Vol. 157, No. 1, 1988 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 30, 1988 Pages 174-182

TRITICONE A: A NOVEL BIOACTIVE LACTAM WITH POTENTIAL AS A MOLECULAR PROBE

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Received October 10, 1988

Triticone A is one member of a family of novel compounds which are spirocyclic lactams produced by several plant pathogenic fungi including Drechslera tritici repentis on wheat. It undergoes racemization to form triticone B and when tested, the enantiomeric mixture causes chlorosis and necrosis on a wide range of plants. Fluorescein diacetate treated protoplasts in conjunction with various triticone treatments allowed for accurate quantitation of the biological activity of the toxin. Various physiological functions of the wheat cell are impaired including the Hill and CO_2 fixation reactions in photosynthesis. In addition, triticone A inhibits enzymes that have SH functional groups as part of their active site, eg., the protease-ficin. Neither triticone C or D had any activity in the enzyme or protoplast assays. It is apparent that triticone A has some potential as a molecular probe in a variety of biological systems.

Fungi are common phytopathogens and many produce phytotoxins, extracellular bioactive compounds which may evoke chlorosis, necrosis, and wilt -- common symptoms of plant disease. Phytotoxins have tremendous potential as screening agents for the selection of disease-resistant crops and as models for bioregulators with herbicidal or phytohormone-like activity (1,2). And, like other microbial toxins, they may have potential as molecular probes.

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0006-291X/88 $1.50

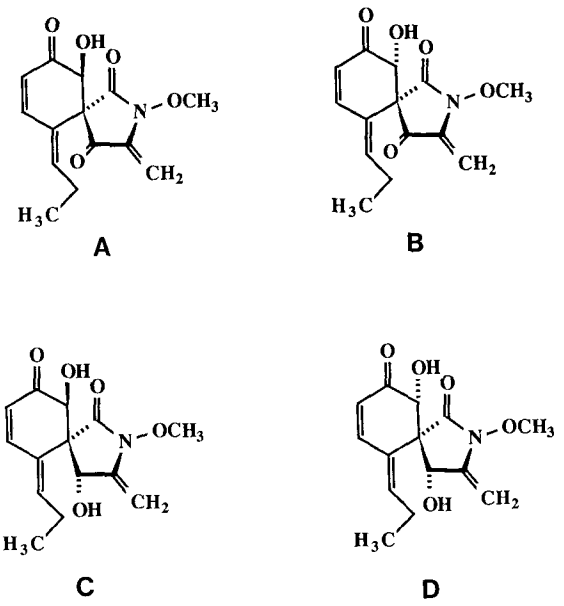

Fig. 1 Structural formulae of triticones A, B, C, and D

Recently, a series of novel molecules containing an unusual spirocyclic lactam moiety was discovered in culture filtrates of Drechslera tritici-repentis which causes tanspot disease of wheat (3). Given the trivial name of triticones, this family of compounds is comprised of analogs which differ only in the stereochemistry of the hydroxyl group on the cyclohexene ring or in the oxidation level of the carbon alpha to the exocyclic methylene (3, Sugawara, unpublished, Fig. 1). This report presents results of preliminary experiments which tested the bioselectivity and molecular reactivity of triticones A, C and D.

METHODS

Triticones. Triticones A, B, C, and D were obtained as described (3, Sugawara unpublished). Because triticones A and B are interconvertible (3), triticone A in this paper will refer to the enantiomeric mixture of A and B.

Protoplasts. Leaves of Triticum aestivum L cultivars Pondera and Red Chief (wheats) were abraded gently with carborundum to remove epidermal layers, then diced with a razor blade (4). Ten grams of leaf pieces were transferred to 25 ml digestive solution containing 500 mM sucrose, 1 mM $CaCl_2$, 5 mM MES, 1% Cellulase (Worthington 67159P), 1% Rhozyme (Genencor HP 150), and 0.3%

Macerozyme (Yakult) at pH 5.8. After shaking at 40 rpm for 3 h, the brei was filtered through muslin and centrifuged at 400 g x 15 min. The floating layer of chloroplasts was removed, washed in 20 ml of 500 mM mannitol, 1 mM $CaCl_2$, 5 mM MES at pH 6.0, and recentrifuged at 400 g x 15 min. The pellet was collected and resuspended in the mannitol solution at a concentration of 10^6 protoplasts/ml.

For assays using flow cytometry, triticones in 2% ethanol were added to protoplast suspensions for 1 to 3 h. Stainability with fluorescin diacetate (FDA) was monitored using a Becton-Dickinson fluorescent activated cell sorter (FACS) 440 with a Spectra-Physics 164-05 argon ion laser tuned to 200 mW at 488 nm (4). Autofluorescence of chlorophyll was monitored with a 650 nm long pass filter. Fluorescence of fluorescein was measured with a 530/30 band pass filter using a 600 nm dichroic mirror. Protoplasts were passed through a 70 μ nozzle at 1000/min. Forward light scatter properties of each particle were used to establish a minimum size threshold. Data were collected in the LIST mode and analyzed using LACEL programs ACQ4, DISP4, and DISP2D with a Becton-Dickinson Consort 40. The experiments were repeated and the results obtained were ±10% of the values shown.

Chloroplasts - Hill Reactions. Chloroplasts of wheat cultivar Pondera and Avena sativum (oats) were obtained as described in (5) and (6), respectively. The assay buffer for wheat was 330 mM sorbitol, 10 mM EDTA, and 25 mM Tricine at pH 8.4. For oats, the assay buffer was 330 mM sorbitol, 2 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, and 50 mM HEPES at pH 7.6. Using appropriate light and dark controls, triticones were added to 0.8 ml of chloroplast suspensions in assay buffer (10 μg chlorophyll/ml) and preincubated 10 min at 22C. Then, 0.2 ml of 2,6 dichlorophenol-indolphenol (DCPIP) (2 mM) was added and the suspension was incubated under cool, white fluorescent light (70 μEinsteins/m^2/sec) for 10 min. Absorbance at 580 nm was measured and the % DCPIP reduced was calculated.

Enzymes. The esterolytic activity of ficin (Sigma #105C-9500) was monitored at 253 nm using BAEE as the substrate (7). Subtilisin BPN' (Sigma P-5255) and acetone powders of mycelia from D. tritici-repentis were assayed using azocasein (8). β-Glucosidase activity and esterase activity of acetone powders of the fungus were monitored spectrophotometrically at 405 nm and 346 nm using p-nitrophenyl-β-glucoside and o-nitrophenyl-acetate, respectively. The esterase activity of acetone powders of

Pondera wheat was measured using FDA and monitoring the reactions with a Spex Fluorolog 2, model F211 equipped with a xenon 150 W lamp and 0.5 mm emission and excitation slits. Fluorescent emission at 515 nm was measured by single photon counting every 60 sec for 12 min. All enzyme assays were optimized and data reported as % of control (no toxin) values. The experiments were repeated at least twice with essentially the same results.

CO_2 Fixation. Dark CO_2 fixation was measured as previously described with the same statistical variation as indicated (9).

RP-HPLC. Chemical modification of triticones A and C by cysteine and serine was evaluated by monitoring peak height and retention time during RP-HPLC on a SenshuPak ODS column (6 x 150 mm) using CH_3CN: H_2O (1:1) and 1 ml/min; the R_t for triticone A is 5 min, triticone C is 6 min.

RESULTS

Triticones A, C, and D were screened for necrogenic activity on excised leaves of several graminicolous and four dicotyledenous plants utilizing a leaf puncture-droplet overlay technique (10,11). Tricicone A caused necroses on all eleven plants when applied at a concentration of 900 µM. At 36 µM triticone A, nine of the eleven developed necrotic lesions. In contrast, triticones C and D, 900 µM, failed to evoke necroses on any of the plants. Pondera, a cultivar of wheat, did develop chlorosis when treated with triticone D at 900 µM.

As a more quantitative alternative to the leaf-puncture method of monitoring biological activity, we developed a sensitive assay using protoplasts. This assay permitted uniform suspension followed by a direct assessment of bioactivity via flow cytometry and an appropriate fluorescent stain (4). Protoplasts were prepared from the wheat cultivars Pondera and Red Chief, incubated in 36 µM triticone A, and fluorescent labeling studied as a function of treatment time. Within 30 minutes, viability of protoplasts decreased by nearly 50% (Fig. 2), and LD_{50} values for triticone A approached 4 to 6 µM after 3 h (Fig. 3). Neither triticone C nor D caused any discernible effects on protoplasts from either cultivar (Fig. 2).

Triticone A may have prevented fluorescent labelling of treated protoplasts by perturbing the plasmalemma, thus precluding retention of fluorescein (the fluorescent product of FDA hydrolysis). However, specific stains that only enter cells

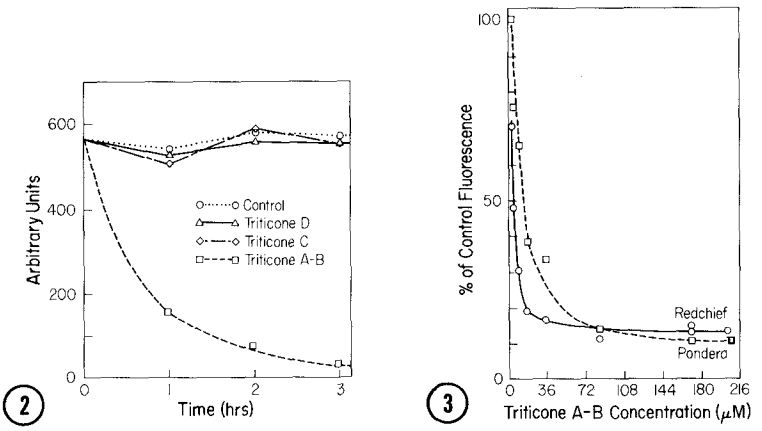

Fig. 2. Protoplasts of Pondera wheat were exposed to triticones (36μM in 2% aqueous EtoH) for 1 to 3 hours. The ordinate represents the number of fluorescent events times the mean fluorescence intensity divided by 1000. Units of fluorescence intensity are arbitrary and logarithmic.

Fig. 3. Concentration dependent reaction of wheat protoplasts to triticone A. Protoplasts of either cultivar Redchief or Pondera were treated for 2 hours with triticone A in 2% EtoH. Stainability was calculated as in (Fig. 2) then expressed as % of the 2% EtoH control. The ordinate is a percentage calculated by multiplying mean fluorescence from FDA times the number of fluorescent events of treated samples, divided by the same calculation for the control (untreated protoplasts).

with damaged membranes such as calcofluor white M2R, and Hoechst 33258 (4) did not enter triticone A treated protoplasts any better than they entered untreated protoplasts. As a positive control, protoplasts were treated with 10 mM trichloracetate, a chaotrope known to disrupt biological membranes (12). Trichloroacetate greatly facilitated staining by calcofluor white and Hoechst reagents.

The effect of triticones on enzymes was tested using protein preparations from several sources. Acetone powders prepared from wheat leaves were optimized for esterase activity using FDA in a fluorescence assay. Within 10 minutes, exposure of the wheat esterase to 36 μM triticone A resulted in 50% inhibition of the enzyme activity as compared to an untreated control preparation (Table 1). Neither triticone C or D had any inhibitory effect (data not shown). In contrast, esterase activity of D. tritici-repentis was stimulated slightly at 3-fold concentrations of triticone A (Table 1). β-Glucosidase activity of the mycelial preparation was unaffected, but the fungal protease activity was extremely sensitive (Table 2). Further tests with commercial

Table 1. Activity of triticone A on various enzyme preparations as contrasted to a control reaction run under optimum conditions*

Enzyme Activity	Activity of Control %	Activity % of Control Triticone A µM			
		36	72	108	
Ficin	100	70	56	47	
Subtilisin	100	100	97	95	
Protease (from D. tritici)	100	26	23	23	
Glucosidase (from D. tritici)	100	98	100	100	
Esterase (from D. tritici)	100	102	117	123	
Esterase (wheat Pondera)	100	50	-	-	
		Triticone A µM			
		.04	.4	4.0	24.0
Fd-oxido-reductase	100	132	177	296	573

*Each enzyme activity that was assayed was optimized for the conditions described (see Methods). Each assay mixture contained between 50 and 500 µg of protein, 1 ml of solution, an incubation time of 5-10 min at 22°C.

proteases revealed that ficin, a known SH protease, was inhibited by triticone A while subtilisin, a serine protease, was relatively unaffected.

Because chlorosis is a predominant symptom in susceptible reactions of wheat to the pathogen, chloroplasts of wheat and oats were isolated and Hill reactions were monitored in the presence and absence of triticone A or C. An examination of the Hill reaction tests the efficiency of electron flow in photosynthetic electron transport. Triticone C was inactive, while triticone A inhibited this process in wheat and oats by approximately 16% and 33% at 22 and 45 µM respectively. The chlorophyll spectrum of chloroplasts treated with triticone A was not appreciably different from that in controls, so the sites of inhibition were suspected to be in the proteins. The terminal enzyme in photosynthetic electron transport is ferredoxin-oxido-

reductase, known to have at least two sulfhydryl groups necessary for catalytic reduction of NADP (13). Ferredoxin, a cofactor for this enzyme has a 2Fe-2S type of electron transfer system. In contrast to its inhibition of ficin, triticone A stimulated ferredoxin oxido-reductase (Table 1). As little as 40 nM triticone A was sufficient to increase reduction of NADP by nearly 30%. CO_2 fixation is another function of chloroplasts. In multiple assays of leaf pieces of Coker and Pondera wheat cultivars, triticone A (32 ± 13 μM) inhibited CO_2 fixation in both by 50%.

At this point certain studies on the chemical reactivity of triticone A were conducted. This compound is unstable at temperatures > 35°C and in the presence of silica gel (3). We presumed that triticone A is active because of its electron-deficient exocyclic methylene group (Fig. 1) and that strong nucleophiles should react with triticone A in a nucleophilic addition. Indeed, cysteine (free base) and serine did react with triticone A in HEPES at pH 7.5. Using RP-HPLC to monitor the level of triticone A, equimolar cysteine caused losses of 87%, 90%, and 94% of detectable triticone A in solution within 1, 5, and 10 minutes after mixing, respectively. No losses were detected if cysteine was omitted from the reaction mixture. Analogous treatment with serine caused losses of 17%, 40%, and 49% in the same respective time periods. (Neither cysteine-HCl nor serine reacted with triticone A in methanol or methanol-water mixtures.) Identical treatment of triticone C with cysteine (free base) or serine caused no losses of triticone C. After isolation and purification of the reaction product by thin layer chromatography, preliminary analyses of the NMR and FAB-MS data suggested that the product of triticone A and an 8-fold excess of cysteine was a cysteinyl adduct of triticone A.

DISCUSSION

At the molecular level, triticone A appears capable of modifying sulfhydryl enzymes. Ficin is inhibited and ferredoxin oxido-reductase is stimulated when treated with triticone A. Subtilisin, however, exhibits little or no sensitivity to this toxin. The facile reaction between cysteine and triticone A is suggestive that triticone A affects SH enzymes via covalent modification. Further, the nonreactivity of triticone C

indicates that the keto group adjacent to the exocyclic methylene is essential for reactivity.

At the physiological level, triticone A is nonselectively toxic towards plant tissues and protoplasts. The site(s) of activity are intracellular and altered permeability of the plasmalemma does not appear to be a factor. Triticone A generally suppresses the photosynthetic processes in cells and chloroplasts, even though it stimulates purified ferredoxin oxido-reductase, the terminal enzyme of PS-ETS. The role of triticones in plant plathogenesis remains to be understood, however the chlorosis-inducing activity of triticone C on Pondera wheat may be involved in the cultivar selectivity of tanspot disease (14). Also interesting is the inhibition of proteases from D. tritici-repentis by triticone A. We must ask, how does this fungus avoid poisoning itself?

While much more work remains to establish the nature of interaction of triticone A with proteins, these initial results demonstrate that this novel lactam has potential as a molecular probe of sulfhydryl enzymes.

ACKNOWLEDGEMENTS

The authors wish to acknowledge the support of the National Science Foundation grant KMDB-8607347 and the Montana Agricultural Experiment Station.

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