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INHIBITION OF TRICHOTHECENE TOXIN BIOSYNTHESIS BY NATURALLY OCCURRING SHIKIMATE AROMATICS

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Key Word Index—Umbelliferae; Leguminosae; furanocoumarins; flavonoids; *Fusaria*; trichothecene toxins.

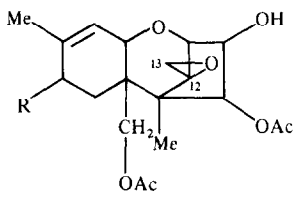
Abstract—Certain naturally occurring flavonoids and furanocoumarins are inhibitors of trichothecene toxin biosynthesis. These compounds block T-2 biosynthesis in liquid cultures of *Fusarium sporotrichioides* NRRL 3299 at concentrations substantially less than required to block fungal growth. Inhibited cultures accumulate variable amounts of trichodiene, the hydrocarbon precursor of the trichothecenes. These inhibitors appear to block the trichothecene biosynthetic pathway after formation of trichodiene and before formation of highly oxygenated trichothecenes. Exposure to these widely occurring plant shikimate aromatics may inhibit trichothecene production during plant pathogenesis.

INTRODUCTION

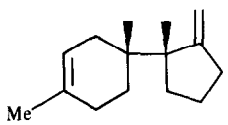
Plants produce a great diversity of secondary metabolites as normal constituents and as phytoalexins induced by fungal infection. Many of these compounds have been shown to inhibit fungal growth *in vitro* and have been postulated to similarly restrict fungal growth in plant tissues [1]. Although most research on phytoalexins and related compounds has concerned their direct fungitoxicity, some plant metabolites have also shown indirect effects such as inactivation of fungal hydrolytic enzymes [2].

Plant pathogenic species of *Fusarium* produce a wide variety of phytotoxic secondary metabolites including trichothecene toxins, potent eukaryotic protein synthesis inhibitors [3]. Evidence for the role of trichothecenes in plant pathogenesis includes their isolation from diseased plant tissue [3] and correlations of virulence with ability to produce toxin *in vitro* [4]. Plants resistant to toxin-producing fungi might produce factors that promote toxin degradation [5] or prevent toxin synthesis.

The trichothecene toxins, including T-2 toxin, neosolaniol and diacetoxyscirpenol (Fig. 1), are synthesized from the hydrocarbon trichodiene (Fig. 1) by a series of oxygenations known to require molecular oxygen [6]. We have investigated the effects on trichothecene biosynthesis of a variety of cytochrome P-450 monooxygenase inhibitors [7], sterol-inhibitory fungicides and plant secondary metabolites, including two classes of shikimate aromatics—flavonoids and furanocoumarins. The ability of flavonoids to modulate cytochrome P-450 dependent monooxygenations *in vivo* and *in vitro* has been well-documented in mammalian systems [8–10]. Similar studies of furanocoumarins have been primarily concerned with their effects on microsomal monooxygenations in insect herbivores [11]. We report here the identification and characterization of flavonoids and furanocoumarins that inhibit trichothecene toxin production in the plant pathogenic fungus *Fusarium sporotrichioides* in liquid cultures.



T-2 toxin R = $OCOCH_2CH(Me)_2$
Neosolaniol R = OH
Diacetoxyscirpenol R = H

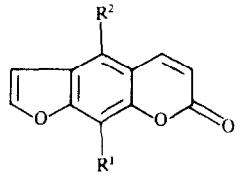


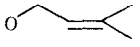
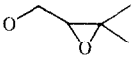
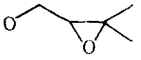
Trichodiene

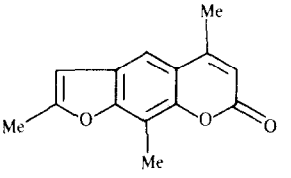
Fig. 1.

RESULTS AND DISCUSSION

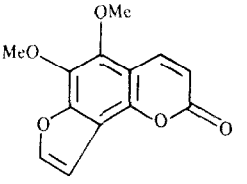
For initial comparisons of toxin inhibition by shikimate aromatics, fungal cultures were treated at approximately 10 mM in microtiter plates and assayed with a monoclonal antibody to T-2 toxin. Fourteen of 15 flavonoids and the three isoflavonoids tested did not inhibit (0%) T-2 toxin production (biochanin A, butein, chrysin, daidzein, eupatorin, fisetin, galangin, hesperetin, kaempferol, luteolin, 7-methoxyflavone, 6-methylflavone, morin, α-naphthoflavone, β-naphthoflavone, quercetin



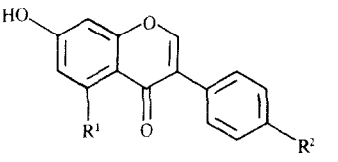
	R^1	R^2
Psoralen	H	H
Xanthotoxin	OMe	H
Xanthotoxol	OH	H
Bergapten	H	OMe
Isopimpinellin	OMe	OMe
Imperatorin		H
Isoimperatorin	H	
Oxypeucedanin	H	
Heraclenin		H
5,8 - Dihydroxy-psoralen	OH	OH



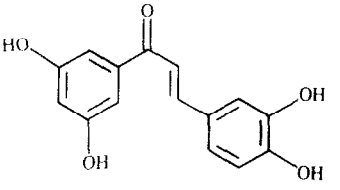
Trioxsalen



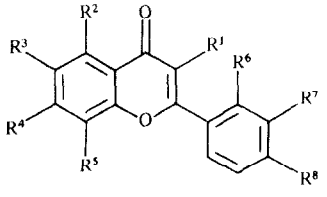
Pimpinellin



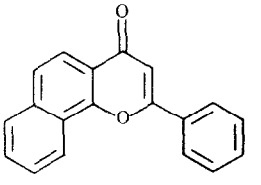
Daidzein R = R^2 = H
Biochanin A R^1 = OH R^2 = OMe



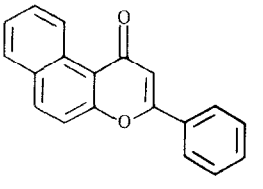
Butein



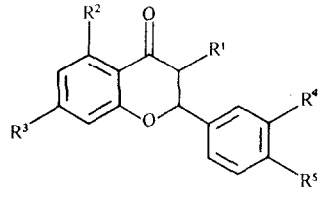
Flavone unsubstituted
7 - Methoxy R^4 = OMe
4' - Methoxy R^8 = OMe
Chrysin R^2 = R^4 = OH
Kaemferol R^1 = R^2 = R^4 = R^8 = OH
Galangin R^1 = R^2 = R^4 = OH
Fisetin R^1 = R^4 = R^7 = R^8 = OH
Luteolin R^2 = R^4 = R^7 = R^8 = OH
Morin R^1 = R^2 = R^4 = R^6 = R^8 = OH
Quercetin R^1 = R^2 = R^4 = R^7 = R^8 = OH
Eupatorin R^2 = R^7 = OH, R^3 = R^4 = R^8 = OMe
6 - Methyl R^3 = Me
6 - Methoxy R^3 = OMe
3 - Methoxy R^1 = OMe
2' - Methoxy R^6 = OMe



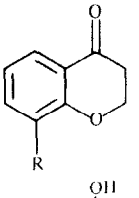
α - naphthoflavone



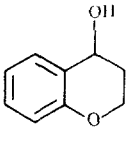
β - naphthoflavone



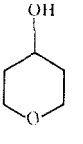
Flavanone unsubstituted
Hesperetin R^1 = H, R^2 = R^3 = R^4 = OH, R^5 = OMe
Taxifolin R^1 = R^2 = R^3 = R^4 = R^5 = OH



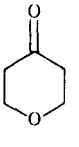
4 - Chromanol



4 - Chromanone R = H
5 - Hydroxy 1 - tetralone R = OH



Tetrahydropyranol



Tetrahydropyranone

Fig. 2.

and taxifolin) (Fig. 2). Flavone strongly (>85%) inhibited T-2 toxin production. Eleven of the 12 furanocoumarins tested strongly inhibited (60–85%) T-2 toxin production in *F. sporotrichioides* (bergapten, 5,8-dihydroxypsoralen, heraclenin, imperatorin, isoimperatorin, isopimpinellin, oxypeucedanin, pimpinellin, psoralen, xanthotoxin and

xanthotoxol). Trioxsalen did not inhibit (0%) toxin production.

Several toxin-inhibitory compounds identified in the initial screen were retested at 1–5 mM in 10 ml cultures to determine whether they inhibited toxin production directly, or indirectly by inhibiting fungal growth. Culture extracts were analysed by gas chromatography. Many of the furanocoumarins, in particular, almost completely inhibited T-2 toxin production at concentrations where fungal growth was much less effectively inhibited (Table 1). These results suggested that the inhibition of trichothecene production was not due solely to direct fungitoxicity.

To further investigate structural requirements for toxin inhibition, various flavone derivatives and related simpler compounds were screened at 5 mM in 25 ml fungal cultures. T-2 toxin production was measured by immunoassay. The substituted flavonoids 2′-methoxyflavone, 6-methylflavone, 7-methoxyflavone; the chromanoids 4-chromanone, 4-chromanol, 5-hydroxy-1-tetralone; and tetrahydropyranol and tetrahydropyranone did not significantly (>20%) inhibit fungal growth or T-2 toxin production. Some toxin inhibition was observed with the flavone derivatives 4-methoxyflavone (35% inhibition), 6-methoxyflavone (39%), 3-methoyxyflavone (58%) and flavanone (73%). Under the same conditions, unsubstituted flavone at 2, 4 or 5 mM inhibited T-2 toxin production by 95–100% (Table 1). Flavone and flavanone decreased fungal growth but, always, to a lesser extent than they affected toxin production.

The lack of activity exhibited by the chromanoids and the pyrano-compounds indicates that the complete flavone structure is necessary for toxin inhibition. Certain positions on the flavone rings (e.g. R^4 and R^6) apparently are important because substituents there diminished activity. Substitution at R^3 gave activity that ranged from proton > methoxy > methyl. Reduction of the 2,3-double bond to flavanone retained much of the activity. All of the highly substituted compounds that are common plant constituents were inactive at 5–10 mM. Specificity in the effects of a variety of flavonoids on mammalian microsomal enzymes has been previously reported [8–10].

A completely different structure-activity correlation was observed in the furanocoumarin series tested. Both unsubstituted compounds such as psoralen and methoxylated analogues such as xanthotoxin and the pimpinellins were very active, while a methylated analogue, trioxalen, had no toxin-inhibitory activity. Substitution with prenyl and epoxyprenyl side chains (imperatorin, isoimperatorin, oxypeucedanin and heraclenin) still failed to diminish activity. Differential effects of the various compounds are probably not due to their differential metabolism by fungal cultures because neither flavone nor the furanocoumarins could be recovered from the media after incubation.

In order to identify the site of toxin inhibition, the treated cultures were analysed by GC and GC-MS for trichothecenes and other sesquiterpene compounds that might be biosynthetic intermediates. Analysis of culture

Table 1. Effect of flavone and furanocourmarins on dry weight, T-2 toxin and trichodiene in *Fusarium sporotrichioides*

Compound	mM	% Inhibition*		Trichodiene(μM)*‡
		Dry wt	T-2 Toxin†	
Flavone	5	85	100	7
	4	55	100	6
	2	62	100	59
Heraclenin A	5	45	96	42
B	5	20	100	0
Imperatorin A	5	23	97	64
B	5	0	100	1
Xanthotoxin A	1.5	46	95	265
B	1.5	46	100	304
Bergapten	5	0	100	0
Pimpinellin	5	0	82	14
Isopimpinellin	5	12	100	39
Psoralen	5	48	100	74

*Each number is the result of a single test in a 10 ml culture as described in the Experimental. Net dry weight and net T-2 toxin were determined by subtracting values at 24 hr (when test compounds were added) from values at day 7. Data are expressed as percent of a DMSO-treated control culture. The letters A and B designate replicate experiments.

†Analysed in duplicate by gas chromatography as described in the Experimental.

‡Analysed in duplicate by gas chromatography-mass spectroscopy as described in the Experimental.

extracts showed that the trichothecene precursor trichodiene accumulated in cultures of *F. sporotrichioides* treated with flavone or furanocoumarins (Table 1). No trichodiene was detected in DMSO-treated control cultures. GC and GC-MS analysis failed to reveal significant amounts of diacetoxyscirpenol, neosolaniol or other highly oxygenated trichothecenes in the flavone- or furanocoumarin-treated cultures. In some experiments addition of the plant secondary metabolites led to a decrease in toxin accumulation without a concommitant increase in trichodiene (Table 1). Variable recovery of trichodiene has been previously observed [7] and may be due to its catabolism or to its conversion to trichothecenes or other sesquiterpene metabolites not detected by our analytical method.

The shikimate aromatics appeared to block *in vivo* conversion of trichodiene to oxygenated trichothecenes. If trichodiene is oxygenated by a cytochrome P-450 dependent enzyme, inhibition could occur at any step of the complex monooxygenation pathway. Inhibition could result from binding to the reductase required for reduction of cytochrome P-450. In preliminary experiments with fungal microsomes, flavone and furanocoumarins at relatively high concentrations (100 μM) were poor inhibitors (< 20%) of NADPH-cytochrome P-450 reductase when cytochrome *c* was used as the electron acceptor. However, it will be necessary to repeat these assays with the natural electron acceptor, cytochrome P-450, to obtain conclusive results. Attempts to obtain trichodiene oxygenation by microsome preparations have been unsuccessful to date.

In summary, flavone and certain naturally occurring furanocoumarins are able to inhibit trichothecene toxin biosynthesis in *F. sporotrichioides*. Most toxin-inhibited cultures accumulate the hydrocarbon trichodiene, a trichothecene biosynthetic precursor, which suggests that trichodiene oxygenation is blocked. The shikimate aromatics appear to block the trichothecene biosynthetic pathway before production of any trichothecenes with a 12,13-epoxy group, a substituent necessary for toxicity. Levels of the furanocoumarins high enough to block toxin production are readily attained in uninfected plants [12, 13], and their concentrations can be greatly increased upon fungal infection [14]. Trichothecene toxin production may be limited during pathogenesis in plants that contain these widely occurring metabolites.

EXPERIMENTAL

Chemicals. Furanocoumarins were purified and analysed as previously described (15).

Cultures. A culture of the T-2 toxin-producing strain *F. sporotrichioides* NRRL 3299 (ATCC 24043) derived from a single spore was used for these studies. Solid-medium cultures were grown under 20 W General Electric Cool White fluorescent tubes (F20 T12-CW) in a growth chamber programmed for an alternating 12 hr, 25° light/12 hr 20° dark schedule. Strains were maintained on V-8 juice agar slants at 4° and as conidial suspensions in 10–15% glycerol at −70°. Liquid cultures were inoculated at a starting density of 1×10^5 conidia per ml YEPD-5G (0.1% yeast extract, 0.1% peptone, 5% glucose (16) with conidia washed from cultures grown on V-8 agar plates for 7–10 days. Liquid cultures were grown in the dark in 50 ml flasks in 10 or 25 ml of medium for 24 hr at 28° on a rotary shaker operating at 180 rpm for 25 ml cultures and 150 rpm for 10 ml cultures.

To initially screen inhibitors, one ml of a 24 hr 25 ml liquid culture was transferred to a well in a 24-well microtiter plate (Falcon). Test compounds were added to duplicate cultures as 100 × stock solutions in DMSO to a final DMSO concentration of 1–2%. Control cultures were treated with DMSO only. The plates, sealed with parafilm to reduce evapn, were incubated at 28° at 50–60 rpm on a minishaker. After 6 days, the cultures were harvested and stored at −20° until the immunoassays were performed. For further testing, compounds were added in DMSO as described above to 10 or 25 ml cultures 24 hr after inoculation. Cultures were analysed for toxin at 24 hr and at 7 days after inoculation. For dry weight measurements at 24 hr and at 7 days, mycelia from 1 ml aliquots of the 10 ml liquid cultures or from 5 ml aliquots of the 25 ml cultures were collected on pre-weighed GF/A (Whatman) filters, dried for 24 hr and weighed.

Immunoassay. CIEIA. Competitive inhibition enzyme-linked immunoassays (CIEIA) were performed with affinity-purified monoclonal antibody 15H6 as previously described (17, 18). Assays were read on an automatic microliter plate reader (Dynatech Laboratories, Inc., Alexandria, VA). The 15H6 monoclonal antibody was a generous gift from Dr K. W. Hunter, Uniformed Services University School of Medicine, Bethesda, Maryland.

GC analysis for trichothecene production. Each whole culture was extracted twice with an equal volume EtOAc. The two extracts were pooled and then passed through a charcoal column (Romer Labs, Inc., Washington, MO) that was pre-washed with 10 ml EtOAc. The eluent was combined with a subsequent wash (20 ml EtOAc) and evapd to dryness. The residue was resuspended in 1 ml EtOAc. An aliquot equivalent to a 1 or 2 ml portion of the original sample was evapd to dryness at 80° under N_2, reacted with 100 to 200 μl trimethyl silating reagent (Tri-Sil/TBT, Pierce Chemical Company, Rockford, IL) for 1 hr at 80° and brought to 1 ml with hexane. Measurements were made by flame ionization detection. Purified samples of T-2 toxin, diacetoxyscirpenol and neosolaniol were purchased from Sigma.

GC-MS analysis for trichodiene. Trichodiene levels in whole culture extracts were measured by GC-MS on a Hewlett Packard 5970 Mass Selective Detector. A 12.5 m × 0.2 mm crosslinked dimethyl silcone fused silica column was used in the analysis. The GC column was coupled directly into the source of the mass spectrometer. The samples were injected in the splitless mode at 120°. The column was held for isothermal for 2 min and then programmed rapidly to 250° (40°/min). Under these conditions, trichodiene elutes at 4.04 min (± 0.05 min). The trichodiene was detected in the selected ion mode by measuring the response at *m/z* 65, 108, and 109, the major fragments observed in the EI spectrum of trichodiene. Quantitation was based on comparision of response for the samples with an external standard curve which was generated by injections of known amounts of authentic trichodiene across the range of 1 ng to 1 μg. Injection of 1 ng of trichodiene yielded a signal-to-noise ratio greater than 20.

NADPH–Cyt c reductase assay. Microsomes were prepared as previously described [19]. Mycelium was homogenized in a Bead Beater in 500 mM sucrose, 10 mM ethylenediaminetetracetic acid, 1 mM phenylmethylsulfonyl fluoride, 100 mM phosphate, pH 7.5. The microsome fraction obtained upon differential centrifugation was suspended in the above buffer without ethylenediaminetetracetic acid. Reductase was assayed with 0.1 mM NADPH, 40 μM horse heart cytochrome *c*, 5 mM KCN, 100 mM phosphate, pH 7.5, using an extinction coefficient of 21 mM^{-1} cm^{-1} at 500 nm. When inhibitors were tested, 10 μl in DMSO (final concentration 100 μM) was added to the spectrophotometer cuvette along with the reaction mixture; control mixtures contained 10 μl DMSO. The activity of control micro-

somes was 55 nmol/min/mg protein and each assay contained 200 μg microsomal protein. Cytochrome *c* was not reduced directly by flavone or any furanocoumarins tested.

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