) **I**
H	glycine	**1**	234–235[a]	> 5000 (0%)	> 100 (0%)
CH_3	alanine	**2**	167–168[a]	> 5000 (0%)	> 100 (0%)
C_2H_5	α-aminobutyric acid	**3**	125–126	> 5000 (17%)	100
C_3H_7	norvaline	**4**	113–115	> 5000 (17%)	> 100 (0%)
C_3H_7 (*i*)	valine	**5** DL	170–172	> 5000 (34%)[b]	4.6
	D-valine	**5** D	165–166[c]	> 5000 (0%)	26
	L-valine	**5** L	165–166[c]	> 5000 (36%)[d]	2.3
C_4H_9	norleucine	**6**	126–128	> 5000 (10%)	> 100 (0%)
C_4H_9 (*i*)	leucine	**7**	161–162	> 5000 (46%)	> 100 (0%)
C_4H_9 (*s*)	isoleucine	**8**	134–136	> 5000 (21%)	100
Ph	phenylglycine	**9**	215–216	> 5000 (0%)	> 100 (0%)
$PhCH_2$	phenylalanine	**10**	163–165	> 5000 (0%)	47

[a] Reported 231–232 °C for **1** [8] and 162 °C for **2** [9].
[b] Inhibition by combination of equimolar amino acids at total concentration of 5000 μM: valine plus leucine 67%; valine plus leucine and isoleucine 56%.
[c] Specific rotation ($CHCl_3$, c = 2) **5** L, S(−), $[-2.1\ °C]_D^{25}$ and **5** D, R(+), $[+2.1\ °C]_D^{25}$. The other compounds are DL.
[d] I_{50} 20 mM (Fig. 1).

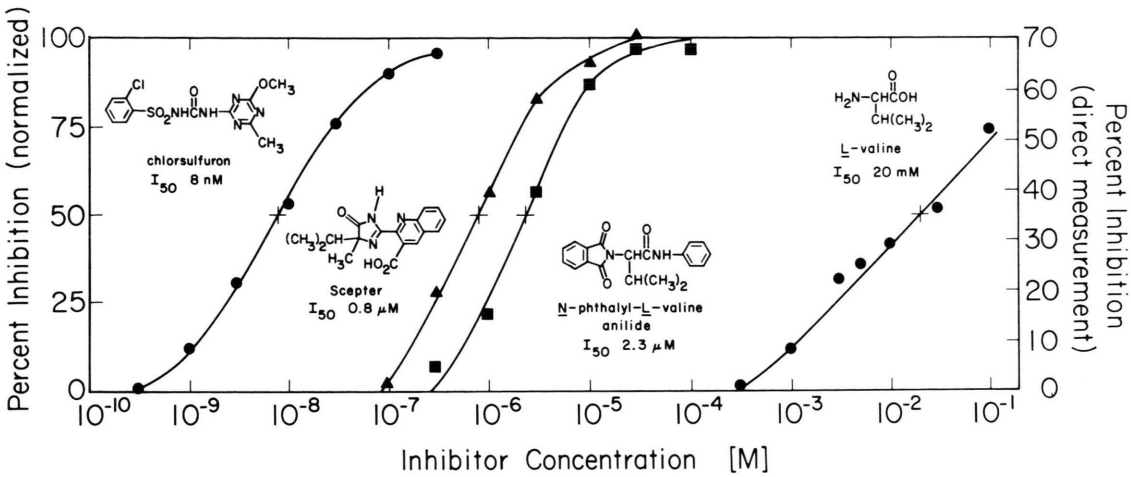

Fig. 1. Relative potency of N-phthalyl-L-valine anilide, L-valine and the herbicides Scepter and chlorsulfuron as inhibitors of *Zea mays* acetohydroxyacid synthase.

phthalyl anilides of the "valine group" (*i.e.*, **3**, **5** and **8**) could not be demonstrated (data not shown), so the cooperative effect may well be specific to combinations of amino acids.

AHAS Inhibition by various dicarboximide derivatives of valyl anilide (Table II)

Valyl anilide (compound **11** from removal of the phthalyl protecting group of **5** DL) is inactive at 100 μM. Little or no activity loss occurs on replacing the phthalimide group with a *cis*-cyclohexane-1,2-dicarboximide or 1-cyclohexene-1,2-dicarboximide group (*i.e.*, **12** and **13**) whereas the isomeric **14** is 10-fold less potent. Compounds without the six-membered ring (*i.e.*, **15** and **16**) show little or no activity at 100 μM. Maleimide **16** is structurally related to N-ethylmaleimide, which is widely used as a sulfhydryl group inhibitor. Although AHAS is sensitive to sulfhydryl reagents [7, 16], the inactivity of maleimide **16** indicates that interference with sulfhydryl group function does not contribute to the activity of substituted-valine derivatives. Moreover, the stepwise increase in activity observed on substitution of the maleimide residue with one (**17**) and two (**18**) methyl groups indicates a specific interaction of these groups with the active site.

Effects on plant growth

N-Phthalyl-L-valine anilide (**5** L) significantly inhibits the root growth of wild mustard and barnyardgrass at 1 and 30 μM, respectively.

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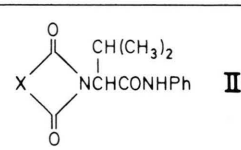
Table II. Physical data and I_{50} values for inhibition of AHAS activity by compounds of general structure II.



II

Compd No.	X	m.p. [°C]	I_{50}, [μM] (or % inhib)
12		94–96	5.8
13		123–124	3.5
14		130–134	37
15		106–107	> 100 (0%)
16		105–106	> 100 (13%)
17		92–93	> 100 (30%)
18		118–119	33

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