Effect of Gliotoxin and Related Compounds on Acetolactate Synthase

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Gliotoxin (**1**), a fungal metabolite, inhibited acetolactate synthase (ALS), one of the targets of commercial herbicides. The inhibitory effect of gliotoxin was more potent against yeast ALS than against tobacco cells. The ALS inhibitory activity of gliotoxin was considered to be associated with the 1,4-dione moiety. *p*-Benzoquinones and naphthoquinones, which have 1,4-dione moiety, also showed ALS inhibition. 2-Methyl-substituted derivatives were especially potent inhibitors of yeast ALS.

Keywords: *Acetolactate synthase; gliotoxin; p-benzoquinone; naphthoquinone*

INTRODUCTION

A number of microbial products show phytotoxic or herbicidal action, and their use as herbicides has been attempted (Strobel et al., 1991; Tanaka and Omura, 1993). Microbes produce a large number of secondary metabolites that possess various biological activities and structures, many of which are potentially biodegradable (Okuda, 1992).

It has been suggested that targets for effective herbicides are enzyme inhibitors (Dodge, 1987; Haworth and Siehl, 1990). The inhibition of amino acid biosynthesis is well established in herbicidally active compounds (Pillmoor, 1989). Inhibitors of essential amino acid biosynthesis are potentially nontoxic to mammals because the reactions are unique to plants and microbes (Kishore and Shah, 1988). Acetolactate synthase (ALS, EC 4.1.3.18) is the first common enzyme in branched-chain amino acid biosynthesis (Umbarger, 1983). This enzyme catalyzes two reactions: condensation of two pyruvate molecules to produce α-acetolactate, a precursor of valine and leucine, and condensation of pyruvate and α-ketobutyrate to form α-acetohydroxybutyrate, a precursor of isoleucine. The sulfonylureas and the imidazolinones have been commercialized as ALS inhibitors (Hawkes et al., 1989; Los, 1987).

Substantial sequence similarities of ALS from bacteria, yeasts, and higher plants have been found (Mazur et al., 1987), suggesting that it has been conserved across species boundaries. Herbicides inhibiting glutamate synthase and tryptophan synthase were discovered by screening using microbial enzymes (Omura et al., 1984; Shuto et al., 1989), and an ALS inhibitor was isolated from microbial broth by assaying with yeast ALS (Haraguchi et al., 1992). The inhibitor produced by the fungus *Aspergillus flavus* was identified as gliotoxin (**1**; Figure 1), and showed potent growth inhibition against lettuce roots (Haraguchi et al., 1992). Gliotoxin inhibited ALS prepared from tobacco cells, and growth inhibition of cultured pea roots by gliotoxin was reversed by valine, leucine, and isoleucine (Haraguchi et al., 1996). The present paper deals with the relationship between structures and ALS inhibitory activity of gliotoxin-related compounds.

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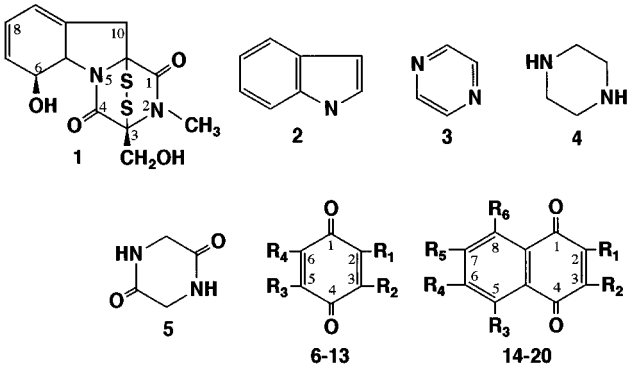

Figure 1. Chemical structures of gliotoxin-related compounds tested for inhibitory activity on acetolactate synthase.

MATERIALS AND METHODS

Chemicals. Gliotoxin (**1**) was isolated from culture broth of *A. flavus* DA-11 as previously described (Haraguchi et al., 1992). *p*-Benzoquinone (**6**), tetrahydroxy-*p*-benzoquinone (**8**), 2,5-diphenyl-*p*-benzoquinone (**13**), duroquinone (**10**), coenzyme Q_0 (**11**), plumbagin (**20**), FAD, and cocarboxylase (thiamine pyrophosphate) were purchased from Sigma Chemical Co. (St. Louis, MO). 2,5-Dihydroxy-*p*-benzoquinone (**7**), *p*-toluquinone (**9**), and naphthazarin (**17**) were purchased from Tokyo Chemical Industry Co. (Tokyo, Japan). Indole (**2**), pyrazine (**3**), piperazine (**4**), glycine anhydride (**5**), 1,4-naphthoquinone (**14**), lawsone (**15**), juglone (**16**), and menadione (**19**) were purchased from Nakarai Tesque Inc. (Kyoto, Japan). Flaviolin (**18**) and 2-hydroxy-6-methoxy-3,5-dimethyl-1,4-benzoquinone (**12**) were isolated from culture broth of *Phoma wasabiae* (Soga et al., 1984; Haraguchi et al., 1986).

Preparation of Plant ALS. Tobacco (*Nicotiana rustica*) cells were cultured in MS medium (Murashige and Skoog, 1962) supplemented with 3% sucrose, 0.1 ppm 2,4-D, and 1 ppm zeatin at 25 °C under continuous fluorescent lighting. The cells were harvested and homogenized in 2 volumes of buffer containing 0.1 M K_2HPO_4, 1 mM sodium pyruvate, 0.5 mM $MgCl_2$, 0.5 mM thiamine pyrophosphate, 10 μM FAD, and 10% glycerol (pH 7.5). The homogenate was filtered through eight layers of cheesecloth. After centrifugation at 27000*g* for 20 min, the crude enzyme was precipitated from the supernatant fluid with $(NH_4)_2SO_4$. Acetolactate synthase was collected at 50% saturation by centrifugation. The pellet was dissolved in buffer containing 0.1 M K_2HPO_4, 20 mM pyruvate, and 0.5 mM $MgCl_2$ (pH 7.5) and desalted on a Sephadex G-25 (Pharmacia LKB) column equilibrated with the same buffer. The enzyme preparation was used immediately for assays (Ray, 1984).

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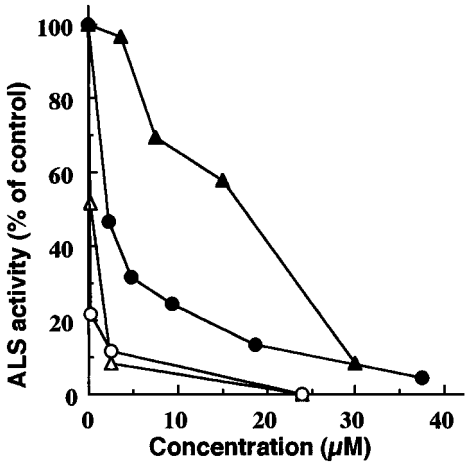

Figure 2. Effect of gliotoxin and bensulfuron methyl on acetolactate synthase. Each plot is the mean of triplicate determinations, with the standard deviation indicated by a vertical bar. (●, ○) Enzyme prepared from *S. cerevisiae*; (▲, △) enzyme prepared from *N. rustica* cells. (●, ▲) Gliotoxin; (○, △) bensulfuron methyl.

Preparation of Yeast ALS. *Saccharomyces cerevisiae* IFO 0203 was cultured in a medium consisting of 2% glucose, 0.3% $(NH_4)_2SO_4$, 0.025% $CaCl_2 \cdot 2H_2O$, 0.005% $ZnSO_4 \cdot 7H_2O$, 0.01% $FeNH_4(SO_4)_2 \cdot 12H_2O$, 0.1% sodium pyruvate, and 0.0001% biotin at 25 °C. The cells were harvested by centrifugation and washed with 1% NaCl. The cell paste was suspended in the same buffer used in the homogenation of plant cells and then disrupted by ultrasonication using a Branson Sonifier (Grimminger and Umbarger, 1979). After centrifugation at 17000*g* for 10 min, the crude enzyme was precipitated from the supernatant fluid with 50% saturated $(NH_4)_2SO_4$. Then the pellet was dialyzed against a half concentration of the same buffer. After centrifugation at 10000*g* for 10 min, the supernatant was used for the ALS assay.

ALS Assay. The enzyme assay was carried out in a total volume of 1 mL at 30 °C for 90 min (Chaleff and Mauvais, 1984). The reaction mixture contained 20 mM phosphate buffer (pH 7.0), 20 mM sodium pyruvate, 0.5 mM thiamine pyrophosphate, 0.5 mM $MgCl_2$, 10 μM FAD, and enzyme solution. Reactions were initiated by the addition of 100 μL of enzyme preparation and terminated by the addition of 100 μL of 6 N H_2SO_4.

Acetolactate was measured colorimetrically by a modification of the procedure described (Westerfield, 1945). The acidified reaction mixtures were incubated at 60 °C for 15 min, after which 1 mL of 0.5% creatine and 1 mL of 5% α-naphthol (in 2.5 N NaOH) were sequentially added. After incubation at 60 °C for 15 min, the absorbances were measured at 525 nm.

RESULTS

Effect of Gliotoxin on Plant and Yeast ALS. The inhibitory effect of gliotoxin (**1**) against acetolactate synthase from *N. rustica* cells was shown in Figure 2. Almost 90% inhibition was observed at 30 μM. A potent commercial ALS inhibitor, bensulfuron methyl, which is not a 1,4-quinone derivative, showed 92% inhibition at 2.4 μM.

Most of the enzymological and kinetic studies on inhibition of ALS by herbicides have been carried out with a *Salmonella typhimurium* enzyme, which resembles the plant enzyme with regard to its sensitivity to herbicides but is different in subunit composition and feedback regulation (Durner et al., 1991). Gliotoxin has been reported to have antimicrobial activity (Taylor, 1971). The inhibition of ALS might be concerned with antimicrobial activity of gliotoxin. Therefore, the effect on ALS from a microbe was examined. The actions of

Table 1. Effect of Gliotoxin-Related Compounds on Acetolactate Synthase

compd[a]		activity[b] (% of control)
control		100
1	gliotoxin	8.0
2	indole	100
3	pyrazine	96.3
4	piperazine	98.4
5	glycine anhydride	100
6	*p*-benzoquinone	34.1

[a] Each compound was added at a final concentration of 30 μM. [b] Activity was determined by using yeast ALS and expressed as the mean of triplicate determinations.

Table 2. Effect of Benzoquinone Derivatives on Acetolactate Synthase

compd	R_1	R_2	R_3	R_4	IC_{50} (μM)[a]
6	H	H	H	H	25.3
7	OH	H	OH	H	>100
8	OH	OH	OH	OH	>100
9	Me	H	H	H	12.8
10	Me	Me	Me	Me	>100
11	OMe	OMe	Me	H	68.1
12	OH	Me	Me	OMe	>100
13	Phe	H	Phe	H	>100

[a] Inhibitory activity was expressed as the mean of 50% inhibitory concentration of triplicate determinations against yeast ALS, obtained by interpolation of concentration−inhibition curves.

ALS inhibitors on microorganisms have been well studied (Yadav et al., 1986; Forlani et al., 1991), especially *S. cerevisiae*, which has been used for genetic analysis on mutants resistant to ALS inhibitors (Falco and Dumas, 1985). As shown in Figure 2, the inhibitory effect of bensulfuron methyl on ALS from *S. cerevisiae* IFO 0203 was similar in extent to that of the plant; the IC_{50} values were 0.12 μM and 0.27 μM against yeast and tobacco ALS, respectively. On the other hand, gliotoxin (**1**) showed potent inhibition against yeast ALS; the IC_{50} values were 2.0 μM and 18.7 μM against yeast and tobacco ALS, respectively.

Effect of Gliotoxin-Related Compounds on Yeast ALS. Gliotoxin (**1**), a member of epithiodiketopiperazine compounds produced by numerous fungi (Howell and Stipanovic, 1983; Leigh and Taylor, 1976), consists of epithiodiketopiperazine and indole parts. The effect of compounds structurally related to gliotoxin on yeast ALS was examined. As shown in Table 1, indole (**2**) did not affect yeast ALS at 30 μM, nor were rings containing nitrogen, pyrazine (**3**), piperazine (**4**), or glycine anhydride (**5**) found to cause ALS inhibition. On the other hand, *p*-benzoquinone (**6**) at 30 μM showed 66% inhibition against yeast ALS. Inhibitory activity on ALS by gliotoxin (**1**) may be attributed to the 1,4-dione part.

As *p*-benzoquinone derivatives, ubiquinones, especially Co Q_0 and Co Q_1, have been reported to inhibit ALS activity (Schloss and Aulabaugh, 1990; Schloss and Van Dyk, 1988). Table 2 shows the effect of various *p*-benzoquinone derivatives on yeast ALS. In the present result, Co Q_0 (**11**) showed ALS inhibition against yeast enzyme, which confirms earlier findings (Schloss and Van Dyk, 1988). The 2-methylation of *p*-benzoquinone (**6**) which showed 50% inhibition at 25.3 μM, forms *p*-toluquinone (**9**), which exhibited increased ALS inhibition. The 50% inhibition was observed at 12.8 μM **9**. In addition, 3-, 5-, and 6-methylation of **9** forms duroquinone (**10**), which was ineffective on ALS activity. Hydroxylation of *p*-benzoquinone (**6**) decreased in ALS inhibitory activity; 2,5-dihydroxy-*p*-benzoquinone (**7**) and tetrahydroxy-*p*-benzoquinone (**8**) were not effective

Table 3. Effect of Naphthoquinone Derivatives on Acetolactate Synthase

compd	R_1	R_2	R_3	R_4	R_5	R_6	IC_{50} (μM)[a]
14	H	H	H	H	H	H	5.8
15	OH	H	H	H	H	H	>100
16	H	H	OH	H	H	H	>100
17	H	H	OH	H	H	OH	12.3
18	OH	H	OH	H	OH	H	>100
19	Me	H	H	H	H	H	16.5
20	Me	H	OH	H	H	H	2.1

[a] Inhibitory activity was expressed as the mean of 50% inhibitory concentration of triplicate determinations against yeast ALS, obtained by interpolation of concentration−inhibition curves.

at 100 μM. 2,5-Diphenyl-*p*-benzoquinone (**13**) was also ineffective on ALS inhibition. 3-Hydroxy-6-methoxy-3,5-dimethyl-1,4-benzoquinone (**12**) is also a fungal metabolite (Haraguchi et al., 1986) and did not inhibit ALS.

The effect of naphthoquinones on ALS activity was also examined. As shown in Table 3, 1,4-naphthoquinone (**14**) showed more potent inhibition on yeast ALS than 1,4-benzoquinone (**6**). Among the naturally occurring 1,4-naphthoquinones, some 2-methylated ones, menadione (**19**) and plumbagin (**20**), showed potent inhibition. Naphthazarin (**17**) was also effective in inhibiting ALS; however, other hydroxylated naphthoquinones, lawsone (**15**), juglone (**16**), and flaviolin (**18**), were ineffective.

Considering that *p*-toluquinone (**9**) showed potent ALS inhibition, 2-methylation of quinone skeletons may account for the increase in the inhibitory activity. 2-Methyl-8-hydroxy substitution of naphthoquinone might be an effective inhibitor, which is an interest of further investigation. However, concerning gliotoxin (**1**), the dione moiety is different from those of quinone compounds. Structure−activity relationships among various diketopiperazines will be studied in the future.

DISCUSSION

Gliotoxin (**1**) inhibited tobacco and yeast ALS, and the inhibitory activity was thought to be associated with the 1,4-dione moiety. On the other hand, antimicrobial activity of gliotoxin was reported to be associated with the disulfide group (Brewer et al., 1966). It has been suggested that epithiodiketopiperazine compounds like gliotoxin may exert their biological effects by redox cycling (Munday, 1982). However, a number of different enzymes have been shown to be inhibited by gliotoxin and both the oxidized (disulfide) and the reduced (dithiol) forms of the compound have been shown to be active (De Clercq et al., 1978; Van Der Pyl et al., 1992). The quinone moiety is also concerned with redox cycling (Afanas'ev et al., 1990; Cadenas, 1995) and is responsible for various biological activities. Indeed, some benzo- and naphthoquinone derivatives showed ALS inhibition as potent as that of gliotoxin. Acetolactate synthase requires flavin nucleotide (Durner and Böger, 1990); however, the catalyzed reaction involves no redox step (Schloss, 1989). Gliotoxin has been reported to be capable of covalently modifying many cellular proteins (Waring et al., 1994). Gliotoxin (**1**) does not have a relatively planar 1,4-benzoquinone moiety within its structure but rather a non-conjugated and nonplanar 1,4-dione. The Michael addition reactivities of these two moieties are very different.

Preliminary experiments, to separate the enzyme and inhibitors in the incubation mixture using gel filtration, revealed gliotoxin and other effective quinone compounds inhibit the ALS activity irreversibility. The mechanisms of ALS inhibition by gliotoxin and related active quinone compounds, including competitive binding studies, are under investigation by using purified enzyme.

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