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Triazofenamide Is a Cellulose Biosynthesis Inhibitor

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Triazofenamide is a triazole carboximide herbicide developed by Kureha. The carboximide triazole herbicides were previously thought to be microtubule polymerization inhibitors. The assignment of this mode of action to these compounds was recently shown to be incorrect. In this communication we demonstrate that triazofenamide is a powerful cellulose biosynthesis inhibitor. The similarity of whole plant symptoms generated by cellulose biosynthesis inhibitors and microtubule biosynthesis inhibitors likely contributed to the earlier, mistaken mode of action assignment for this class of chemistry. *Arabidopsis* mutants resistant to either of two other classes of cellulose biosynthesis inhibitors, isoxaben and dichlobenil, are not cross-resistant to triazofenamide.

INTRODUCTION

The triazole carboxamides are potent herbicidal agents developed by Kureha as pre- and postemergent herbicides for the cereal and rice markets. O'Keefe and Klevorn classified the mode of action of this chemical series as mitotic disrupters based on the combination of root and foliar symptoms elicited by a specific member of this class of chemistry, flupoxam (Fig. 1), 1-[4-chloro-3-[(2,2,3,3,3-pentafluoro-propoxy) methyl] phenyl]-5-phenyl-1H-1,2,4-triazole-3-carboximide (1). However, *via* a thorough microscopic examination of flupoxam-treated watercress roots (*Lepidium sativum*), Hoffman and Vaughn concluded that this molecule is not a mitotic disrupter herbicide (2). These investigators did not propose an alternative mode of action for flupoxam.

A second member of this chemical class, triazofenamide (Fig. 1), 1-(3-methylphenyl)-5-phenyl-1H-1,2,4-triazole-3-carboximide, was tested at Dow AgroSciences. As an adjunct to this evaluation, standard biochemistry tests were conducted (see Methods and Materials). The combination of data from these tests provided us with information that suggested that this molecule was a cellulose biosynthesis inhibitor (CBI). This hypothesis was confirmed by demonstrating that triazofenamide treatment significantly reduced the incorporation of [^{14}C-] glucose into *Arabidopsis* acid-insoluble cell wall components at physiologically relevant rates.

In this report we clearly demonstrate that triazofenamide is a specific, powerful inhibitor of cellulose biosynthesis. Furthermore, neither dichlobenil- nor isoxaben-resistant mutants display any significant measure of cross-resistance, indicating that the mode of binding of triazofenamide to the cellulose synthesizing apparatus is different from that of dichlobenil or isoxaben.

METHODS AND MATERIALS

Chemicals

Triazofenamide was synthesized by Dr. T. Thibault (Dow AgroSciences). Isoxaben was supplied by Dr. K. W. Burow (Dow AgroSciences). Dichlobenil was from Chem Service Inc. Radiolabeled glucose was obtained from DuPont NEN Research Products (Boston, MA). DMSO, chloroform, acetone, HPLC grade methanol, nitric acid, acetic acid, agarose, and KOH were from Fisher Scientific (Pittsburgh, PA). Yeast extract was from Difco Laboratories (Detroit, MI). All other standard laboratory chemicals were from Sigma Chemical Co. (St. Louis, MO).

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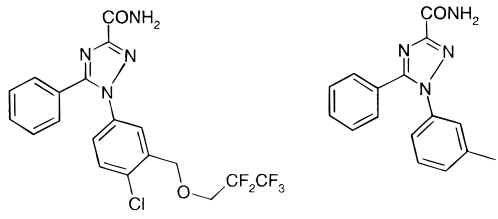

Flupoxam Triazofenamide

FIG. 1. *Triazole carboxamides mentioned in this report.*

Biochemistry Assays

Triazofenamide was characterized using a battery of tests which enable us to classify compounds according to their probable mode of action. These tests, which constitute what we call Tier I, are composed of: (i) an *Arabidopsis*/*Agrostis* plate test; (ii) root measurements in a short-term *Arabidopsis* aqueous test; (iii) an electrolyte leakage test; and (iv) a simple fluorometric photosynthesis assay.

Both *Arabidopsis* and *Agrostis* seeds were sterilized by agitating for 10 min in 10 ml of a 50% bleach solution with one drop of Triton X-100 added as a wetting agent. These seeds were then washed four times with sterile water. They were subsequently dispensed for treatments using a micropipetter with a wide-bore tip.

The *Arabidopsis*/*Agrostis* plate test utilized a nutrient growth medium (3) supplemented with 0.6% sucrose and solidified with 0.5% agarose. A 10-ml aliquot of this melted medium, maintained at 55°C, was used for each treatment. Experimental compounds were added to the medium at various doses as concentrated solutions in DMSO. Controls had the appropriate amount of DMSO added without the inhibitor; the final concentration of DMSO never exceeded 0.8%. We have established that DMSO concentrations in this range do not alter either *Arabidopsis* or *Agrostis* growth, development, or response to common herbicidal agents (data not shown). Half of the treatments also contained the addition of yeast extract to a final concentration of 0.025% to determine whether a complex mix of nutrients would ameliorate the phytotoxic effects. After thorough mixing, 8 ml of the treated medium was transferred into a 60X 15-mm sterile petri dish (Falcon 1007; Becton Dickinson, Lincoln Park, NJ) and allowed to solidify. Approximately 20 μl of sterilized seed was then applied onto the solidified medium and spread evenly over the plate using the remaining 2 ml of the treatment medium. These plates were incubated for 7 days at 23°C under continuous fluorescence lighting (50 μ $m^{-2}s^{-1}$).

Root measurements in *Arabidopsis* and total length measurements in *Agrostis* were recorded to determine the degree of phytotoxicity of test compounds, while a variety of visual symptoms were recorded in both test species and compared to those elicited by well-known standards to obtain information relative to the possible mode of action. We have demonstrated that herbicidal agents with the same mode of action typically elicit similar symptoms (data not shown).

The short-term aqueous test utilized *Arabidopsis* seedlings that had been pregrown on half-strength Murashige and Skoog basal salt medium (4), with 20 g/L sucrose and 8 g/L agarose, in a vertical orientation at 23°C for 7 days. Treatment solutions were prepared in 6-well dishes with full-strength Murashige and Skoog medium and 30 g/L sucrose to give final concentrations of 2.5 and 2.5 ppm of the test compound. The pregrown seedlings were then dropped into the treatment solutions. The dishes were incubated at 26°C in the dark for 2 days. After this culture period seedlings were observed using an inverted microscope at 40X magnification. Observations were made on root tip diameter and other aspects of seedling morphology. Root body and root tip expansion were measured with

an eyepiece reticle. Changes from control morphology were noted for root pericycle and epidermis, root hairs and root tips, petiole length, and leaf hairs in newly expanded seedling leaves. Root growth or its absence was noted. Measurements were compared to those of well-known standards (data not shown).

The electrolyte leakage assay was conducted as described by Duke and Kenyon (5) with slight modification to allow detection of light-enhanced phytotoxicity. This test identifies compounds that injure plants by causing rapid membrane damage, for example by causing free radical formation and subsequent lipid peroxidation.

The photosynthesis assay is a standard test for inhibition of photosystem II (PSII) activity by measuring enhanced fluorescence (6). Comparisons are made in fluorescence measurements between treated mustard leaves and controls utilizing a plant productivity fluorometer (Model SF-20; Richard Brancker Research Ltd., Ottawa, Canada).

Cell Wall Fractionation

The cell wall fractionation procedure was a modification (7) of that described by Carpita and Kanabus (8). The cell wall was fractionated into eight different fractions: (i) unincorporated material and small molecules; (ii) fatty acids; (iii) starch; (iv) pectins; (v) hemicelluloses and pectins; (vi) remaining hemicelluloses; (vii) everything else that is not cellulose; and (viii) cellulose. All treatments consisted of at least three repetitions within each experiment. Inhibitor concentration was 1 μM. This concentration was chosen because it is well above the I_{50} and is expected to provide complete inhibition by both isoxaben and triazofenamide. Incubation time was 1 h in the dark at 27°C. Since this is a very time-consuming assay only one concentration and time point were done.

Cross-Resistance Testing

Seeds from the various *Arabidopsis thaliana* strains (see Table 1) were sterilized, grown, and treated as described above for the *Arabidopsis*/*Agrostis* plate test. Root growth was measured in wild type and compared to that in the dichlobenil- and isoxaben-resistant mutants. A triazofenamide root growth inhibition dose–response curve was established for each of the isoxaben-resistant mutants (DH1, DH47, and DH48) as well as for the dichlobenil-resistant mutant (DH75) and wild type.

TABLE 1
Strains Used in this Study (7)

Strain	Description
Arabidopsis thaliana var. Columbia	Wild-type strain and progenitor of all the mutants described here
DH1	Isoxaben-resistant mutant; resistance not allelic to DH47, DH48, or DH75
DH47	Isoxaben-resistant mutant, resistance allelic to DH48, not allelic to DH1 or DH75
DH48	Isoxaben-resistant mutant, resistance allelic to DH47, not allelic to DH1 or DH75
DH75	DCB-resistant mutant, not allelic to DH1, DH47, or DH48

RESULTS

Tier I Biochemistry

Triazofenamide was evaluated in the Tier I panel of tests. This molecule did not cause electrolyte leakage and was negative in the PSII assay; however, it did produce a distinctive morphology in the short-term *Arabidopsis* aqueous test. The seedling root tips swelled and developed in a manner characteristic of CBIs. The swelling of seedling root tips which occurred is generally characteristic of both microtubule polymerization inhibitors (MTIs) and CBIs; however, a detailed examination of the symptomology distinguishes between these two modes of action (summarized in Table 2). The diameter of the treated root tips was approximately twofold that of the controls, and root hairs had continued to differentiate on the root epidermis so that they appeared to cover the root tip. This combination of symptoms is indicative of CBIs rather than MTIs (9).

TABLE 2
Symptoms Distinguishing between CBIs and MTIs in the Biochemistry Tests

	Solvent control	Cellulose biosynthesis inhibitor	Microtubule polymerization inhibitor
Arabidopsis root tip diameter	125 μm	250–350 μm	450–600 μm
Root hair development	Begins in zone of differentiation 750 μm from root tip	Although root elongation ceases, development of abundant, long root hairs continues to within 130 μm of root tip, appearing in cross section as if the tip itself has grown hairs. Root hairs may be swollen in a goblet shape.	A few root hairs may differentiate near root tip but this is not a prominent feature.
Condition of root epidermis	Root epidermis integrity is normal	Root epidermis integrity is maintained.	Root epidermis near tip is profoundly disordered as each cell rounds up, giving the appearance of a mass of bubbles.
Lateral root initials	Normal	Lateral roots initiate normally.	No lateral root initials are formed.
Additional symptoms	Not applicable	Distorted development of leaf hairs in Arabidopsis; often selective for dicots.	Distinctive grass seedling morphology; typically selective for grasses.

Cell Wall Fractionation

Cell wall fractionation revealed that the overall pattern of [^{14}C]-glucose incorporation resulting from a brief exposure to triazofenamide treatment was essentially identical to that elicited by isoxaben, a known CBI (Table 3). Both compounds displayed their greatest inhibitory effect on the incorporation of radioactive glucose into cellulose (>80% inhibition). This table also shows that both compounds also significantly reduced the incorporation of label into the hemicellulose fraction. This is a nonspecific effect that has been previously observed when CBIs are used at rates that greatly exceed the levels necessary to obtain root growth inhibition (data not shown).

TABLE 3
Cell Wall Fractionation of Wild-Type Arabidopsis Treated with either Triazofenamide (TF) or Isoxaben (dpm)

Fraction	Control	TF	Isoxaben
Soluble	245,400	262,800	210,700
Lipid	6,000	6,800	6,700
Starch	6,000	6,400	5,000
Pectin	10,900	13,400	12,800
Hemicellulose	102,000	51,000	49,000
Acid	22,100	16,100	14,600
Cellulose	11,200	1,900	1,200

Mutant Cross-Resistance

A triazofenamide dose–response curve was established for root growth inhibition for each of the isoxaben-resistant *Arabidopsis* mutants (DH1, DH47, and DH48), as well as for the dichlobenil-resistant mutant (DH75) and the wild type. The I_{50} values generated from these curves (Table 4) did show modest variability; however, they clearly indicate that these mutants are as sensitive to triazofenamide as is wild-type *Arabidopsis.* Therefore there is no cross-resistance between isoxaben- or dichlobenil-resistant mutants and triazofenamide.

DISCUSSION

Triazofenamide is a highly active CBI. This compound was synthesized by Kureha as a prospective rice herbicide. Recently, we became

TABLE 4
Dose–Response I_{50}s of Triazofenamide (TF), DCB, and Isoxaben on Various Herbicide-Resistant Arabidopsis Strains

	I_{50} (nM)		
Strain	TF	Isoxaben	DCB
Wild-type	39	1	400
DH75	45	2	1600
DH1	23	23	300
DH47	45	>1000	300
DH48	41	285	400

aware that a close analog of triazofenamide, flupoxam, a herbicide targeted for broadleaf control in wheat, had been tentatively classified as a microtubule polymerization inhibitor (1). Hoffman and Vaughn (2) reinvestigated this issue with a combination of staining and microscopic techniques and concluded that this compound was not a microtubule polymerization inhibitor; however, they did not postulate what the actual mode of action might be. These studies motivated the present investigation in order to provide the missing information.

We postulated that triazofenamide was a cellulose biosynthesis inhibitor due to the symptoms elicited in the *Arabidopsis* short-term aqueous test (Table 2). The diameter of the treated root tips was approximately twofold that of the controls, and root hairs had continued to differentiate on the root epidermis so that they appeared to cover the root tip. This combination of symptoms is indicative of CBIs rather than MTIs (9). MTI-treated root tips would have a larger diameter and be devoid of root hairs. We then showed that this compound specifically inhibits both root elongation (Table 4) and glucose incorporation into cellulose (Table 3) in a manner similar to isoxaben, a known CBI (7). These data confirmed that triazofenamide was indeed a cellulose biosynthesis inhibitor.

It is clear from the studies outlined in this report that triazofenamide is a specific cellulose biosynthesis inhibitor. Furthermore, its lack of cross-resistance with isoxaben- or dichlobenil-resistant mutants (Table 4) suggests a unique mode of binding for this molecule. These data add credence to the hypothesis that cellulose biosynthesis involves a highly complex enzymatic target containing several nonoverlapping binding sites with the ability to accommodate a variety of different chemistries. These nonoverlapping sites could be in different cellulose synthases; recent sequencing efforts in *Arabidopsis* have revealed that at least in this plant cellulose synthase is a large multige family (10). However, these data do not rule out the possibility that these molecules inhibit different steps in the cellulose biosynthetic pathway. This latter case would be analogous to the situation found in the branched-chain amino acid pathway, where the inhibition of several different enzymes (ALS, KARI, IPMD) all lead to herbicidal consequences, albeit at differing inhibitor concentrations (11). Investigation of this question is difficult due to the current absence of an *in vitro* enzyme assay for cellulose synthase.

In conclusion, by the criteria of plant symptoms and specific inhibition of glucose incorporation into cellulose, triazofenamide is a cellulose biosynthesis inhibitor. It would be surprising if its close analog, flupoxam, was not.

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