

# Isomerization and Peroxidizing Phytotoxicity of Thiadiazolidine-thione Compounds

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Peroxidizing Herbicides, Glutathione *S*-Transferase, *Echinochloa*, *Scenedesmus*, Synthesis of Thiadiazolidine-2-thiones

Eight 5-arylimino-3,4-tetramethylene-1,3,4-thiadiazolidine-2-thiones and eight 4-aryl-1,2-tetramethylene-1,2,4-triazolidine-3,5-dithiones were synthesized and their phytotoxic activities were investigated using sawa millet (*Echinochloa utilis*), green microalgae (*Scenedesmus acutus*) and protoporphyrinogen-IX oxidase isolated from etiolated corn (*Zea mays*) seedlings. 5-Arylimino-3,4-tetramethylene-1,3,4-thiadiazolidine-2-thiones showed strong phytotoxic activities and the same herbicidal mode of action as known for peroxidizing herbicides. 5-Arylimino-3,4-tetramethylene-1,3,4-thiadiazolidine-2-thiones were not or very little converted into 4-aryl-1,2-tetramethylene-1,2,4-triazolidine-3,5-dithiones either with *E. utilis* seedlings present for 7 days, with *S. acutus* cells, or using glutathione *S*-transferase (GST) and glutathione (GSH). The phytotoxic activities of 4-aryl-1,2-tetramethylene-1,2,4-triazolidine-3,5-dithiones were stronger than those of 5-arylimino-3,4-tetramethylene-1,3,4-thiadiazolidine-2-thiones [cf. Sato, Y., *et al.*, Z. Naturforsch. **49c**, 49–56 (1994)].

## Introduction

Peroxidizing compounds, cyclic imides and *p*-nitrodiphenyl ethers, are known as inhibitors of protoporphyrinogen-IX oxidase (protox) (Matringe *et al.*, 1989; Nicolaus *et al.*, 1993a). This protox inhibition causes accumulation of protoporphyrin-IX and leads to a rapid degradation of cell membranes in the light to produce ethane. Subsequently, chloroplast constituents such as chlorophyll, acyllipids *etc.* are destroyed (Böger and Sandmann, 1990; Wakabayashi and Böger, 1993; Nandihalli and Duke, 1993). Peroxidizing compounds include a variety of chemical structures (Wakabayashi and Böger, 1993). At the moment, it is difficult to define the chemical structural parts important for protox inhibition and the parts of the structure which may be needed for uptake into the plant.

During our study on structural requirements of protox inhibition it became apparent that some new peroxidizing compounds are converted into

active isomers in plants. We have previously reported that *N*-aryl-3,4,5,6-tetrahydroisophthalimides were converted into *N*-aryl-3,4,5,6-tetrahydrophthalimides (Hoshi *et al.*, 1993 a) and 5-arylimino-3,4-tetramethylene-1,3,4-thiadiazolidin-2-ones (thiadiazolidin-ones) are converted into corresponding 4-aryl-1,2-tetramethylene-1,2,4-triazolidin-3-one-5-thiones (triazolidin-one-thiones) by an enzymatic reaction in the plants (Hoshi *et al.*, 1992; Hoshi *et al.*, 1993 b; Sato *et al.*, 1994 a, 1994 b).

It is well known that some 4-aryl-1,2-tetramethylene-1,2,4-triazolidine-3,5-dithiones (triazolidine-dithiones) are strong peroxidizing herbicides (Wakabayashi and Böger, 1993). However, their isomers, 5-arylimino-3,4-tetramethylene-1,3,4-thiadiazolidine-2-thiones (thiadiazolidine-thiones) have also herbicidal activity (Yamaguchi *et al.*, 1987) but the mode of action has not been elucidated yet.

*S*-Ethyl-*N,N*-di-*n*-propylthiocarbamate (EPTC) is degraded by means of glutathione *S*-transferase (GST) and glutathione (GSH) (Lay and Casida, 1976). A partial structure similarity exists between EPTC and 5-arylimino-3,4-tetramethylene-1,3,4-

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thiadiazolidin-2-ones. Recently, Shimizu *et al.* have briefly reported that 5-(4-chloro-2-fluoro-5-methoxycarbonylmethylthiophenylimino)-3,4-tetramethylene-1,3,4-thiadiazolidin-2-one is converted into 4-(4-chloro-2-fluoro-5-carboxymethylthiophenyl)-1,2-tetramethylene-1,2,4-triazolidin-3-one-5-thione in the presence of the impure GST from velvetleaf (Shimizu *et al.*, 1994). The importance of GSH in the reaction with GST prompted us to study the role of GST and GSH in the conversion reaction.

## Materials and Methods

### Synthesis of compounds

4-Aryl-1,2-tetramethylene-1,2,4-triazolidine-3,5-dithiones and 5-arylimino-3,4-tetramethylene-1,3,4-thiadiazolidine-2-thiones were synthesized according to Wakabayashi *et al.* (1976 a, 1976 b, 1976 c, 1978) and Yamaguchi *et al.* (1987). Typical procedures were carried out as follows: 1-(4-Bromophenylthiocarbamoyl)-2-ethoxycarbonylhexahydropyridazine : *N*-Ethoxycarbonylhexahydropyridazine (7.85 g, 0.05 mol) were dissolved in 20 ml benzene and mixed with 4-bromophenyl isothiocyanate (10.7 g, 0.05 mol), then stirred for 3 h at room temperature. The solvent was evaporated *in vacuo* and the remaining residue was recrystallized from benzene-hexane yielding 16.1 g of colorless crystals, m. p. 150–151°C (148–149.5°C, Wakabayashi *et al.*, 1976 a; Wakabayashi *et al.*, 1976b). IR  $\nu_{\max}$  (KBr)  $\text{cm}^{-1}$ : 3260, 2950, 1710, 1520.

5-(4-Bromophenylimino)-3,4-tetramethylene-1,3,4-thiadiazolidine-2-thione (no. **5** of Table I) : A

solution of 1-(4-bromophenylthiocarbamoyl)-2-ethoxycarbonylhexahydropyridazine (14.8 g, 0.04 mol) in 5% KOH-ethanol (60 ml) was refluxed for 4 h. The precipitated potassium carbonate was separated by filtration and washed with hot ethanol. The combined filtrate was concentrated *in vacuo*. The remaining residue was washed with water yielding colorless crystals (10.9 g, 90.0% yield) of 1-(4-bromophenylthiocarbamoyl)-hexahydropyridazine (Wakabayashi *et al.*, 1978). One g (3.3 mm) of this compound was dissolved in a mixture of 0.71 g of pyridine (9.0 mm) and 15 ml of dichloromethane. After dropwise addition of a mixture of thiocarbonyl chloride (0.46 g, 4.0 mm) in dichloromethane (5 ml) at 0°C, the solution was stirred for 3 h at room temperature and then added to water. The dichloromethane layer was separated, dried with  $\text{Na}_2\text{SO}_4$ , and evaporated *in vacuo*. The residue was chromatographed over silica gel (50 g, chloroform as eluent) to give 0.66 g of brown crystals, m. p. 130–134°C (Yamaguchi *et al.*, 1987). IR  $\nu_{\max}$  (KBr)  $\text{cm}^{-1}$ : 2900, 1640, 1240, NMR  $\delta_{\text{H}}$  ( $\text{CDCl}_3$ ): 1.87 (4H, d), 3.76 (2H, m), 4.10 (2H, m), 6.73 (2H, d), 7.36 (2H, d).

4-(4-Bromophenyl)-1, 2-tetramethylene-1, 2, 4-triazolidine-3, 5-dithione (no. **6** of Table I) : A mixture of 1-(4-bromophenylthiocarbamoyl)-hexahydropyridazine (2.0 g), carbon disulfide (0.5 g) and potassium hydroxide (0.36 g) in ethanol (50 ml) was refluxed for 3 h. After evaporation, diluted hydrochloric acid was added to the residue. The resulting solid material was collected and washed with water. Recrystallization from ethanol gave 1.32 g colorless crystals, m. p. 225–227°C (230–232°C, Wakabayashi *et al.*, 1976 c), IR  $\nu_{\max}$  (KBr)  $\text{cm}^{-1}$ : 1310, 1280, NMR  $\delta_{\text{H}}$  ( $\text{CDCl}_3$ ): 2.10 (4H, d), 4.10 (4H, m), 7.28 (2H, d), 7.67 (2H, d).

The chemical structures of the reaction products were confirmed by IR- and NMR-spectroscopy, and elementary analysis. Melting points of the products are listed in Table I (see Fig. 2 for synthetic route of nos. **5** and **6**).

Analytical-grade chemicals for algal cultivation were purchased from Kanto Chemicals Co., Inc., Tokyo. Fine chemicals, such as glutathione (GSH), protoporphyrin-IX, and equine glutathione S-transferase (GST) were purchased from Sigma, München.

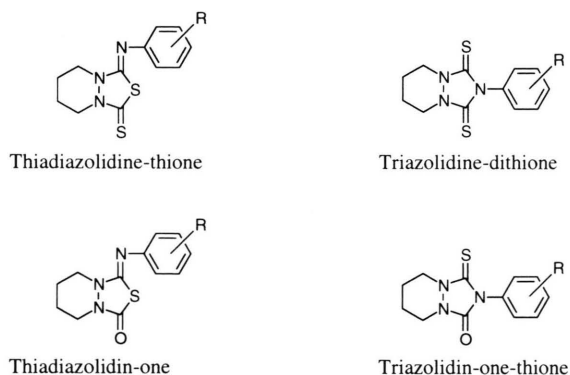


Fig. 1. Structure of thiadiazolidines and triazolidines.

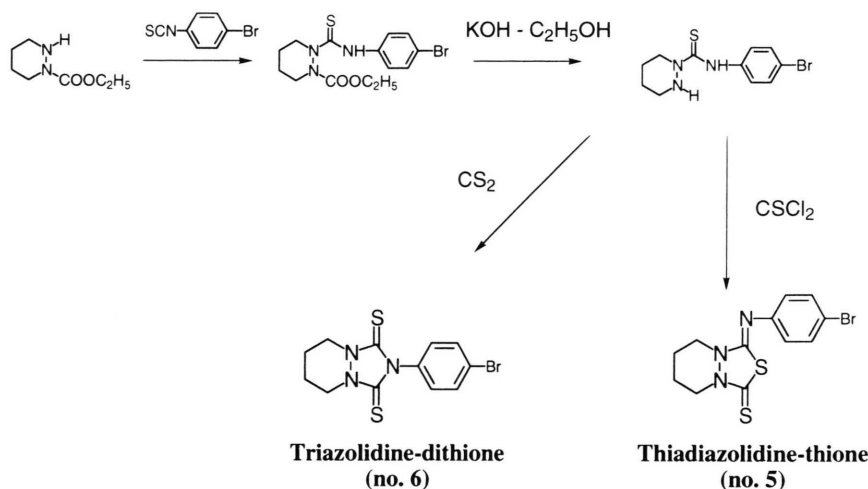


Fig. 2. Synthesis pathway of thiadiazolidine-thiones and triazolidine-dithiones.

#### Root growth inhibition of *Echinochloa utilis*

For stock solutions 5 mg of compounds were mixed with 2.5 mg of talcum powder, 2.5 mg of Tween 20 and 4 droplets of ethanol. After vigorous shaking for 30 min the solution was filled up with water to a final volume of 100 ml. This stock solution was diluted to the appropriate concentration required for the test solution used for the incubation with *E. utilis* seedlings. The seeds were collected in the fields close to Tamagawa University. The seeds germinated at 27°C for 24 h in the dark on wet filter papers in petri dishes using sterile waters. Compounds nos. **1** to **16** were used in this experiment. Root growth inhibition of *E. utilis*, expressed as pI<sub>50</sub> (E), was determined according to the method reported by Sato *et al.* (1994 a).

#### Cultivation of *Scenedesmus acutus*; determination of chlorophyll decrease and ethane formation

Compounds nos. **1** to **16** were used in these experiments. For the measurement of chlorophyll decrease autotrophic *S. acutus* was grown according to Sandmann *et al.* (1979), and chlorophyll decrease, expressed as pI<sub>50</sub> (Chlorophyll), was measured by our method reported elsewhere (Wakabayashi *et al.*, 1988). Ethane formation, indicated as pI<sub>50</sub> (Ethane), was determined using autotrophic *S. acutus* culture according to Sato *et al.* (1994 a). The pI<sub>50</sub> (Ethane) is the logarithmic "activity value" of our previous publication (Lambert *et al.*, 1983, called pK<sub>a</sub> in that publication).

#### Determination of protoporphyrinogen-IX oxidase activity

Preparation of protoporphyrinogen-IX and determination of protoporphyrinogen-IX oxidase (protox) were carried out according to Nicolaus *et al.* (1993). The protox inhibition activity of the compounds is expressed as pI<sub>50</sub> (Protox), the negative logarithm of the I<sub>50</sub> value of protox inhibition.

#### Conversion of thiadiazolidine-thiones into triazolidine-dithiones

The test solutions and *Echinochloa* seedlings were prepared as mentioned above for the root growth inhibition assay. Twenty seedlings placed on filter paper in dishes of 8 φ x 10 cm were soaked with 10 ml of the test solution. The conversions were followed for a period of 7 days at 27°C. 100 μl aliquots of the assay solution were analyzed every 24 h by a Shimadzu LC-4 HPLC system equipped with Senshu Pak ODS-1251-120K column (4.5 φ x 250 mm; Senshu Scientific Co., Tokyo, Japan). A solvent mixture of acetonitrile-distilled water (3:2, v/v) was used as the mobile phase (flow rate 1 ml/min) and the eluates were continuously monitored by a UV detector (SSC 3000B, Senshu Scientific Co., Tokyo, Japan) at 210 nm. The amounts of thiadiazolidine-thiones and triazolidine-dithiones were calculated as follows: Peaks showing up in the blank experiment were subtracted from the peaks in the chart of the

compound tested. It was assumed that the remaining peaks were produced by the modified test compound. The amounts equivalent to these peaks were expressed in percent of the peak areas of thiadiazolidines or of triazolidines.

Conversion experiments were run also with *Scenedesmus acutus* cells in the dark. To 100 ml of a 24-h old *S. acutus* culture (containing 0.5 g of glucose and 0.25 g of yeast extract per liter in addition to the mineral medium) the test sample dissolved in 100  $\mu$ l acetonitrile was added and cultured for 20 h at 22°C in the dark. The final sample concentration was 5 ppm, the final acetonitrile concentration below 1%. After incubation a 10-ml culture aliquot was extracted in three steps with 10-ml aliquots of benzene. The combined benzene extract was evaporated *in vacuo* and re-dissolved in 500  $\mu$ l of acetonitrile. This solution was analyzed by HPLC to determine the content of thiadiazolidine-thiones and triazolidine-dithiones. The procedure of HPLC analysis was the same as in the conversion experiment with *E. utilis* seedlings.

#### Cofactors for conversion by GST

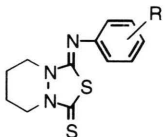
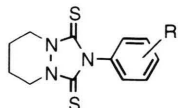










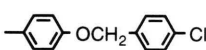
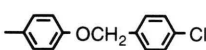
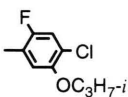
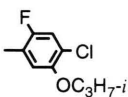
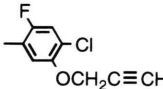
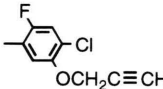
0.1 mM of no. **5**, 0.1 mM of cofactor and 0.1 mg protein (= 5–10 units) / ml of equine GST in potassium phosphate buffer (0.05 M, pH 6.8) was incubated for 2 h at 30°C. GSH, dithiothreitol (DTT) and other SH-compounds were used as cofactors. Conversion of no. **5** was determined by HPLC as mentioned in conversion with *E. utilis*. 1 unit of equine GST will conjugate 1  $\mu$ mol of 1-chloro-2,4-dinitrobenzene (CDNB) per min at pH 6.5, 25°C.

## Results and Discussion

The structures of synthesized thiadiazolidine-thiones (nos. **1**, **3**, **5**, **7**, **9**, **11**, **13**, and **15**), triazolidine-dithiones (nos. **2**, **4**, **6**, **8**, **10**, **12**, **14**, and **16**), and their melting points are listed in Table I. Melting points and IR- and NMR data agreed well with those of the literature (Wakabayashi *et al.*, 1976 a, 1976 b, 1976 c, 1978; Yamaguchi *et al.*, 1987).

Root growth inhibition with sawa millet (*Echinochloa utilis*), which correlates with the herbicidal activity in the field, is shown in Table II. All compounds exhibited root growth inhibition. Among them, four compounds (nos. **13** - **16**), which have a 4-chloro-2-fluoro-5-substituted

Table I. Thiadiazolidine-thiones and triazolidine-dithiones synthesized.

				
		Thiadiazolidine-thione (Thia)	Triazolidine-dithione (Tria)	
No		Aryl moiety		M.P. (°C)
1	Thia			Oil
2	Tria			248–250
3	Thia			105–108
4	Tria			204–206
5	Thia			130–134
6	Tria			225–227
7	Thia			101–105
8	Tria			156–159
9	Thia			83–86
10	Tria			204–206
11	Thia			174–176
12	Tria			183–186
13	Thia			108–111
14	Tria			172–173
15	Thia			134–136
16	Tria			211–215

phenyl moiety, had extremely strong activity. Apparently, the structural requirement for the strong herbicidal activity is similar to that of cyclic imide-type peroxidizing herbicides (Hoshi *et al.*, 1993 a).

The  $pI_{50}$  values of chlorophyll decrease, ethane formation by *Scenedesmus acutus* and corn protox inhibition are shown in Table II. All compounds (nos. **1–16**) induced chlorophyll decrease and produced ethane. Comparing these activities within each thiadiazolidine-thione and triazolidine-dithione set, e.g. no. **5** and **6**, triazolidine-dithiones were always found more active than thiadiazolidine-thiones. This holds for all peroxidative parameters measured, namely for protox inhibition as well as for the cellular markers like ethane pro-

Table II. Phytotoxic activities of thiadiazolidine-thione and triazolidine-dithione peroxidizers (for compounds see Table I).

Compound No.	Prottox	Chlorophyll	$PI_{50}$ Ethane	<i>Echinochloa</i>
Thiadiazolidine-thiones				
<b>1</b>	<4.50	4.86	5.36	4.82
<b>3</b>	5.96	6.63	6.26	6.39
<b>5</b>	6.13	6.83	6.26	6.61
<b>7</b>	5.12	6.56	6.29	5.54
<b>9</b>	5.96	7.17	7.04	6.80
<b>11</b>	6.09	7.70	6.92	5.26
<b>13</b>	6.21	7.95	6.78	7.55
<b>15</b>	6.96	8.26	7.10	8.23
Triazolidine-dithiones				
<b>2</b>	5.49	5.20	5.69	5.45
<b>4</b>	8.17	7.36	7.30	7.05
<b>6</b>	8.14	7.43	7.33	7.18
<b>8</b>	6.82	7.07	7.07	5.65
<b>10</b>	8.14	7.74	7.56	7.04
<b>12</b>	7.92	7.81	7.64	6.06
<b>14</b>	8.92	8.28	7.17	7.91
<b>16</b>	8.05	8.77	8.14	8.77

duction in the light or inhibition of root growth. All compounds inhibited the prottox although strength of inhibition was different depending upon the aryl-substituents of the compounds. Triazolidine-dithiones generally showed a 10 to 100 times stronger activity than thiadiazolidine-thiones. This tendency is similar for peroxidizing thiadiazolidin-ones and triazolidin-one-thiones (Sato *et al.*, 1994 a, 1994 b). These findings indicate that thiadiazolidine-thiones are peroxidizing herbicides like triazolidine-dithiones.

As we reported in a previous paper, thiadiazolidin-ones isomerized to triazolidin-one-thiones during incubation with *E. utilis* and spinach homogenate. Thiadiazolidin-ones showed strong phytotoxic activity by their isomers, triazolidin-one-thiones, depending, however, on the structure of the *N*-aryl residue (Sato *et al.*, 1994 a, 1994 b). The conversion of thiadiazolidine-thiones (nos. **1**, **3**, **5**, **7**, **9**, **11**, **13** and **15**) and triazolidine-dithiones (nos. **2**, **4**, **6**, **8**, **10**, **12**, **14** and **16**) into their isomers by incubation with *E. utilis* seedlings was investigated. The compounds were detected by retention time on HPLC analysis and the amounts determined by the percent of the total peak area. When thiadiazolidine-thiones had got in contact with *E. utilis*, the thiadiazolidine-thione peaks found were 97.6, 92.9, 97.4, 83.9, 83.7, 94.8, 95.6, 90.5%

for nos. **1**, **3**, **5**, **7**, **9**, **11**, **13** and **15**, respectively, and no peaks for triazolidine-dithiones were found on the same chart. Starting the experiment with triazolidine-dithiones (nos. **2**, **4**, **6**, **8**, **10**, **12**, **14** and **16**) only triazolidine-dithione peaks were observed. These results indicate that the conversion of thiadiazolidine-thiones and triazolidine-dithiones into their isomers did not occur by incubation with *E. utilis* (see Table III).

The conversion of thiadiazolidine-thiones (nos. **5**, **7**, and **11**) and triazolidine-dithiones (nos. **6**, **8**, and **12**) into their isomers during a 20-hr incubation with *S. acutus* cells was also investigated. Detection of the compounds was qualitatively and quantitatively carried out as in the conversion experiment using *E. utilis*. In each HPLC chart 93.1% of no. **5**, 93.8% of no. **7** and 90.2% of no. **11** were found, and the peaks of their isomers were not observed. When starting the experiment with triazolidine-dithiones, only triazolidine-dithione peaks were found. These results show that the conversion of thiadiazolidine-thiones and triazolidine-dithiones into their isomers did not occur during the 20-hr incubation of *S. acutus* (see Table III).

The mechanism of conversion of thiadiazolidin-ones by an isomerase was hypothesized in previous papers (Hoshi *et al.*, 1993b; Sato *et al.*, 1994 a, 1994 b). At first, nucleophilic addition should take

Table III. Conversion of thiadiazolidine-thiones and triazolidine-dithiones by *Echinochloa* seedlings during a 7-day incubation period.

Compound present at start*	Thiadiazolidine-thione form	Triazolidine-dithione form
Thiadiazolidine-thiones		
<b>1</b>	97.6	n.d.**
<b>3</b>	92.9	n.d.
<b>5</b>	97.4 (93.1)	n.d.
<b>7</b>	83.9 (93.8)	n.d.
<b>9</b>	83.7	n.d.
<b>11</b>	94.8 (90.2)	n.d.
<b>13</b>	95.6	n.d.
<b>15</b>	90.5	n.d.
Triazolidine-dithiones		
<b>2-16</b>	no conversion	

The data in parenthesis refer to conversion assays with *Scenedesmus acutus*.

\* For compounds assigned to the numbers see Table I.

\*\* n.d., not detected.



Table IV. Conversion of thiadiazolidine-thione and thiadiazolidin-one by glutathione *S*-transferase (GST).

Compounds	Additions	Thiadiazolidine form	Triazolidine form
<b>Thiadiazolidin-one</b>			
	<b>GST + GSH</b>	5.2	94.8
	<b>GST + DTT</b>	3.8	96.2
<b>Thiadiazolidine-thione</b>			
 (no.5)	<b>GST + GSH</b>	98.2	n.d.*)
	<b>GST + DTT</b>	98.2	n.d.
	<b>GST + L-Cys</b>	92.8	n.d.
	<b>GST + Thioglycollate</b>	92.4	n.d.
	<b>GST + Ethylmercaptan</b>	98.5	n.d.
<b>SH Compounds</b>			
	<b>GST</b>	98.6	n.d.
	<b>GSH</b>	98.8	n.d.
	<b>DTT</b>	99.4	n.d.
	<b>L-Cys</b>	98.3	n.d.
	<b>Thioglycollate</b>	99.1	n.d.
	<b>Ethylmercaptan</b>	98.7	n.d.

\*n.d. : not detected

place at the sulfur atom at the 1,3,4-thiadiazolidine ring followed by a keto-enol type rearrangement. The nucleophile is subsequently eliminated and the ring closed. GST with GSH as a cofactor (Lay and Casida, 1976; Shimizu *et al.*, 1994) was one of candidates of isomerase activity. Thiadiazolidin-ones were converted into triazolidin-one-thiones by bovine and equine GST in the presence of GSH (Iida *et al.*, 1994). For example, about 95% of 5-(4-bromophenylimino)-3,4-tetramethylene-1,3,4-thiadiazolidin-2-one was isomerized to the corresponding triazolidin-one-thione in the presence of the equine GST and SH-substrates (see Table IV). The results of conversion of no. **5** into no. **6** by GST in the presence of GSH, dithiothreitol, L-cysteine, thioglycollate, or ethylmercaptan as SH compounds are shown in Table IV. Nos. **5** and **6** were determined by HPLC as mentioned in the conversion experiments using *E. utilis*. The following percent data of no. **5** were detected on the HPLC chart: 98.2% with GST + GSH, 98.2% with GST + DTT, 92.8% with GST + L-cysteine, 92.4% with GST + thioglycollate, 98.5% with GST + ethylmercaptan, and 98.6% with GST, 98.8% with GSH, 99.4% with DTT, 98.3% with L-cysteine,

99.1% with thioglycollate and 98.7% with ethylmercaptan. There were no peaks of no. **6** found on the HPLC chart. Accordingly, no. **5** was not converted into no. **6**.

Thiadiazolidine-thiones were neither converted by *Echinochloa* seedlings, *Scenedesmus* cells nor by equine GST with GSH, under the same conditions as applied for thiadiazolidin-ones. The difference may be due to their structures, and the GST may have a specific affinity to a carbonyl but not to a thiocarbonyl group.

The isomerization of thiadiazolidin-ones reported may be catalyzed by an enzyme (isomerase) whose action is similar to GST. Experiments are under way to isolate the converting enzyme, to check whether it is an authentic GST, and to clarify the mechanism of isomerization.

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