Interactions of the Bleaching Herbicide Clomazone with Reduced Glutathione and Other Thiols

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Z. Naturforsch. **45c,** 498–502 (1990); received November 2, 1989

Clomazone, Bleaching Herbicide, Reduced Glutathione, Cysteine, Mercaptoethanol

The reactivity of the bleaching herbicide clomazone with reduced glutathione (GSH) was studied under *in vitro* conditions. Clomazone at 1.3 mM and GSH at 100 mM were reacted in a phosphate buffer (pH 8.8) at 25 °C for 3 days. The resulting synthetic product was identified as a GS-conjugate of clomazone by means of TLC, HPLC, and mass spectrometry (chemical ionization) procedures. Exogenous application of 0.5 mM of GSH and cysteine reduced significantly the bleaching effect of clomazone on leaf disks of velvetleaf (*Abutilon theophrasti* Medik.). Mercaptoethanol was not effective in antagonizing the bleaching effect of clomazone on velvetleaf leaf disks. These results show that clomazone can react with selected thiol compounds under *in vitro* conditions. The conjugation of clomazone with GSH may be involved in the metabolic detoxication of this herbicide in plants.

Introduction

Clomazone (2-[2-chlorophenyl)methyl]-4,4-dimethyl-3-isoxazolidinone) is a selective herbicide used for the control of many grass and broadleaf weeds in soybeans [1, 2]. Velvetleaf (*Abutilon theophrasti* Medik.) is extremely sensitive to clomazone. Clomazone reduces carotenoid and chlorophyll levels causing a bleaching appearance in treated susceptible plants [3, 4]. Sandmann and Böger [5, 6] reported that clomazone inhibits the enzymes isopentenyl pyrophosphate isomerase and prenyltransferase and disrupts the formation of key components of the isoprenoid pathway needed for the synthesis of carotenoid and chlorophyll pigments in treated plants.

The basis of the observed differential sensitivity of crop and weed species to clomazone is presently unknown. A number of recent reports have indicated that differential metabolism alone could not explain the crop selectivity of clomazone [7–9]. The exact identity of clomazone metabolites formed in plants has not been determined. Nevertheless, the potential conjugation of clomazone with endogenous plant substances such as sugars [7, 8] or thiols [9] has been proposed.

Reduced glutathione (GSH) is the most important non-protein thiol peptide found in higher plants. GSH functions mainly as: a) a protectant of chloroplast membranes from oxidative damage, b) a reductant in the metabolic detoxication of herbicides and other xenobitoics, and c) a storage or long-distance transport form of reduced sulfur in plant cells [10, 11]. Devlin and Koszanski [12] reported recently that the herbicide safener dichlormid (2,2-dichloro-N,N-di-2-propenylacetamide) was effective in protecting corn (*Zea mays* L.) against bleaching injury caused by clomazone. This safener is known to protect grass crops from herbicide injury by enhancing the levels of GSH and the activity of glutathione S-transferase enzymes [EC 2.5.1.18] involved in herbicide detoxication [13, 14].

Therefore the objectives of the studies reported in this publication were to examine: a) the chemical reactivity of clomazone with GSH under *in vitro* conditions and b) the potential influence of exogenously applied thiols such as GSH, cysteine, and mercaptoethanol on the bleaching effect of clomazone on leaf disks of velvetleaf.

Materials and Methods

Chemicals

Technical (95% pure), analytical (99% pure) and radiolabeled samples of clomazone were provided by FMC Chemical Corporation, Princeton,

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Verlag der Zeitschrift für Naturforschung, D-7400 Tübingen
0341–0382/90/0500–0498 $ 01.30/0

New Jersey. Radiolabeled clomazone was uniformly labeled with ^{14}C on the aromatic ring and had a specific activity of 28.0 mCi/mmol. Clomazone was dissolved in acetone and made up to volume with distilled water. Reduced glutathione (GSH), cysteine and mercaptoethanol were purchased from Sigma Chemical Co., St. Louis, Missouri. L-(glycine-2-^{3}H)glutathione was purchased from New England Nuclear and had a specific activity of 1,300 mCi/mmol.

Chemical synthesis of GS-clomazone conjugate

A synthetic conjugate of clomazone with GSH was prepared according to the method reported by Brown and Neighbors [15] for the synthesis of a GS-chlorimuron-ethyl conjugate. Analytical and ^{14}C-labeled clomazone (0.1 μCi) were mixed to a final concentration of 100 mM. Unlabeled and ^{3}H-labeled GSH (0.1 μCi) were mixed to a final concentration of 100 mM. The herbicide and GSH samples were then reacted without stirring in a phosphate buffer (pH 8.8) at 25 °C for 3 days. The reaction mixture was concentrated to 0.1 ml and a 50 μl aliquot was spotted on silica-gel thin-layer chromatography (TLC) plate (Whatman Silica Gel 150A LK5DF, Whatman Inc., Clifton, New Jersey). The plates were developed in butanol:acetic acid:water (12:3:5, v/v/v) and co-chromatographed with standards of [^{14}C]clomazone and ^{3}H-GSH. Spots on developed TLC plates corresponding to standard clomazone and to the reaction product of clomazone and GSH were scraped and analyzed by high performance liquid chromatograph (HPLC).

HPLC chromatography

Twenty μl of standard clomazone and of the synthetic GS-clomazone product were injected into a Hewlett-Packard HPLC (model 1090) equipped with a Hewlett-Packard 200 mm by 46 mm Hypersil ODS 5 mm C-18 column and a Hewlett-Packard 100 mm by 46 mm C-18 pre-column. The chemicals were analyzed by gradient elution chromatography. The procedure included a 1 min elution in a solution of water: methanol (85:15, v/v) followed by a gradient change to a solution of water:methanol (90:10, v/v) over a 25 min period. The flow rate was 1.0 ml/min and the column temperature was 40 °C. The chemicals were detected by a diode array detector monitoring at 254 nm. Clomazone and the synthetic GS-clomazone conjugate were separated by their absorption spectra and retention times.

Mass spectrometry analysis

A Varian VG 7070E analytical mass spectrometer equipped with a chemical ionization (CI) mode was used. One μl samples of clomazone and the synthetic GS-clomazone conjugate were analyzed by using isobutane as a reagent gas according to the procedures described by Rosen and Dziedzic [16].

Interactions of clomazone with thiols on velvetleaf leaf disks

Disks (6 mm in diameter) were cut from leaves of 3–4 week old velvetleaf plants grown under greenhouse conditions. The disks were placed in 9 cm diameter polystyrene petri dishes (10 disks/dish) with 15 ml of a solution containing 1% sucrose, 1 mM MES buffer (pH 6.5) and clomazone and the thiol compounds. Clomazone was used at 0, 0.5, 5, and 50 μM. All thiols were used at 0.5 mM and included GSH, cysteine, and mercaptoethanol. Seven days after the initiation of these experiments, chlorophyll was extracted from velvetleaf disks according to the method of Hiscox and Israelstam [17]. The disks were soaked for 24 h in darkness in centrifuge tubes containing 5 ml of DMSO (dimethyl sulfoxide) at room temperature. Sample tubes were then centrifuged at $500 \times g$ for 10 min and the spectrophotometric absorbance of the supernatant was measured at the appropriate wavelengths for determining total chlorophyll according to the equations of Arnon [18]. Two replications of all treatments were made and each experiment was replicated twice in time.

Results and Discussion

In vitro conjugation of clomazone with GSH

TLC analysis showed that the synthetic product of the reaction of clomazone with GSH had an R_f value of 0.4 in the butanol:acetic acid:water (12:3:5, v/v/v) developing system. The R_f value for clomazone standard was 0.95. Data from HPLC analysis confirmed that the GS-clomazone reaction product was a different compound than

the parent clomazone (Fig. 1). Mass spectrometry analysis in the chemical ionization mode provided information on the molecular weight of standard clomazone (M + 1 = 240) and of the GS-clomazone product (M + 1 = 511) as well as fragmentation data that is useful for structural assignments (Fig. 2). The mass spectrum of the conjugate shows that this product is a molecule larger than clomazone. In addition, a comparison of the two mass spectra shown in Fig. 2 indicates that the 204-ion peak corresponding to clomazone minus the chlorine ion is missing from the spectrum of the GS-clomazone product. Thus, it seems that GSH displaces the chlorine ion on the phenyl ring of clomazone to form the GS-clomazone conjugate (Fig. 2).

In recent years, fast atom bombardment (FAB) mass spectrometry has become a widely utilized tool for the identification of glutathione conjugates of many pesticides isolated from plants [19]. Therefore we attempted to further analyze the obtained synthetic GS-clomazone conjugate by means of FAB mass spectrometry in collaboration with Dr. G. L. Lamoureux of the USDA Biosciences Research Laboratory at Fargo, North Dakota, U.S.A. The results of these studies, however, were not as conclusive as those obtained from the chemical ionization mass spectrometry studies. The failure of FAB mass spectrometry analysis in characterizing the structure of the GS-clomazone conjugate may be due to one or more of the following reasons [19]: a) the GS-clomazone conjugate may be labile, b) the GS-clomazone metabolite was not sufficently pure for analysis by FAB, and c) FAB may not work well with isoxazolidinone herbicidal derivatives such as clomazone. It

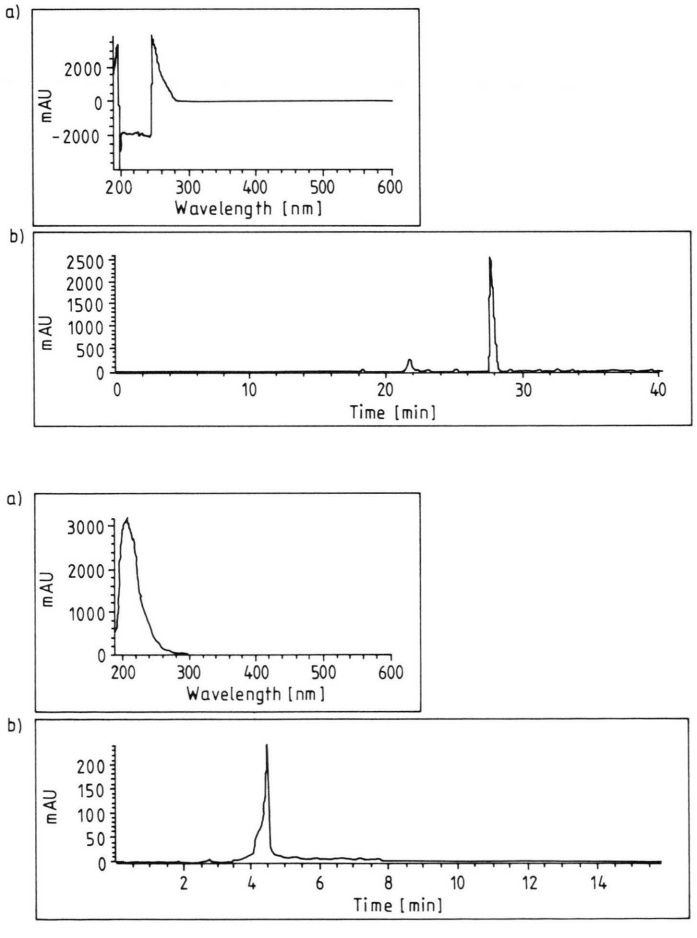

Fig. 1. Absorption spectra and HPLC chromatographs of clomazone (above) and of the synthetic GS-clomazone conjugate (below).

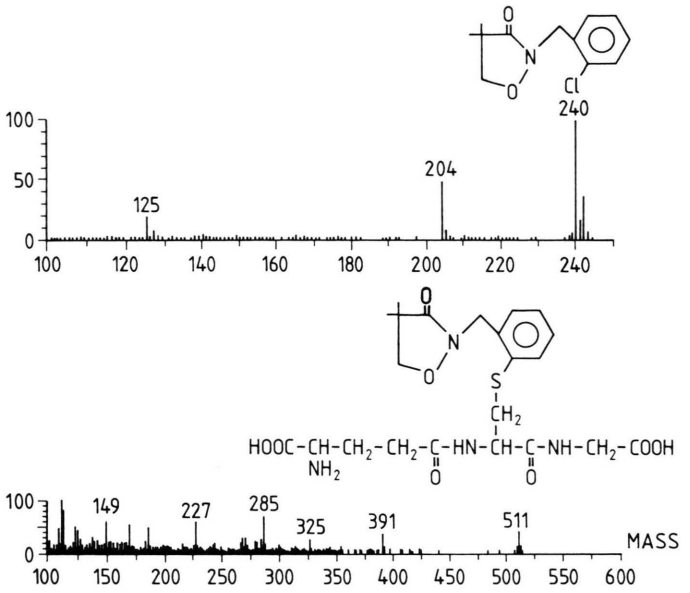

Fig. 2. Mass spectra and corresponding structures of clomazone (above) and of synthetic GS-clomazone conjugate (below).

should be noted here that FAB analysis of the GS-clomazone conjugate was attempted several months after the CI mass spectrometry analysis and it is likely that the conjugate could have separated into its components during storage. Indeed, FAB analysis of the metabolite produced a mass spectrum similar to that obtained for the standard clomazone.

Therefore, because the results of the aforementioned studies are not entirely conclusive, the proposed conjugation of the herbicide clomazone with GSH under *in vitro* conditions should be viewed with caution and awaits further experimentation in the future. Utilization of NMR spectroscopy to conclusively identify the chemical nature of the obtained GS-clomazone product was not possible because of the low quantities available for such analysis.

An unknown metabolite of clomazone detected in extracts from tolerant and susceptible plant species [11] appeared to have similar TLC chromatographic properties to those of the synthetic GS-clomazone conjugate discussed in this study. Additional studies are needed, however, to conclusively characterize the chemical nature of these metabolites.

Interactions of clomazone with thiols on velvetleaf leaf disks

Clomazone reduced significantly the total chlorophyll content of velvetleaf disks at every concentration used (Fig. 3). The bleaching effect of clomazone, however, was more pronounced at 5 and 50 μM, reducing chlorophyll by 50 and 90%, respectively.

Mercaptoethanol appeared to be phytotoxic when used alone and decreased the total chlorophyll content of velvetleaf disks by more than 50% (Fig. 3). In contrast, GSH and cysteine antagonized the effect of clomazone on total chlorophyll content of velvetleaf disks. The antagonism of the clomazone bleaching effect by these two thiols was more pronounced when clomazone was used at the high concentration of 50 μM (Fig. 3). Exogenously applied glutathione and other thiol compounds have been also reported to reverse the phytotoxic activity of other herbicidal classes such as the chloroacetamides [20] and DBMIB (2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone) [21].

In conclusion, data presented in this paper demonstrated that clomazone is capable of reacting with reduced glutathione under *in vitro* conditions, forming a product which was tentatively identified

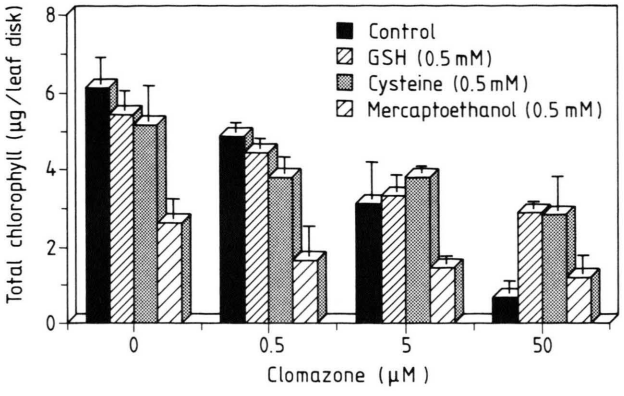

Fig. 3. Influence of exogenously applied thiols on the effect of clomazone on total chlorophyll content of velvetleaf leaf disks. Bars on each histogram represent the Standard error of each mean.

as the GS-clomazone conjugate. Indirect evidence for the potential reactivity of clomazone with GSH was provided by the reversal of the clomazone bleaching effects on velvetleaf leaf disks by exogenously applied GSH and cysteine.

Acknowledgements

This study was supported by the FMC Corporation and by the Virginia Soybean Board. We thank Mrs. Sue A. Meredith for technical assistance in parts of this study. The mass spectrometry analysis (chemical ionization mode) of clomazone and his metabolite were conducted at the Mass spectrometry Laboratory of the Virginia Polytechnic Institute and State University, Blacksburg, Virginia. Gratitude is also expressed to Dr. G. L. Lamoureux of the USDA Biosciences Laboratory at Fargo, North Dakota for his collaboration in the FAB mass spectrometry studies.

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