Isomerization and Peroxidizing Phytotoxicity of Thiadiazolidine Herbicides

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Certain 5-(arylimino)-3,4-tetramethylene-1,3,4-thiadiazolidin-2-ones (thiadiazolidines) are peroxidizing bleaching herbicides which interrupt chlorophyll biosynthesis, inhibit the activity of protoporphyrinogen oxidase, lead to accumulation of protoporphyrin IX, and induce ethane formation in the light. The same effects are caused by their isomers, the 4-aryl-1,2-tetramethylene-1,2,4-triazolidin-3-one-5-thiones (triazolidines). Couples of thiadiazolidines and corresponding triazolidine isomers were synthesized. Thiadiazolidines with a 4-bromophenylimino, 4-chlorophenylimino, 4-chloro-2-methylphenylimino, 4-chloro-2-fluorophenylimino, 4-chloro-2-fluoro-5-propargyloxyphenylimino and 4-chloro-2-fluoro-5-isopropoxyphenylimino moiety were converted to the corresponding triazolidines both with *Echinochloa* seedlings or a spinach homogenate present, depending on the 5-arylimino moiety. The 5-[4-(chlorobenzyloxy)phenylimino]-3,4-tetramethylene-1,3,4-thiadiazolidin-2-one analogue did not convert to the corresponding triazolidine under both conditions. Thiadiazolidines as well as triazolidines having a 4-chloro-2-fluoro-5-methoxycarbonylmethylthiophenyl moiety were converted to an unidentified compound whose structure is assumed to be 4-(4-chloro-2-fluoro-5-carboxymethylthiophenyl)-1,2-tetramethylene-1,2,4-triazolidin-3-one-5-thione. Apparently, the general conversion mechanism is caused by enzymatic hydrolysis of thiadiazolidines to an unstable intermediate which rapidly and spontaneously changes to the corresponding triazolidine isomer.

Introduction

Peroxidizing compounds interfere with chlorophyll biosynthesis as was first shown with green algal cultures [1, 2]. Furthermore, in the light a rapid degradation of cell constituents is observed as can be measured by membrane leakage, loss of chlorophyll, or ethane production originating from polyunsaturated fatty acids. These effects are caused by protoporphyrin IX (proto IX) accumulating in the plant cells in which chlorophyll biosynthesis is blocked. This tetrapyrrole is sensitized by light and leads to phytotoxic radical formation (for overviews see [3, 4]). Enrichment of protoporphyrin IX, on the other hand, depends on inhibition of protoporphyrinogen oxidase (protox) by peroxidizing compounds [5].

Peroxidizing xenobiotica have different chemical core structures and a rapid development in the agrochemical industry came up with a plethora of new compounds in recent years (see *e.g.* [6, 7]). Most of these compounds relate to *p*-nitrodiphenyl ethers, aryl-substituted (hetero)cyclic imide types, arylpyrazoles as well as carbamoylpyridine derivatives. Little is known about structure-activity relationships. Evidence was presented, that an active peroxidizer must be a competitive inhibitor of protoporphyrinogen (the substrate for protox), representing about half the fraction of the natural substrate, and must be bicyclic [8]. The length of the inhibitor molecule should be in the 12 Å-range. Nothing is known about essential structural elements.

To obtain some insight into molecular requirements of protox inhibitors we conducted experiments on a new group of peroxidizing compounds in which apparently a less active form is converted to an active inhibitor provided *Echinochloa* seedlings or a spinach homogenate is present.

We have previously reported that N-aryl-3,4,5,6-tetrahydrophthalimides (imides) and N-aryl-3,4,5,6-tetrahydrophthalamic acids (amide acids) are interconverted by incubating them with sawa millet *(Echinochloa utilis)*. N-Aryl-3,4,5,6-tetrahydroisophtha-limides (isoimides), which show strong herbicidal activities like imides, were trans-

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formed to amide acids, followed by cyclization to imides and/or hydrolysis to corresponding anilines and 3,4,5,6-tetrahydrophthalic acid during incubation with *E. utilis*. Due to the above interconversions, imides, isoimides and amide acids exhibit identical phytotoxic effects although their activity was found very different [9–11].

4-Aryl-1,2-tetramethylene-1,2,4-triazolidin-3-one-5-thiones are strong bleaching and peroxidizing herbicides. However, their isomers, 5-(arylimino)-3,4-tetramethylene-1,3,4-thiadiazolidin-2-ones have also been shown to be active herbicides but the mode of action of these compounds have not been elucidated yet. In this paper, we present data on thiadiazolidines suggesting an enzymic conversion of these compounds to strong inhibitors of protoporphyrinogen oxidase and therefore to active peroxidizers in plant cells.

Materials and Methods

Synthesis of compounds

Thiadiazolidines and triazolidines were prepared as described [12–16]. Typical procedures were carried out as followed: 1-(4-bromophenylthiocarbamoyl)-2-methoxycarbonylhexahydropyridazine: 20.2 g of N-methoxycarbonylhexahydropyridazine (0.14 mol) were dissolved in 100 ml benzene and gently mixed with 30 g of *p*-bromophenyl isothiocyanate (0.14 mol), then stirred for 3 h at room temperature. The solvent was evaporated *in vacuo* and the remaining residue recrystallized from benzene–hexane yielding 29.6 g of colorless crystals, m.p. 160–161 °C. IR ν_{max} (KBr) cm^{-1}: 3250, 2950, 1710, 1510.

5-(4-Bromophenyl)-3,4-tetramethylene-1,3,4-thiadiazolidin-2-one (No. **2** of Table I): A solution of 1-(4-bromophenylthiocarbamoyl)-2-methoxycarbonylhexahydropyridazine (25.08 g, 0.07 mol) in 5% KOH-methanol (30 ml) was refluxed for 4 h. The precipitated carbonate was separated by filtration, washed with hot ethanol and concentrated *in vacuo*. The remaining residue was washed with water yielding colorless crystals (12.6 g, 60.3%) of 1-(4-bromophenylthiocarbamoyl)-hexahydropyridazine.
8.98 g (0.03 mol) of this compound was added to 5.05 g of pyridine (0.06 mol) and dissolved in 70 ml of dichloromethane. After dropwise addition of a mixture of trichloromethyl chloroformate (2.97 g, 0.015 mol) in dichloromethane (30 ml) at 0 °C the solution was stirred for 18 h at room temperature, followed by decantation into ice-water. The dichloromethane layer was separated, dried over Na_2SO_4 and the solvent evaporated. The residue was chromatographed over silica gel (250 g, dichloromethane as elution solvent) to give 8 g of colorless crystals, m.p. 70–72 °C (69–70 °C in ref. [16]), IR ν_{max} (KBr) cm^{-1}: 2930, 1700, 1630, NMR δ_H ($CDCl_3$): 1.86 (4 H, m), 3.75 (4 H, m), 6.83 (2 H, d, J = 0.9 Hz), 7.45 (2 H, d, J = 0.9 Hz).

4-(4-Bromophenyl)-1,2-tetramethylene-1,2,4-triazolidin-3-one-5-thione (No. **11** of Table I): A mixture of 1-(4-bromophenylthiocarbamoyl)-2-methoxycarbonylhexahydropyridazine (3.26 g) and sodium acetate (0.2 g) in xylene (40 ml) was refluxed for 5 h. After evaporation the residue was washed with water and recrystallized from benzene–ethanol to give colorless crystals (2.42 g), m.p. 152–153 °C (ref. [15], 149–152 °C), IR ν_{max} (KBr) cm^{-1}: 2950, 1730, 1490, 1440, NMR δ_H ($CDCl_3$): 2.00 (4 H, m), 3.37 (2 H, m), 4.05 (2 H, m), 7.35 (2 H, d, J = 0.9 Hz), 7.75 (2 H, d, J = 0.9 Hz).

All reaction products were purified by recrystallization and/or column chromatography, and their structures confirmed by melting point, IR and NMR spectroscopy. Melting points of the products are listed in Table I. See Fig. 1 for the synthetic route of compounds No. **2, 11.**

Root growth inhibition

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 resulting in the test solution used for the incubation procedures with *Echinochloa* (see below).

Sterilized seeds of sawa millet *(Echinochloa utilis)* germinated at 27 °C for 24 h in the dark. Twenty seedlings were placed on a double filter layer in a petri dish (8 × 10 cm) and soaked with 10 ml of the test solution. Each dish was kept under a light/dark regime (12/12 h), light intensity of 11,000 lux at 27 °C. After 7 days the root length was measured and the inhibition rate relative to the untreated control was calculated.

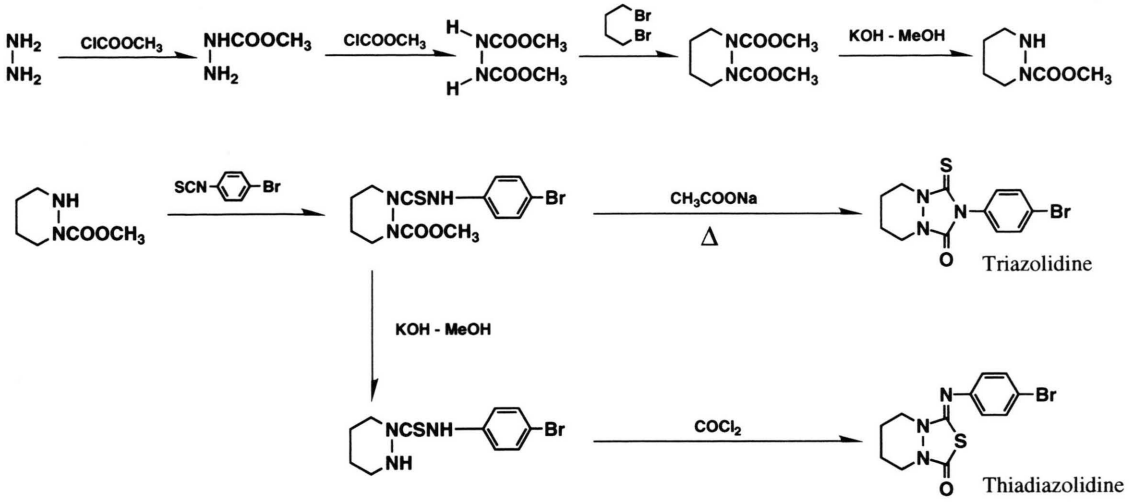


Fig. 1. Synthesis pathway of thiadiazolidines and triazolidines. See Materials and Methods for details.

The molar I_{50} values were calculated from the dose response relationship by means of a Probit analysis. Root growth inhibition indices are given as pI_{50} representing the negative logarithm of the molar concentration which resulted in a 50% inhibition.

Cultivation of Scenedesmus acutus; ethane determination

Autotrophic *Scenedesmus acutus* was grown in sterile liquid culture at 22 °C under continuous illumination by fluorescent light (4000 lux), and gassed with air enriched with 4% CO_2 (v/v) [17]. Every second day 40 ml of the culture were adjusted to a cell density of 5 µl packed cell volume (pcv)/ml cell suspension and transferred into 60 ml of sterilized fresh mineral medium. For the ethane bioassay, a culture grown for 24 h up to a density of 2 µl pcv/ml was incubated with the compounds dissolved in ethanol. The ethanol content was kept below 0.1% (v/v) in the assay medium. For ethane determination 70 ml algae suspension were placed into a 100 ml flask, 0.3 g of $NaHCO_3$ and an adequate amount of the compound added, then sealed with a silicon stopper. Incubation with the herbicide was carried out under continuous fluorescent light (15,000 lux) at 22 °C for 17 h. The ethane content was measured with a Shimadzu GC-6 A gas chromatography system equipped with a flame ionization detector (Shimadzu, Kyoto) using a Unipack S glass column (3 mm × 2 m) as the analytical column. Double reciprocal plots of ethane formation *vs.* herbicide concentration yielded the "activity values" which are presented as pI_{50} values (comp. [18, 19] for details).

Determination of protoporphyrin IX

After a 24 h cultivation period *Scenedesmus* cells were incubated with 1 µM of the herbicides for 1 h. 25 ml of the treated algae suspension were filtered, washed twice with ice-cold water and extracted twice by incubation for 10 min with 3 ml of a solution containing methanol, tetrahydrofuran and a 5 mM aqueous solution of trifluoroacetic acid (30:16:5, v/v/v) at 55 °C. Protoporphyrin IX was determined by separation on a Shimadzu LC-9A HPLC system equipped with a Senshu Pak C_6H_5-1252-N column (4.6 × 250 mm, Senshu Scientific Comp., Tokyo, Japan) and a Senshu Pak C_6H_5-1032-N precolumn (4.6 × 30 mm). The elution was carried out with a mixture of methanol, tetrahydrofuran and a 5 mM aqueous solution of trifluoroacetic acid (30:16:5, v/v/v). The protoporphyrin content was measured by a fluorescence-HPLC monitor (RF 550, Shimadzu) using an excitation and emission wavelength of 405 and 633 nm, respec-

tively. The amount expressed as nmol/ml pcv was calculated by comparison with an authentic proto IX standard.

Determination of protoporphyrinogen oxidase activity

I_{50} values of protoporphyrinogen oxidase inhibition were obtained with the enriched maize enzyme according to [20] (see that ref. also for preparation of protoporphyrinogen). Maize etioplasts were prepared by differential centrifugation steps directly before measurements of protoporphyrinogen oxidase activity. Protoporphyrin IX formation was followed spectrofluorometrically for 5 min using an excitation and emission wavelength of 405 and 632 nm, respectively. The standard reaction mixture contained 0.1 M Tris/HCl (pH 7.3), 1 mM EDTA, 1 mM dithiothreitol, 0.03% Tween 80 (w/v), 3–5 μM protoporphyrinogen, and about 0.5 mg of etioplast protein depending on the enzyme activity. Autoxidation of protoporphyrinogen was determined in presence of an aliquot of a heat-denatured etioplast fraction to correct the enzymic activity. Inhibitors were dissolved in DMSO and added to a final solvent concentration below 0.2% (v/v). The I_{50} values documented are means of three independent sets of experiments.

Conversion of thiadiazolidines into triazolidines

The test solutions and seedlings were prepared as mentioned above for the root growth inhibition assay. Twenty seedlings placed on filter paper in dishes of 8 × 10 cm were soaked with 10 ml of a test solution. The chemical changes were followed for a period of 7 days at 27 °C. 100 μl of the assay solution were analyzed every 24 h by a Shimadzu LC 4 HPLC system equipped with Senshu Pak ODS-1251-120 K column (4.5 × 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 at 210 nm. The amounts of compounds separated by HPLC and showing up as areas on the recorder strip were taken as 100%. The areas covered by the isomers represent their amount in % thereof. In Tables III and IV these amounts are expressed as "molar percent" (= percent figures divided by the corresponding molecular weight).

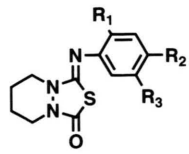
Spinach leaves from the market were kept in the refrigerator before use. After removing petioles and midribs, 60 g of leaf tissue were homogenized in a blendor 4 times for 2–3 sec each with 160 ml of 50 mM Tricine/NaOH, pH 8. The debris was removed by filtering through four layers of cheesecloth and one layer of nylon gauze (*cf.* [22]). 5 ml of the homogenate containing 10^{-5} M of herbicide was kept in the dark at 27 °C for 24 h, then extracted in three steps with 5 ml aliquots of EtOAc. The combined EtOAc solution was dried over Na_2SO_4 and evaporated. The residue was dissolved in 500 μl of acetonitrile and subjected to HPLC analysis. To check for non-enzymic conversion of the compounds the spinach homogenate was heat-denatured for 30 min at 60 °C.

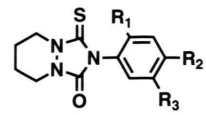
Conversion of thiadiazolidine into triazolidine was also assayed by using buffers at pH 5, 7 and 9, namely 0.01 M acetic acid/AcONa, 0.01 M KH_2PO_4/NaOH and 0.01 M KCl–H_3BO_3/NaOH, respectively [21]. 5 mg of 5-(4-bromophenylimino)-3,4-tetramethylene-1,3,4-thiadiazolidin-2-one (compound No. **2** of Table I) and 4-(4-bromophenylimino)-1,2-tetramethylene-1,2,4-triazolidin-3-one-5-thione (No. **11**) were dissolved in 10 ml of acetonitrile, respectively. This solution was diluted 100-fold with the buffers mentioned above, and kept in the dark at 27 °C for 7 days. A 10 ml sample was withdrawn every 24 h and extracted three times with 10 ml of EtOAc. The extracts were dried over Na_2SO_4, concentrated *in vacuo* and dissolved in 600 μl of acetonitrile followed by HPLC analysis.

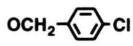
Results and Discussion

Nine sets of thiadiazolidines (No. **1–9**) and triazolidines (No. **10–18** of Table I) were prepared and biological activities of the compounds determined by light-induced ethane evolution, protoporphyrin IX formation and *Echinochloa* root growth inhibition (Table II). As has been discussed by Watanabe *et al.* [17] for a series of cyclic imides all these parameters correlate quantitatively, indicative of the phytotoxic activity of the peroxidizing compound examined. The short-term accumulation of protoporphyrin IX in autotrophic *Scenedesmus* cells suggest that the chlorophyll biosynthesis pathway is interrupted by both thiadiazolidine as well as triazolidine derivatives. This

Table I. Thiadiazolidine and triazolidine compounds synthesized.


(Thia)


(Tria)

No.		R_1	R_2	R_3	M.p. [°C]
1	Thia	H	H	H	97 ~ 99
10	Tria				155 ~ 156
2	Thia	H	Br	H	70 ~ 72
11	Tria				152 ~ 153
3	Thia	H	Cl	H	80 ~ 83
12	Tria				161 ~ 163
4	Thia	CH_3	Cl	H	103 ~ 106
13	Tria				91 ~ 96
5	Thia	F	Cl	H	71 ~ 75
14	Tria				102 ~ 106
6	Thia	F	Cl	$OCH_2C{\equiv}CH$	126 ~ 128
15	Tria				169 ~ 171
7	Thia	F	Cl	OC_3H_7-*i*	oil
16	Tria				oil
8	Thia	H		H	162 ~ 164
17	Tria				163 ~ 164
9	Thia	F	Cl	SCH_2COOCH_3	99 ~ 101
18	Tria				oil

was confirmed for four compounds (No. **2, 4, 11** and **13**) by determining their inhibitory activity on isolated maize protoporphyrinogen oxidase (Table I). Inhibition of chlorophyll biosynthesis at this step leads to subsequent accumulation of protoporphyrin IX, the product of protoporphyrinogen oxidase.

Proto IX is either formed by non-enzymic protoporphyrinogen oxidation with molecular oxygen, or catalyzed by inhibitor-insensitive protoporphyrinogen-oxidizing enzymes present in the plasma membrane or in microsomes [3, 23, 24]. The proto IX accumulation observed by treatment of algae cells with thiadiazolidines and triazolidines is caused by protox inhibition. The same holds for light-induced ethane evolution with autotrophic *Scenedesmus* cells as a marker for thylakoid degradation.

No marked differences in the pI_{50} values between thiadiazolidines and triazolidines have been found when using the *Echinochloa* root test (see couples No. **2** and **11** or **4** and **13** of Table II, col. 4). The same holds for the ethane assay with *Scenedesmus* (col. 3). These are long-term experiments. When assaying for short-term proto IX formation, however, a substantial difference was found, the thiadiazolidines being the less active forms (col. 2). This difference of inhibition is even stronger in the cell-free enzymological protox inhibition assay (col. 2, No. **2, 11** and **4, 13;** data in parentheses).

These findings may be due either to a different uptake velocity of both compound types into the cells, or to a conversion of a less active form into an active one taking place during the bioassays with *Echinochloa* and *Scenedesmus*. Indeed a unidirectional conversion occurs between thiadiazolidines and triazolidines as shown in Table III using 7 day old *Echinochloa* seedlings. No other peaks except the sets of thiadiazolidines and triazolidines used were detected in quantitative HPLC analysis. Starting the conversion experiment with thiadiazolidines, 98% of No. **1,** 96% of No. **2,** 95% of No. **3,** 74% of No. **4,** 95% of No. **5,** and 65% of No. **6** were converted to the corresponding triazolidines No. **10–15,** respectively. Thiadiazolidine No. **8** was not converted to triazolidine No. **17.** Triazolidines No. **10–17,** however, did not change to corresponding thiadiazolidines at all under these conditions. These findings indicate that thiadiazolidines exhibit their herbicidal activities after they iso-

Table II. Protoporphyrin IX formation and phytotoxic activities of thiadiazolidine and triazolidine peroxidizers (for compounds see Table I).

(1) Compounds	(2) Proto IX[a] [nmol/ml pcv]	(3) pI_{50} Ethane[b]	(4) pI_{50} *Echinochloa*[c]
Thiadiazolidines			
No. **1**	12.13	4.76	4.42
2	27.79 (5.3)[d]	6.43	6.60
3	65.11	6.53	6.47
4	34.46 (5.5)	6.30	5.53
5	61.06	6.41	6.83
6	75.07	7.64	8.09
7	80.57	7.26	7.56
8	79.25	7.23	5.37
9	52.80	6.02	6.65
Triazolidines			
No. **10**	14.63	4.70	4.49
11	73.74 (7.9)	6.57	6.79
12	66.78	6.56	6.85
13	82.20 (6.7)	6.53	5.89
14	66.50	6.71	6.93
15	67.30	7.50	8.28
16	92.83	7.98	7.56
17	85.00	7.46	6.98
18	66.54	6.40	7.43

[a] Protoporphyrin IX accumulation in *Scenedesmus* after a 1 h incubation in the presence of 1 μM of the compounds indicated.
[b] pI_{50} (ethane) = -log K_a ("activation value", see ref. [19]).
[c] Root growth inhibition of *Echinochloa utilis*.
[d] Values in parentheses show the pI_{50} for maize protoporphyrinogen oxidase.

merized to triazolidine structures. The thiadiazolidine No. **9** and triazolidine No. **18** were modified to a compound resembling 4-(4-chloro-2-fluoro-5-carboxymethylthiophenyl)-1,2-tetramethylene-1,2,4-triazolidin-3-one-5-thione as evidenced by HPLC analysis (data not documented).

To define whether this conversion proceeds biologically or chemically, only buffer solution was used omitting *Echinochloa* seedlings. No structural changes of thiadiazolidines and triazolidines were observed independent on the pH applied. These results indicate that the conversion of thiadiazolidines to triazolidines does not occur chemically. In a spinach homogenate, 37% of No. **2** was converted to triazolidine No. **11,** but – as expected –

Table III. Conversion of thiadiazolidines into triazolidines by *Echinochloa* seedlings during a 7 day incubation period. Data are molar percent of the compounds present after incubation.

Compound present at start[a]	Thiadiazolidine form	Triazolidine form
Thiadiazolidines		
No. **1**	1.9	98.1
2	4.1	95.9
3	5.3	94.7
4	26.2	73.8
5	4.6	95.4
6	15.6	84.4
7	34.6	65.4
8	100	0
9[b]	0	0
Triazolidines		
No. **10**	0	100
11	0	100
12	0	100
13	0	100
14	0	100
15	0	100
16	0	100
17	0	100
18[b]	0	0

[a] For compounds assigned to the numbers see Table I.
[b] See explanation in text.
The concentrations at start were 50 ppm for compounds **1–18** equivalent to the incubation assay for *Echinochloa* root growth inhibition.

Table IV. Conversion of thiadiazolidines into triazolidines by a spinach homogenate. Data are in molar percent of compounds found after incubation.

Compound present at start	Thiadiazolidine form	Triazolidine form
Thiadiazolidines		
No. **2**	51.0	49.0
8	100	0
9	0	0
Triazolidines		
No. **11**	0	100
17	0	100
18	0	0

For No. see Table I; the concentration of each compound was 10^{-5} M at start.

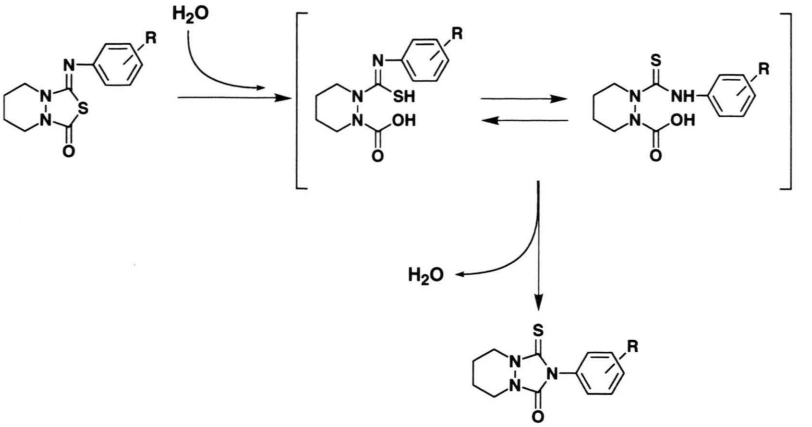


Fig. 2. Proposed mechanism of isomerization of thiadiazolidines into triazolidine peroxidizers.

No. **11** did not convert to the thiadiazolidine No. **2,** and thiadiazolidine No. **8** and triazolidine **17** were not converted. Thiadiazolidine No. **9** and triazolidine **18** changed to an unknown compound similar to the result obtained with *Echinochloa* seedlings present. In a boiled spinach homogenate, thiadiazolidines No. **2, 8** and **9** did not convert to the corresponding triazolidines (data not documented). These results indicate that isomerization of the thiadiazolidines to triazolidines is catalyzed by an enzyme. As indicated in Fig. 2 it appears that thiadiazolidines are hydrolyzed enzymatically to an unstable intermediate (compounds in brackets) which can rapidly change nonenzymatically to the corresponding triazolidine derivative. Lack of isomerization of compound No. **8** can be explained by a specificity of the isomerizing enzyme for binding of certain of thiadiazolidines only. The structure of compound **8** may not fit into a binding niche therefore excluding itself as a substrate for isomerization. Studies are underway to clarify the mechanism of the isomerization, as well as to confirm the biologically active compound of No. **9** and **18.**

In conclusion we like to mention that the "pro drug" principle as exemplified with the thiadiazolidines/triazolidines may have some bearing on selectivity. The conversion process is an enzymic reaction in the plant, and inactive thiadiazolidine analogs may be developed which are converted to herbicidal isomers by weeds but not by certain crops.

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