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The Interaction of Barley Acetolactate Synthase with 4,6-Dimethoxypyrimidine Inhibitors

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Abstract: Acetolactate synthase (ALS) catalyzes the first common step in the biosynthesis of valine, leucine, and isoleucine. ALS is the target enzyme for several classes of structually diverse herbicides. We have synthesized 4,6-dimethoxypyrimidine derivatives as ALS inhibitors, and their inhibitory activities on barley ALS were determined. IC_{50} values for the derivatives are 0.2~200 μM. K11570, the most potent ALS inhibitor with IC_{50} of 0.2 μM, showed mixed-type inhibition with respect to substrate pyruvate, and the progress curves for ALS inhibition by K11570 indicated that the amount of inhibition increased with time. Inhibition-competition experiments were carried out and indicated that three different classes of inhibitors, K11570, a sulfonylurea Ally, and leucine, bind to ALS in a mutually exclusive manner. Chemical modification of tryptophanyl and tyrosyl residues of ALS decreased the sensitivity of ALS to K11570, while cysteine modification did not affect the sensitivity. These results suggest that tryptophanyl and tyrosynyl residues are probably located at or near the inhibitor binding site.
Key words: acetolactate synthase, barley, 4,6-dimethoxypyrimidines.

Acetolactate synthase (ALS, EC 4.1.3.18 : also referred to as acetohydroxyacid synthase) catalyzes the first common step in the biosynthetic pathway for the production of Val, Leu, and Ile in plants and microorganisms. ALS catalyzes the condensation of two molecules of pyruvate to form 2-acetolactate in the first step of the Val and Leu synthetic pathway, and the condensation of one molecule of pyruvate and 2-ketobutyrate to form 2-aceto-2-hydroxybutyrate as the second step of Ile biosynthesis.

The enzyme requires thiamine pyrophosphate (TPP), flavin adenine dinucleotide (FAD), and a divalent cation for its catalytic activity (Schloss, 1984; Schloss *et al.*, 1985). The biosynthesis of branched-chain amino acids is regulated in part by the control of ALS activity. In entericbacteria, isozyme I and III, but not II, are sensitive to feedback inhibitor Val (DeFelice *et al.*, 1982). In higher plants, feedback inhibition of pea ALS by Val, Leu, and Ile singly, and synergistically by the pairs of the amino acids, has been reported (Lee *et al.*, 1991). In addition, cooperative inhibition of barley enzyme by the pairs of the amino acids, Leu plus Val, and Leu plus Ile, has been reported (Miflin, 1969).

ALS has attracted a great deal of attention as the target for several structurally distinct classes herbicides (Fig. 1), including sulfonylureas (Chaleff and Mauvais, 1984; LaRossa and Schloss, 1984; Ray, 1984), imidazolinones (Shaner *et al.*, 1984), triazolpyrimidines (Gerwick *et al.*, 1990), and pyrimidyl-oxy(thio)-benzoates (Choi *et al.*, 1993). Herbicide-resistant mutant ALS has been shown to possess an altered ALS which is less sensitive to inhibition (Chaleff and Ray, 1984; Hall and Devine, 1990). ALS enzymes from a wide range of organisms are sensitive to these herbicides. The selective action of these herbicides to weeds is due to their metabolism in crop species (Kearney and Kaufman, 1987).

Because of the interest in new herbicide design, efforts have been made to explore herbicide binding sites on ALS. The initial binding of sulfonylurea on ALS from *S. typhimurium* is partly competitive with pyruvate (LaRossa and Schloss, 1984; Hawkes *et al.*, 1989), but pyruvate is apparently required for the slow conformational change to tightly bound inhibitor-enzyme complex (LaRossa and Schloss, 1984). Imidazolinone was shown to be a noncompetitive or mixed-type inhibitor of ALS from corn (Shaner *et al.*, 1984) or peas (Ahn *et al.*, 1992), respectively. The inhibition of pea ALS by pyrimidyl-oxy(thio)-benzoate was also shown to be mixed-type (Choi *et al.*, 1993). Schloss *et al.* (1988) have demonstrated that sulfonylurea, triazolopyrimidine,

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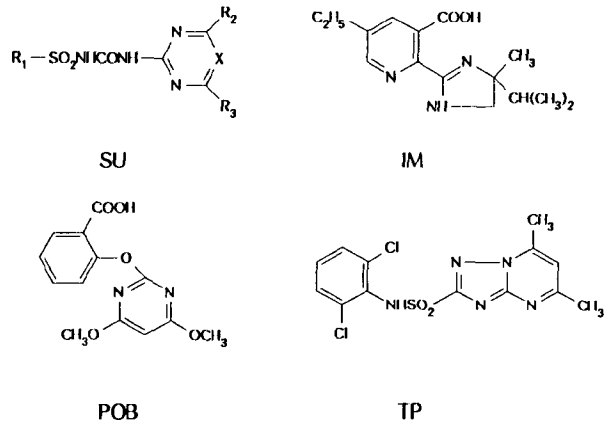


Fig. 1. The chemical structures of herbicides. SU : sulfonylureas; IM : imidazolinone (imazethapyr); POB : pyrimidyl-oxy-benzoate; TP : triazolopyrimidine sulfonanilide.

and imidazolinone compete with each other for binding to bacterial ALS. Genetic studies also support the contention that the three classes of herbicides have overlapping binding site on ALS (Saxena and King, 1988; Shaner *et al.*, 1988). ALS from tobacco and cotton mutants resistant to triazolopyrimidine was cross-resistant to inhibition by sulfonylurea, imidazolinone, and pyrimidyl-oxy-benzoate (Suhramanian *et al.*, 1990).

In this study, we have synthesized 4,6-dimethoxypyrimidine derivatives as a new chemical class of herbicide, and examined their inhibitory activity and mode of inhibition on barley ALS.

Materials and Methods

Materials

4,6-Dimethoxypyrimidine derivatives and sulfonylurea Ally were synthesized at Korea Research Institute of Chemical Technology (Taejon, Korea). Sodium pyruvate, thiamine pyrophosphate, flavin adenine dinucleotide, dithiothreitol (DTT), ethylenediaminetetraacetic acid (EDTA), L-leucine, creatine, α-naphthol, N-bromosuccinimide (NBS), 5,5′-dithio-nitrobenzoic acid (DTNB), N-acetylimidazole, leucin-agarose, and Sephadex G-25 were purchased from Sigma Chemical Co. (St. Louis, USA). DEAE-cellulose was from Whatman. All other chemicals used were reagent grade and were obtained from usual sources.

Enzyme preparation and activity assay

Barley ALS was prepared from one week old etiolated barley shoots which were grown under nonsterile conditions in vermiculate at 25℃. The shoots (80 g) were homogenized in 320 ml buffer containing 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 1 mM EDTA, 50 μM FAD, 1 mM DTT, and 15% (v/v) glycerol. The homogenate was filtered through 8 layers of cheesecloth and centrifuged at 20,000×*g* for 20 min.

ALS enzyme was collected at 30 to 50% saturation of ammonium sulfate by centrifugation. The enzyme was further purified through DEAE-cellulose and leucine-agarose chromatography as described previously (Lee *et al.*, 1991).

The enzyme was assayed colorimetically as described previously (Lee *et al.*, 1991) by quantifying the amount of acetoin formed from acetolactate, using creatine and α-naphthol. The final reaction mixture contained 20 mM potassium phosphate (pH 7.0), 20 mM sodium pyruvate, 0.5 mM $MgCl_2$, 0.5 mM TPP, and various concentrations of inhibitors. Stock solutions of inhibitors were prepared in dimethyl sulfoxide (1 mg/ml) and diluted with phosphate buffer, pH 7.0, prior to addition to the reaction mixture.

Chemical modification of ALS

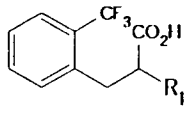
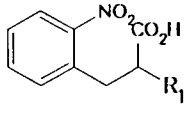
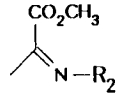
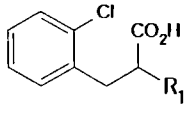
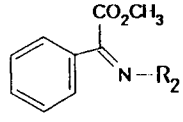
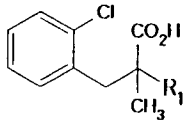
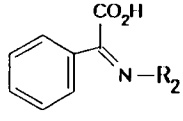
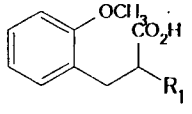
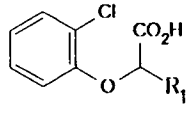
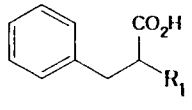
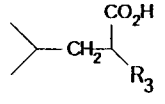
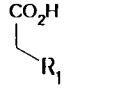
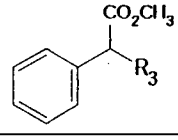
To modify tryptophanyl residues of ALS, the enzyme was treated with 0.63 mM NBS in 20 mM phosphate buffer (pH 7.0) in the presence of 0.15 M sodium pyruvate at 25℃ for 20 min. To remove unreacted NBS, pyruvate, and enzyme reaction product (acetolactate), the reaction mixture was loaded on Sephadex G-25 column (0.5×5 cm), and then centrifuged for 4 min at 20,000×*g* (Penefsky, 1977). The modification of cysteinyl residues of ALS was carried out with 2.5 mM DTNB in a similar manner. Tyrosyl residues of ALS were modified by the reaction with 10 mM N-acetylimidazole.

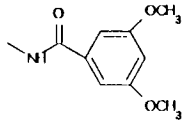
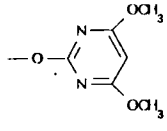
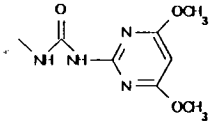
Results and Discussion

Inhibitory activity of 4,6-dimethoxypyridines

ALS is believed to play an important role in the regulation of biosynthesis of the amino acids since the enzyme is subject to feedback inhibition by end products, Val, Leu, and Ile. ALS is one of most prominent and attractive targets of herbicides since the level of ALS in plants is very low and its inhibition by herbicides simultaneously blocks the biosynthesis of three essential amino acids, Val, Leu, and Ile. ALS is a unique herbicide target in that structually unrelated classes of herbicides inhibit the enzyme. Pyrimidine ring appeared to be an important component of sulfonylurea herbicides and pyrimidyl-oxy-benzoate. Thus, we have synthesized various types of pyrimidine derivatives to develop a new class of ALS-inhibiting herbicide. The biological activity of new pyrimidine derivatives was determined by measuring their inhibition on barley ALS. The structures and IC_{50} values of 4,6-dimethoxypyrimidine deriv-

Table 1. The structures and IC_{50} values of 4,6-dimethoxypyrimidine derivertives

Name	Structure	IC_{50}	Name	Strucuture	IC_{50}
K11570		0.20 μM	K11343		ND
K11566		11.0 μM	K11607		ND
K11559		0.14 mM	K11643		ND
K11560		0.20 mM	K11645		ND
K11568		0.17 mM	K12037		ND
K11330		0.17 mM	K12046		ND
K11342		ND[a]	K12047		ND

R_1	R_2	R_3
		

[a]Not detectable at 1 mM.

atives are shown in Table 1. The IC_{50} values for inhibition is defined as the concentration of inhibitor which inhibits ALS enzyme activity 50%. The following equation was used to calculate the IC_{50} value (Ray, 1984):

$$\% \text{ Activity} = 100/(1+[I]/IC_{50})$$

where % activity equals the amount of activity in the presence of various inhibitor concentrations as percent of an untreated control, and [I] equals the inhibitor concentration. The IC_{50} was calculated by a least-squares method using the kinetics program by Dr. Stephen P. J. Brooks (Carleton University, Ottawa, Canada).

Among pyrimidine derivatives synthesized, K11570 was the most potent ALS inhibitor with IC_{50} of 0.2 μM. In activity-structure relationship the active inhibitors appeared to have a o-substituted or unsubstitued phenyl group and an amide bridge between pyrimidine and phenyl group. The strong inhibitory activity occurs when phenyl group contains a strong electron withdrawing substituent (e.g. $-CF_3$, $-NO_2$) ortho to the bridge. The pyrimidine derivatives without phenyl group do not show any detectable inhibitory activity. Therfore, phenyl group appears to be an essential component of active pyrimidine derivatives.

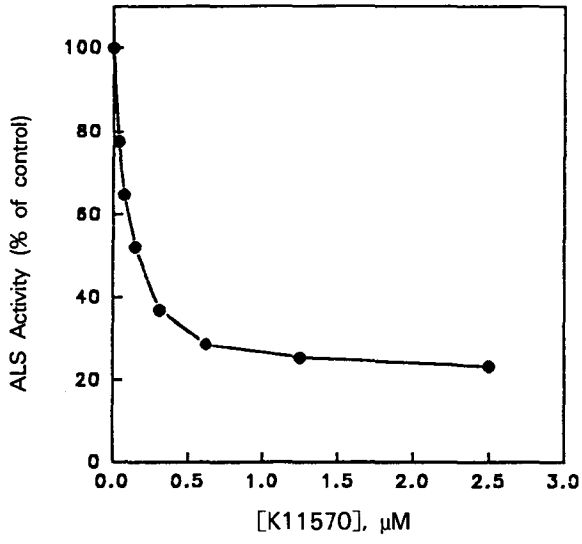


Fig. 2. Dose-response for the inhibition of barley ALS by K11 570.The values are given as the percent of an untreated control. The acetolactate formed was measured as acetoin as described in "Material and Methods".

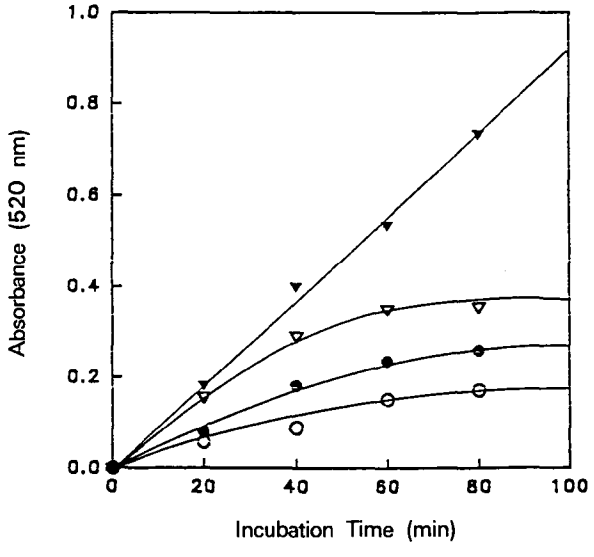


Fig. 3. Rate curves for acetolactate formation by ALS in the presence of K11570. The concentrations of K11570 were 0 (▲), 0.1 (△), 0.2 (●), and 0.4 (○) μM.

A dose-response curve for the inhibition of ALS by K11570 is shown in Fig. 2. The inhibitory activity of K11570 is lower than those of commercial sulfonylurea but is higher than those of an imidazolinone Cadre and pyrimidyl-oxy-benzoate. IC_{50} values of a sulfonylurea Glean, an imidazolinone Cadre, and pyrimidyl-oxybenzoate for the inhibition of pea ALS were reported to be 50 nM, 0.5 μM, and 0.18 mM, respectively (Ahn *et al.*, 1992; Choi *et al.*, 1993). IC_{50} values of an imidazolinone Imazaquin for inhibition of barley and corn ALS were reported to be 3.8 μM and 12.0 μM, respectively (Durner *et al.*, 1991). Among 14 compounds tested, K11570 and K11566 appeared to be potent candidates for ALS-inhibiting herbicide.

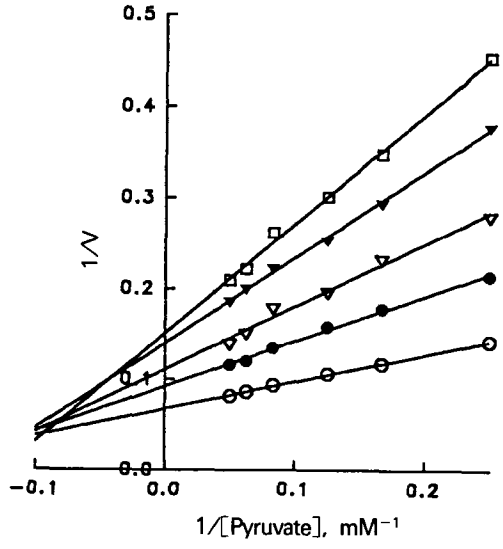


Fig. 4. The inhibition of ALS by K11570. The concentrations of K11570 were 0 (○), 0.1 (●), 0.2 (△), 0.25 (▲), and 0.5 (□) μM.

Inhibition mode of ALS by 4,6-dimethoxypyrimidine K11570

The progress curves for the inhibition of ALS by K11570 are shown in Fig. 3. The amount of inhibition is a function of the inhibitor concentration, and increases with incubation time. Similar kinetics has been reported for the inhibition of pea ALS by sulfonylurea and imidazolinone (Ahn *et al.*, 1992; Ray, 1984). However, the inhibition of pea ALS by pyrimidyl-oxy-benzoate, a weak inhibitor, was independent at the time of incubation (Choi *et al.*, 1993). Slow-binding is recognized as a typical feature of potent, reversible inhibitors (Schloss and Cleland, 1982). Slow development of inhibition of enzymatic activity may be explained in a mechanism that there is rapid formation of an enzyme-inhibitor complex (EI) which then slowly isomerizes to a second tighter complex EI*.

The inhibition pattern of barley ALS by K11570 was determined by Lineweaver-Burk plotting of reaction rates with varing concentrations of substrate at several fixed concentrations of K11570 (Fig. 4). The inhibition pattern is a mixed-type with respect to substrate pyruvate. In a mixed-type inhibition the inhibitor binds to both the free enzyme and the enzyme-substrate complex, but with different dissociation constants (Segel, 1975). Apparent dissociation constant (K_i) for the enzyme-inhibitor complex was determined to be 1.1 μM, and the dissociation constant (K_i') for the enzyme-substrate-inhibitor complex was calculated to be 2.2 μM

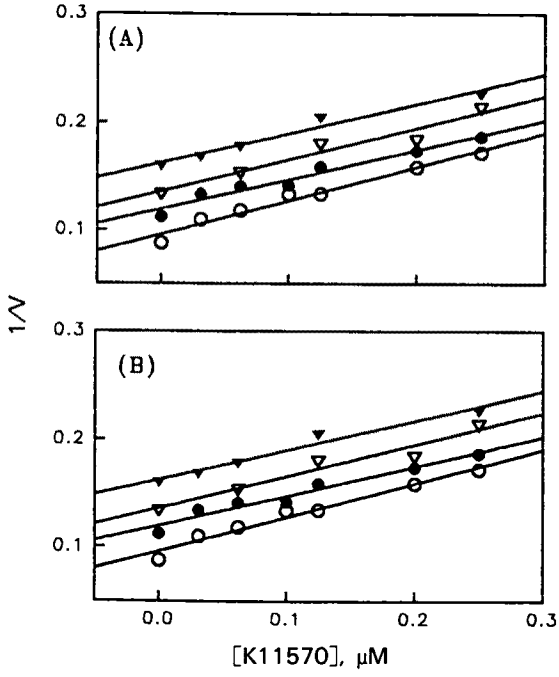


Fig. 5. Dual inhibition analysis of ALS at different concentrations of K11570 versus Ally and leucine at different fixed levels. The concentration of sodium pyruvate was 20 mM. (A) The concentrations of Ally were 0 (○), 5 (●), 10 (▽), 50 (▼) nM. (B) The concentrations of leucine were 0.0 (○), 0.075 (●), 0.15 (▽), and 0.6 (▼) mM (B).

using the following kinetic equation (Voet, 1990):

$$\frac{1}{v}=\frac{\alpha K_m}{V_{max}}\frac{1}{[S]}+\frac{\alpha'}{V_{max}}$$

If we plot reaction rates against substrate concentrations in a double reciprocal manner, the slope and the intercept are $\alpha K_m/V_{max}$ and α'/V_{max}, respectively. Dissociation constants K_i and K_i' could be obtained from the following relationships:

$$\alpha=1+[I]/K_i,\quad \alpha'=1+[I]/K_i'$$

The inhibition of pea ALS by both sulfonylurea and imidazolinone was reported to be noncompetitive (Hawkes *et al.*, 1989). Another class of inhibitor, pyrimidyloxy-benzoate showed mixed-type inhibition for pea ALS (Choi *et al.*, 1993). The inhibition of pea ALS by the end products, branched-chain amino acids, is also mixed-type with respect to pyruvate (Lee *et al.*, 1991).

The relationship between binding sites of K11570 and a sulfonylurea Ally (and Leu) on ALS was examined by dual inhibition (inhibition-competition) analysis. The concentration of K11570 was varied at different fixed levels of Ally or Leu under saturating substrate concentration. The plots of reciprocal rates versus K 11570 concentrations at several fixed levels of Ally best fit a family of parallel lines (Fig. 5A). Similar results

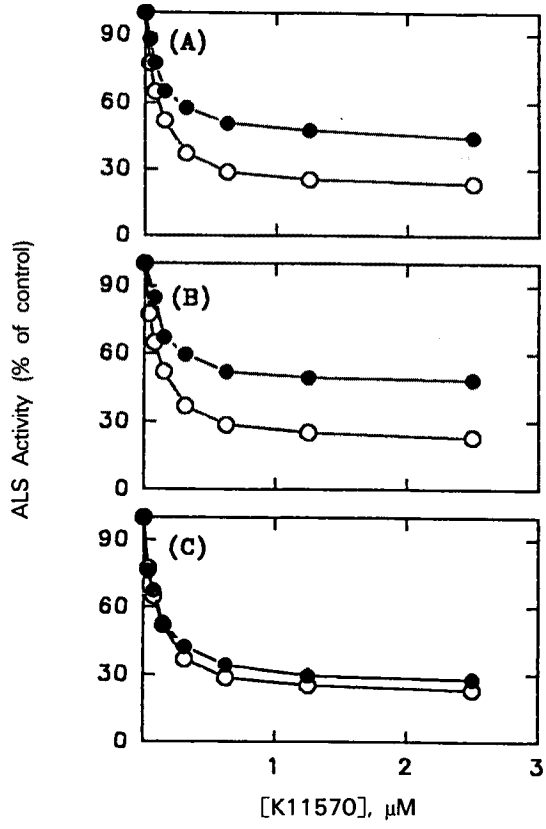


Fig. 6. The effect of chemical modification on the sensitivity of ALS to K11570. Barley ALS was treated with 0.63 mM NBS (A), 10 mM N-acetylimidazole (B) or 1 mM DTNB (C) in the presence of 0.15 M pyruvate for 20 min at 25℃ in 0.05 M potassium phosphate, pH 7.0. Excess reagent, pyruvate, and enzyme reaction product were removed as described in "Material and Methods" and their activity of untreated (○) and treated (●) ALS was assayed in the presence of K11570.

were obtained when Leu was substituted for Ally (Fig. 5B). This kinetic pattern indicates that the binding of the herbicides and Leu to barley ALS is mutally exclusive (Segel, 1975). The binding site of K11570 on barley ALS may overlap at least partially with those of Ally and Leu. Schloss *et al.* (1988) have demonstrated that sulfonylurea, imidazolinone, and triazolopyrimidine sulfonanilide compete with each other for binding to bacterial ALSII. The cross-resistance studies of tobacco and cotton mutants also suggests that binding sites of herbicides, triazolopyrimidines and sulfonylureas on the ALS enzyme overlap at least partially with that of Leu (or Val) (Subramanian *et al.*, 1991).

Amino acid residues at inhibitor binding site

In order to elucidate the amino acid residues which are involved in the binding of K11570, we carried out chemical modification of ALS. Since most herbicides have an aromatic ring as an essential component, it is expected that there are interactions between the her-

bicide and tryptophanyl and/or tyrosyl residues of ALS. The treatment of barley enzyme with tryptophan-specific reagent, NBS, and tyrosine-specific reagent, N-acetylimidazole, decreased the sensitivity of ALS to K11570 (Fig. 6A, B). The reaction mixtures included substrate pyruvate to protect tryptophanyl and tyrosyl residues at the active site. However, modification of cysteinyl residues of ALS by DTNB did not affect the sensitivity of ALS to K11570 (Fig. 6C). The results implicate the involvement of tryptophanyl and tyrosyl residues in the binding of K11570 to the barley ALS. Recently, Lee *et al.* (1988) have demonstrated that tobacco ALS becomes resistant to a sulfonylurea, sulfometuron methyl, by sustituting Trp-573 to Leu.

Acknowlegement

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