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Structure−Activity Relationships of Trichothecene Toxins in an *Arabidopsis thaliana* Leaf Assay

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Many *Fusarium* species produce trichothecenes, sesquiterpene epoxides that differ in patterns of oxygenation and esterification at carbon positions C-3, C-4, C-7, C-8, and C-15. For the first comprehensive and quantitative comparison of the effects of oxygenation and esterification on trichothecene phytotoxicity, we tested 24 precursors, intermediates, and end products of the trichothecene biosynthetic pathway in an *Arabidopsis thaliana* detached leaf assay. At 100 μM, the highest concentration tested, only the trichothecene precursor trichodiene was nontoxic. Among trichothecenes, toxicity varied more than 200-fold. Oxygenation at C-4, C-8, C-7/8, or C-15 was, on average, as likely to decrease as to increase toxicity. Esterification at C-4, C-8, or C-15 generally increased toxicity. Esterification at C-3 increased toxicity in one case and decreased toxicity in three of eight cases tested. Thus, the increase in structural complexity along the trichothecene biosynthetic pathway in *Fusarium* is not necessarily associated with an increase in phytotoxicity.

KEYWORDS: Trichothecenes; *Arabidopsis thaliana*; *Fusarium*; phytotoxicity

INTRODUCTION

Many plant pathogenic species of the genus *Fusarium* produce trichothecenes (**1**−**24**; **Table 1** and **Figure 1**), a large group of sesquiterpene epoxides that are inhibitors of eukaryotic protein synthesis. *Fusarium graminearum*, *Fusarium sporotrichioides*, and other trichothecene-producing *Fusarium* species are common pathogens of cereal grains, especially in temperate climates in Europe, Asia, and the Americas, where trichothecene contamination of wheat, barley, rye, and maize can be a serious problem. The trichothecene biosynthetic pathway in *F. graminearum* ultimately produces the 8-keto, 7-hydroxy trichothecenes deoxynivalenol **18** and nivalenol **22**, and in *F. sporotrichioides*, it produces the 8-*O*-isovalerate ester T-2 toxin **16**. These compounds and other trichothecenes cause feed refusal, ill thrift, and emetic syndromes in swine and other animals and are associated epidemiologically with chronic and fatal toxicoses in humans (*1*).

The trichothecene skeleton contains a carbon position-1 (C-1) pyran group and a C-12,13 epoxide, but trichothecenes differ otherwise in patterns of extraskeletal oxygenation and esterification at C-3, C-4, C-7, C-8, and C-15 and in the presence of a keto group at C-8. *Fusarium* trichothecenes contain only acetate and other short-chain esters and lack the complex macrocylic esters found in some other trichothecene-producing fungi. Determination and quantification of biological effects of trichothecenes in animal systems began shortly after the discovery of T-2 toxin **16**, deoxynivalenol **18**, nivalenol **22**, and other trichothecenes in the 1960s and 1970s. Among *Fusarium* trichothecenes, animal toxicity is higher among compounds with C-3, C-4, C-8, and C-15 oxygenations, such as T-2 toxin **16** and nivalenol **22**, than among compounds with only C-3 and C-15 oxygenations, such as calonectrin **8** (*2*). Relative biological activities of the more-oxygenated trichothecenes in a wide range of animal systems have been extensively studied and recently reviewed (*1*, *3*).

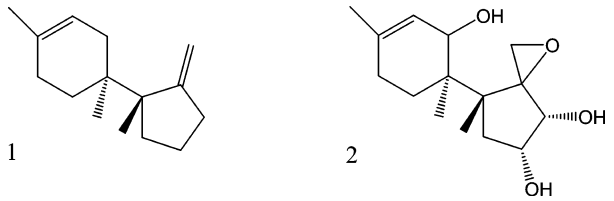

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Figure 1. Structures of trichodiene **1** and isotrichotriol **2**.

As compared to animal systems, assessment of the effects of trichothecenes in plant systems has been rather neglected, although toxicity of 4,15-diacetoxyscirpenol **11** to peas, lettuce, and other plants was first reported more than 40 years ago (*4*). Comparative studies of trichothecene phytotoxicity have been limited, in large part, by the difficulty and expense of obtaining trichothecenes in highly purified form in quantities sufficient for phytotoxicity assays. Typically, one or two compounds have been isolated in different laboratories and then tested with different assay methods and plant species, thus making quantitative comparisons very difficult. The fragmentary studies available have found differences in toxicity of trichothecenes between plant and animal systems, which might have been expected, but have also found differences in relative toxicity of trichothecenes in different plant systems (*5*−*8*).

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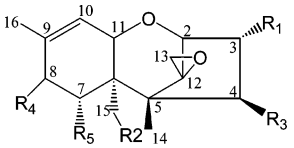
Table 1. Structures and Lethality (LD_{50}) of Trichothecenes



		substituents[a]					
no.	compound	R_1	R_2	R_3	R_4	R_5	$LD_{50} \pm SE$ (μM)[b]
3	isotrichodermol	OH	H	H	H	H	9.0 ± 1.5
4	isotrichodermin	OAc	H	H	H	H	16.1 ± 5.1
5	3,15-dideacetylcalonectrin	OH	OH	H	H	H	>100
6	15-deacetylcalonectrin	OA	OH	H	H	H	>100
7	3-deacetylcalonectrin	OH	OAc	H	H	H	26.6 ± 2.9
8	calonectrin	OAc	OAc	H	H	H	2.6 ± 1.9
9	scirpentriol	OH	OH	OH	H	H	>100
10	15-acetoxyscirpenol	OH	OAc	OH	H	H	3.7 ± 1.1
11	4,15-diacetoxyscirpenol	OH	OAc	OAc	H	H	1.5 ± 0.2
12	3,4,15-triacetoxyscirpenol	OAc	OAc	OAc	H	H	27.6 ± 3.5
13	neosolaniol	OH	OAc	OAc	OH	H	13.0 ± 1.4
14	3-acetylneosolaniol	OAc	OAc	OAc	OH	H	14.6 ± 5.6
15	HT-2 toxin	OH	OAc	OH	Iso	H	1.0 ± 0.8
16	T-2 toxin	OH	OAc	OAc	Iso	H	0.5 ± 0.05
17	3-acetyl-T-2 toxin	OAc	OAc	OAc	Iso	H	4.1 ± 0.2
18	deoxynivalenol	OH	OH	H	=O	OH	23.0 ± 6.8
19	3-acetyldeoxynivalenol	OAc	OH	H	=O	OH	18.7 ± 12.4
20	15-acetyldeoxynivalenol	OH	OAc	H	=O	OH	4.0 ± 2.8
21	3,15-diacetyldeoxynivalenol	OAc	OAc	H	=O	OH	28.2 ± 0.4
22	nivalenol	OH	OH	OH	=O	OH	>100
23	4-acetylnivalenol	OH	OH	OAc	=O	OH	79.7 ± 13.6
24	3,15-diacetylnivalenol	OAc	OAc	OH	=O	OH	4.3 ± 1.4

[a] Iso = isovaleryl ester. [b] LD_{50} was the concentration at which 50% of the *A. thaliana* leaves were dead following treatment.

During the course of long-term research on the biosynthesis of deoxynivalenol **18** and nivalenol **22** by *F. graminearum* and of T-2 toxin **16** by *F. sporotrichioides*, we have used gene-disruption mutants, precursor feeding experiments, and other approaches to create a library of trichothecene biosynthetic pathway precursors, intermediates, and end products. The cruciferous weed *Arabidopsis thaliana* has been used for assay of fungal phytotoxins and is sensitive to trichothecenes (*9*−*12*). In this study, we combined our compound library and *A. thaliana* for a comprehensive and quantitative analysis of structure−activity relationships of trichothecenes biosynthesized by *Fusarium*.

MATERIALS AND METHODS

Trichothecenes. Nivalenol **22** and 4-acetylnivalenol **23** were purchased from Sigma Chemical Co. (St. Louis, MO). The remaining compounds were isolated from wild-type or mutant strains of *F. sporotrichioides* NRRL3299 or *F. graminearum* Z-3639 or were produced by synthetic modification or by biotransformation. All compounds were checked for greater than 95% purity with Gas chromatography−mass spectrometry (GC-MS). Just prior to phytotoxicity assays, each test compound was completely solubilized in acetone to produce a 0.1 M master stock solution, which was serially diluted in acetone to produce additional stock solutions. For phytotoxicity assays, each stock solution was added to sterile distilled water to a final concentration of 0.1% acetone by volume. Control assays contained water with 0.1% acetone.

Trichodiene **1** was isolated from yeast extract−peptone−dextrose liquid (YEPD) cultures of *F. sporotrichioides* mutant strain F15 (a trichodiene oxygenase *Tri4*$^-$ mutant) (*13*). Isotrichotriol **2** was isolated from rice grain cultures of *F. sporotrichioides* strain MB2972 (a C-15 acetyltransferase *Tri3*$^-$ mutant) (*14*). Isotrichodermol **3** was isolated from YEPD cultures of *F. sporotrichioides* strain Tri101-3D (a C-3 acetyltransferase *Tri101*$^-$ mutant) (*15*). Isotrichodermin **4** was isolated from YEPD cultures of *F. sporotrichioides* strain A11b (a C-15 oxygenase *Tri11*$^-$ mutant) (*16*). 3,15-Didecalonectrin **5** and 15-decalonectrin **6** were isolated from YEPD cultures of *F. sporotrichioides* strain MB2972 (*14*). 3-Decalonectrin **7** and calonectrin **8** were isolated from cornmeal cultures of *F. graminearum* strain LH1-41 (a calonectrin oxygenase *Tri1*$^-$ mutant) (*17*). 4,15-Diacetoxyscirpenol **11** was isolated from YEPD cultures of *F. sporotrichioides* strain 1716cos 9-1#1 (a trichothecene overexpression *Tri1*$^-$ mutant) (*18*). 3,4,15-Triacetoxyscirpenol **12**, 3-acetylneosolaniol **14**, and 3-acetyl T-2 toxin **17** were isolated from YEPD cultures of *F. sporotrichioides* strain NA-476 (a C-3 esterase *Tri8*$^-$ mutant) (*19*). Neosolaniol **13** and T-2 toxin **16** were isolated from YEPD cultures of *F. sporotrichioides* strain 5493cos9-1#11 (a trichothecene overexpressing *Tri4*$^-$ mutant) (*18*). HT-toxin **15** was isolated from YEPD cultures of *F. sporotrichioides* strain 7-4-7 (a C-4 acetyltransferase *Tri7*$^-$ mutant) (*20*). 15-Acetyldeoxynivalenol **20** was isolated from YEPD cultures of *F. graminearum* strain B4-1 (a mutant with an extra copy of trichodiene synthase, *TRI5*, and a regulatory protein, *TRI6*) (*21*). 3,15-Diacetyldeoxynivalenol **21** was isolated from rice grain cultures of *F. graminearum* strain NA-8b01 (a C-8 esterase *Tri8*$^-$ mutant) (*19*). 3-Acetyldeoxynivalenol **19** was prepared by feeding deoxynivalenol **18** to liquid cultures of a *Saccharomyces cerevisiae* transformant expressing *F. sporotrichioides* genes *TRI101* (C-3 acetyltransferase) and *TRI12* (a transporter) (*15*). Scirpentriol **9** and 15-monoacetoxyscirpenol **10** were made by hydrolysis of **11** with 0.1 N sodium hydroxide. Deoxynivalenol **18** was made by hydrolysis of 15-acetyldeoxynivalenol **20** with 0.1 N sodium hydroxide. 3,15-Diacetylnivalenol **24** was prepared by mixing 10 mg of **22** in 2 mL of pyridine and acetic anhydride (1:1) and identified with GC-MS and NMR. Mass spectra were recorded with a Hewlett-Packard 5890

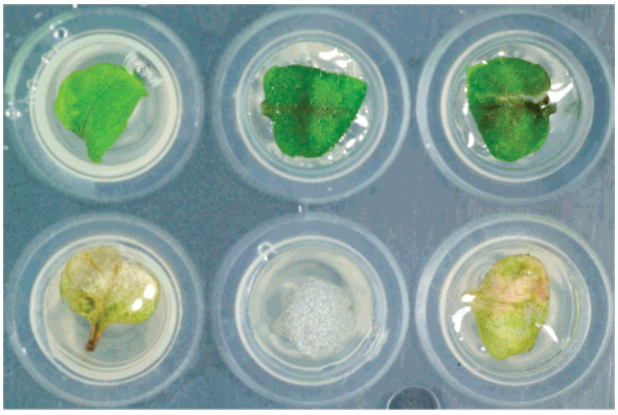


Figure 2. Representative healthy (top row) and dead (bottom row) detached leaves of *A. thaliana* after 7 days of incubation with a trichothecene.

Table 2. Toxicity (ED_{50}) of Selected Trichothecenes and Precursors

	compounds	ED_{50} (±SE) (μM)[a]
1	trichodiene	>100
2	isotrichotriol	>100
3	isotrichodermol	3.5 (±1.9)
4	isotrichodermin	8.6 (±2.2)
5	3,15-dideacetylcalonectrin	90.1 (±7.7)
6	15-deacetylcalonectrin	>100
7	3-deacetylcalonectrin	7.6 (±1.4)
9	scirpentriol	53.6 (±4.4)
12	3,4,15-triacetoxyscirpenol	6.7 (±1.7)
18	deoxynivalenol	3.8 (±3.0)
19	3-acetyldeoxynivalenol	3.4 (±2.6)
20	15-acetyldeoxynivalenol	1.3 (±1.1)
21	3,15-diacetyldeoxynivalenol	10.0 (±1.5)
22	nivalenol	>100
23	4-acetylnivalenol	16.1 (±1.9)
24	3,15-diacetylnivalenol	2.2 (±0.6)

[a] ED_{50} is the concentration at which 50% of the *A. thaliana* leaves showed symptoms of chlorosis, shriveling, or death following treatment.

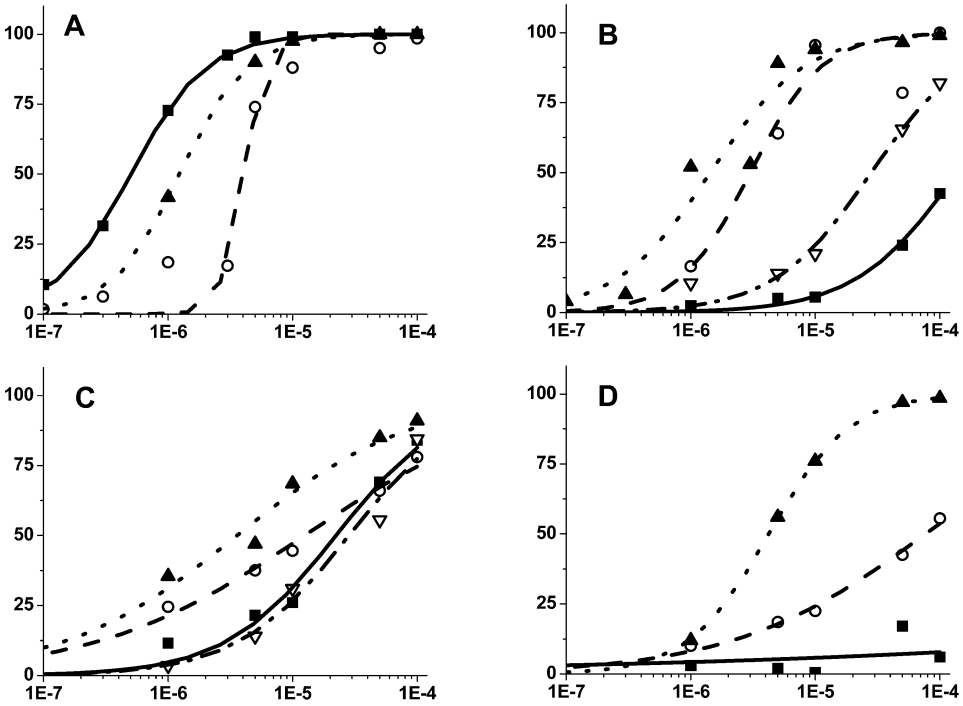


Figure 3. Percentage of dead leaves at concentrations of selected trichothecenes. Each data point is the mean of two replicate experiments. (**A**) T-2 series: ■ = T-2 toxin, ○ = 3-acetyl T-2 toxin, and ▲ = HT-2 toxin. (**B**) Scirpentriol series: ■ = scirpentriol, ○ = 15-acetoxyscirpenol, ▲ = 4,15-diacetoxyscirpenol, and ▽ = triacetoxyscirpenol. (**C**) Deoxynivalenol series: ■ = deoxynivalenol, ○ = 3-acetyldeoxynivalenol, ▲ = 15-acetyldeoxynivalenol, and ▽ = 3,15-diacetyldeoxynivalenol. (**D**) Nivalenol series: ■ = nivalenol, ○ = 4-acetylnivalenol, and ▲ = 3,15-diacetylnivalenol.

MSD spectrometer equipped with a GC 5890 with a DB-5MS column (30 m × 0.25 mm film thickness). The oven temperature was programmed from 120 °C at injection to 210 °C at 15 °C/min, held for 1 min, then heated to 260 °C at 5 °C/min, and held for 8 min with helium as the carrier gas. NMR spectra were recorded with a Bruker 500 MHz instrument.

3,15-Diacetylnivalenol. *m/z* 396 (M^+, 0.5), 336 (M-60, 2.5), 305 (55), 277 (81), 189 (100). ^{13}C NMR (Bruker 500 MHz) in $CDCl_3$: δ7.14 (C14); 15.28 (C16); 20.58 (C3 AcMe); 20.90 (C15 AcMe); 45.82 (C13); 49.47 (C5); 52.24 (C6); 61.70 (C15); 63.88 (C12); 69.56 (C11); 72.89 (C7); 78.14 (C4), 78.34 (C2); 83.15 (C3); 136.02 (C9); 137.88 (C10); 170.20 (C15Ac); 171.73 (C3Ac); 198.89 (C8). ^1H NMR (Bruker 500 MHz) in $CDCl_3$: δ1.17 (s, C14Me); 1.92 (s, C15Me); 1.93 (s, C16Me); 2.24 (s, C3AcMe); 3.10 (d, J = 4.2 Hz, H13a); 3.12 (d, J = 4.2 Hz, H13b); 3.98 (d, J = 4.7 Hz, H2); 4.27 (d, J = 12.2 Hz, H15a); 4.40 (d, J = 12.2 Hz, H15b); 4.40 (d, J = 5.9 Hz, H11); 4.43 (d, J = 3.1 Hz, H4); 4.87 (s, H7); 4.96 (dd, J = 4.7 Hz, H3); 6.59 (dd, J = 5.9 and 1.5 Hz, H10).

Phytotoxicity Assay. Seeds of *A. thaliana* Columbia (Col-4) ecotype were obtained from Lehle Seeds (Round Rock, TX). For surface disinfestation, seeds were placed on filter paper in a Buchner funnel, rinsed for approximately 15 s each with 0.5% sodium hypochlorite and sterile distilled water, and dried with suction. For stratification, seeds were sown on the surface of Petri plates of Murashige−Skoog mineral medium (Sigma, St. Louis, MO) adjusted to pH 5.9 in 1.5% agar, sealed with Parafilm, and incubated for 3 days in the dark at 4 °C. Plates were unsealed, placed in glass trays lined with moist toweling, transferred to a biological incubator (Percival Scientific Inc., Perry, IA), and incubated for 11 days under 24 h fluorescent lights at 25 °C. Agar blocks with uniform, healthy plants were transferred to 24 well tissue culture-treated multiwell plates (Becton Dickson Labware, Franklin Lakes, NJ), and the 24 well plates were returned to the incubator without covers for one more week with additional sterile distilled water added to the wells as needed.

Uniform, healthy leaves with petioles (approximately 1 cm long and 0.5 cm wide) were cut from the rosettes of 3 week old plants and floated on sterile distilled water. Leaves were transferred from the leaf pool into individual wells of 96 well, nonskirted polymerase chain reaction (PCR) microplates (Fisher Scientific, Pittsburgh, PA). Each well contained 250 μL of freshly prepared test solution in water with 0.1% acetone by volume. The uncovered 96 well plates were returned to the incubator in glass trays loosely covered with plastic wrap to maintain humidity. Leaves were observed daily and scored for symptoms after 1 week. All compounds were tested twice in a dilution series of 0, 1.0, 5.0, 10, 50, and 100 μM. As necessary, highly toxic compounds were tested twice again in a dilution series of 0, 0.1, 0.3, 1.0, and 3.0 μM. For each of these tests, 96 leaves were treated and scored for each dilution of each compound. For objectivity and uniformity, assays were blind and all assays were scored by the same individual. Control leaves remained healthy, green, and unshriveled throughout the duration of the assay; apparently healthy control leaves (and leaves on intact plants) occasionally developed areas of dark reddish coloration. Leaves were individually rated for shriveling, chlorosis, and death on a scale of 1−4, where 1 = healthy leaves (green or reddish) with no visible shriveling or chlorosis; 2 = no shriveling, <50% chlorosis or necrosis; 3 = shriveling, up to 90% chlorosis or necrosis; and 4 = dead. For calculating LD_{50} values, categories 3 and 4 were combined as "dead" leaves. For calculating ED_{50} values, categories 2−4 were combined as "symptomatic" leaves.

Statistical Analysis. Statistical analysis of the phytotoxicity assay results was performed using OriginPro 7.5 software (OriginLab, Northampton, MA). Dose−response data were analyzed by the logistic fitting function with the upper and lower asymptotes fixed at 100 and 0, respectively. LD_{50} and ED_{50} values are reported as means ± standard errors.

RESULTS

Phytotoxicity. Twenty-two trichothecenes and two trichothecene precursors were screened for toxicity to detached leaves of *A. thaliana*. Toxicity was expressed as an LD_{50}, the concentration at which one-half of the leaves were dead (**Table**

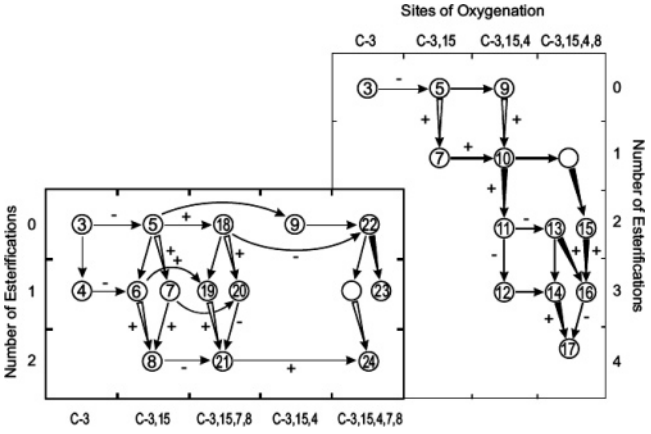


Figure 4. Stepwise changes in toxicity of T-2 toxin **16** (right side) and related trichothecenes and of nivalenol **22** and related trichothecenes (left side). Nodes represent compounds, nodes with numbers represent compounds described in **Table 1**, and nodes without numbers represent compounds not tested in this study. Arrows from left to right represent single oxygenations. Arrows from top to bottom represent single esterifications: Thin black arrows indicate C-3, broad white arrows indicate C-15, and broad black arrows indicate C-4 and C-8. Plus (+) and minus (–) signs on an arrow indicate the direction of the change in toxicity, as the ratio of the substrate toxicity to product toxicity (LD_{50}). A plus sign indicates a two-fold or greater increase in toxicity, and a minus sign indicates a two-fold or greater decrease in toxicity from substrate to product. Arrows represent stepwise changes between compounds but not necessarily the major endogenous biosynthetic pathways.

1) and (**Figure 2**). For less toxic compounds, toxicity was also expressed as an ED_{50}, the concentration at which one-half of the leaves showed symptoms of chlorosis (**Table 2**). At 100 μM, the highest concentration tested, the trichothecene precursor trichodiene **1** was essentially nontoxic, producing mild symptoms of chlorosis in 10% and death in 3% of the leaves. Isotrichotriol **2**, an epoxide of **1**, was slightly more toxic, producing mild symptoms of chlorosis in 10% and death in 10% of the leaves treated at 10, 50, or 100 μM, with no dose−response effect. In more than 50 independent tests, a mean of 4% (range = 0−9%) of control leaves treated with water showed mild symptoms of chlorosis and fewer than 1% of control leaves were dead.

All 22 trichothecenes tested were toxic to *A. thaliana*, but they varied more than 200-fold in toxicity as measured by LD_{50} and ED_{50} (**Tables 1** and **2**). The nine most toxic compounds (LD_{50} < 10 μM) (**3**, **8**, **10**, **11**, **15**−**17**, **20**, and **24**) were structurally diverse and included highly oxygenated compounds such as 3,15-diacetylnivalenol **24** and T-2 toxin and its derivatives **15**−**17**, along with the less-oxygenated intermediates isotrichodermol **3** and calonectrin **8**. The eight moderately toxic compounds (**4**, **7**, **12**−**14**, **18**, **19**, and **21**) were similar in toxicity, with LD_{50} values of 13−28 μM. The five least toxic compounds (LD_{50} > 80 μM) (**5**, **6**, **9**, **22**, and **23**) included highly oxygenated nivalenol **22** and its derivative **23**, as well as less-oxygenated calonectrin **5** and its derivative **6**. Even at 100 μM, **6** produced symptoms in fewer than half of the leaves and **22** produced symptoms in fewer than 10% of the leaves. Dose−response curves for toxicity (percent dead) of four agriculturally important trichothecenes, 4,15-diacetoxyscirpenol **11**, T-2 toxin **16**, deoxynivalenol **18**, nivalenol **22**, and their acetylated derivatives are shown in **Figure 3**.

Effect of Oxygenation on Phytotoxicity. The effect of trichothecene oxygenation on toxicity was examined by comparing 13 pairs of compounds (**Tables 1** and **2**). Eight pairs of compounds differ in hydroxylation at a single site, C-4, C-8, or C-15. Five pairs of compounds differ in both hydroxylation at C-7 and the presence of a keto-group at C-8. The early intermediates isotrichodermol **3** and its derivative **4** were quite toxic, with LD_{50} values of 9−16 μM, but toxicity was reduced >10-fold by C-15 hydroxylation (**3** → **5**, **4** → **6**). C-4 hydroxylation had diverse effects: a slight increase in toxicity (3,15-dideacetylcalonectrin **5** → **9**); >5-fold increases in toxicity (3-deacetylcalonectrin **7**→ **10** and 3,15-diacetyldeoxynivalenol **21**→ **24**); or a >5-fold decrease in toxicity (deoxynivalenol **18** → **22**). C-8 hydroxylation also had diverse effects: a slight increase in toxicity (3,4,15-triacetoxyscirpenol **12** →**14**) or a >10-fold decrease in toxicity (4,15-diacetoxyscirpenol **11**→ **13**). Last, concurrent oxygenation at both C-7 (hydroxyl) and C-8 (keto) had diverse effects: >5-fold increases in toxicity (calonectrin derivatives **5** → **18**, **6** → **19**, **7** → **20**) or at least 10-fold decreases in toxicity (calonectrin **8** → **21** and scirpentriol **9** → **22**).

The effect of multiple oxygenations on toxicity was examined by comparing three series of compounds that differ only in sequential oxygenations at C-4, C-15, and C-7/C-8. In one series from isotrichodermol to nivalenol, **3** → **5** → **18** → **22**, toxicity first decreased 10-fold, then increased 5-fold and decreased 10-fold, resulting in a >10-fold decrease in toxicity overall. In the second series, **3** → **5** → **9** → **22**, toxicity first decreased >10-fold, then remained very low. In the series from calonectrin to 3,15-diacetylnivalenol **8** → **21** → **24**, toxicity was unchanged overall.

Effect of Esterification on Phytotoxicity. The effect of trichothecene esterification on toxicity was examined by comparing 18 pairs of compounds that differ in esterification at a single site, C-3, C-4, C-8, or C-15 (**Tables 1** and **2**). C-3 acetylation had diverse effects: a 10-fold increase in toxicity (3-deactylcalonectrin **7** → **8**); at least 10-fold decreases in toxicity (4,15-diacetoxyscirpenol **11** → **12**, T-2 toxin **16** → **17**, and 15-acetyldeoxynivalenol **20** → **21**); or little effect on toxicity (isotrichodermol **3** → **4**, 3,15-diacetylcalonectrin **5** → **6**, neosolaniol **13** → **14**, and deoxynivalenol **18** → **19**). C-4 acetylation increased toxicity (15-acetoxyscirpenol **10** → **11**, 4-hydroxy-T-2 toxin **15** → **16**, and nivalenol **22** → **23**). C-8 esterification with isovalerate increased toxicity (neosolaniol **13** → **16** and its derivative **14** → **17**). Last, C-15 esterification had no effect on toxicity (3-acetyldeoxynivalenol **19** → **21**) or increased toxicity (two calonectrin derivatives **5** → **7**, **6** → **8**, scirpentriol **9** →**10**, and deoxynivalenol **18** → **20**).

The effect of multiple esterifications on toxicity was examined by comparing two series of compounds that differed only in sequential esterifications at C-3, C-4, and C-15. Among scirpentriol and its derivatives **9** → **10** → **11**→ **12**, toxicity first increased >50-fold and then decreased, resulting in an 8-fold increase overall. Among deoxynivalenol and its derivatives **18** → **19** → **21**, toxicity was unchanged.

The overall effect of oxygenation and esterification on toxicity of the 22 trichothecenes in this study is summarized in network diagrams of T-2 toxin **16** and related trichothecenes (**Figure 4**, right side) and of nivalenol **22** and related trichothecenes (**Figure 4**, left side). Nodes represent compounds, and arrows connecting nodes represent stepwise oxygenations and esterifications. Two-fold or greater changes in toxicity, as assessed by LD_{50} values, are indicated by a plus sign for an increase in toxicity and a minus sign for a decrease in toxicity from substrate to product.

DISCUSSION

For a comprehensive and quantitative analysis of structure−activity relationships of trichothecenes biosynthesized by *Fusar-*

ium, we used an *A. thaliana* detached leaf assay to compare phytotoxicity of 24 biosynthetic pathway precursors, intermediates, and end products. All trichothecenes and intermediates with an epoxide group were toxic to *A. thaliana*; only the nonoxygenated precursor trichodiene **1** was nontoxic. Three previous studies have demonstrated toxicity of trichothecenes to the Col-0 ecotype of *A. thaliana*, but none of these studies included a large number of compounds or a dose−response analysis. In the first study (*12*), deoxynivalenol **18** at 50 μM inhibited seed germination on an agar medium. In the second study (*11*), lesions developed in leaves injected with 1 μM diacetoxyscirpenol **11**, HT-2 toxin **15**, and T-2 toxin **16** or with 10 μM **18**. In the third study (*9*), lesions developed in leaves injected with 75 μM **18**. Our dose−response assay also found that **11**, **15**, and **16** were similar in toxicity ($LD_{50} = 0.5-1.5$ μM) and that all were more toxic than **18** ($LD_{50} = 23$ μM).

Trichothecene biosynthesis is characterized by a generally ordered sequence of oxygenations catalyzed by cytochrome P450s followed by esterifications. First, trichodiene **1** is oxygenated, and then, isotrichotriol **2** cyclizes to isotrichodermol **3**, which has a C-3 hydroxyl group. After acetylation of **3** to isotrichodermin **4**, C-15 oxygenation and esterification form calonectrin and its derivatives **5**−**8**. After calonectrin, the biosynthetic pathway diverges. One pathway leads to nivalenol and its derivatives **22**−**24** via C-4, C-7, and C-8 oxygenations and esterifications. Another pathway leads to T-2 toxin and its derivatives **15**−**17** via C-4 and C-8 oxygenations and esterifications (*1*). Our compound library has made possible a survey of the trichothecene biosynthetic pathways from trichodiene **1** to T-2 toxin **16** and nivalenol **22**. During trichothecene biosynthesis, there is a considerable increase in structural complexity. In our *A. thaliana* assay, however, this increased structural complexity was not necessarily associated with an increase in phytotoxicity. Investigation into the conformational preferences of selected trichothecenes from this study is in progress.

Although T-2 toxin **16**, a structurally complex, pathway end product, was the most toxic compound, isotrichodermol **3**, the first and simplest trichothecene in the pathway, also was quite toxic. In fact, **3** was more toxic than 67% of the 21 more-highly oxygenated or esterified compounds that occur later in the biosynthetic pathway. To our knowledge, this is the first study of the toxicity of **3** or isotrichodermin **4** in any higher plant or animal system. Isotrichodermol **3** appears to contain the minimal structural features necessary for phytotoxicity of *Fusarium* trichothecenes, at least to *A. thaliana*. In this regard, it should be noted that only two trichothecene biosynthetic pathway structural genes should be needed for the synthesis of **3**. *TRI5* encodes trichodiene synthase, which cyclizes farnesyl pyrophosphate to produce trichodiene **1**, and *TRI4* encodes a cytochrome P450 that oxygenates **1** to produce **2**, which cyclizes to **3** (*1*, *22*). *TRI4* and *TRI5* flank the regulatory gene *TRI6* at the center of the trichothecene biosynthetic gene cluster in *F. graminearum* and *F. sporotrichioides*, possibly comprising an evolutionarily ancient module of trichothecene biosynthetic genes.

To our knowledge, trichothecene toxicities have been compared in dose−response assays in only two plant species, wheat and the present study of *A. thaliana* (*6*−*8*, *23*, *24*). For three of the six compounds (**11**, **16**, **18**−**20**, and **22**) tested in both systems, relative toxicity to *A. thaliana* was similar to relative toxicity to wheat. For example, toxicity of T-2 toxin **16** and 15-acetyldeoxynivalenol **20** was high and toxicity of nivalenol **22** was low in both systems. On the other hand, 4,15-diacetoxyscirpenol **11**, but not deoxynivalenol **18** or 3-acetyldeoxynivalenol **19**, was highly toxic to *A. thaliana*. Conversely, **18** and **19** were more toxic than **11** to wheat.

Trichothecene toxicity to the unicellular green alga *Chlamydomonas reinhardtii* was previously assessed by measuring the change in cell density at a single dose of 80 μM (*5*). In that study, the effect of C-3 acetylation on toxicity was examined by comparing seven pairs of compounds that differ only at C-3: **3**−**8**, **11**−**14**, and **16**−**19**. In *C. reinhardtii*, C-3 acetylation decreased toxicity of five of seven compounds tested as follows: **3** → **4**, **7** → **8**, **11** → **12**, **16** → **17**, and **18** → **19**. In contrast, in *A. thaliana*, C-3 acetylation decreased toxicity of only two of these compounds, 4,15-diacetoxyscirpenol **11** → **12** and T-2 toxin **16** → **17**. Both **11** and **16** were highly toxic to *C. reinhardtii*, *A. thaliana*, and wheat, but deoxynivalenol **18** was less toxic to *C. reinhardtii* and *A. thaliana* than to wheat.

The relationship between phytotoxicity and chemical structure is complicated by the fact that toxicity in plant systems can be affected by complex interactions, including uptake and metabolism, as well as intrinsic activity. The extent of oxygenation and esterification may not only influence the recognition and stability of particular trichothecenes but also have an effect on their absorption, distribution, and possible metabolism in *A. thaliana*. Documented metabolism of trichothecenes by plants includes the 3-glycosylation of **18** by maize callus cultures, the deacetylation of **11** by potato tuber tissue, and the deacylation and hydroxylation of **16** by *Baccharis* species (*1*). Initial observations of the reduced phytotoxicity of C-3 acetylated trichothecenes in *C. reinhardtii* and yeast systems initiated plant biotechnology programs to transfer *TRI101*, the gene for trichothecene 3-*O*-acetyltransferase, from *Fusarium* into cereal crop plants (*15*, *25*). The rationale for this approach was that reduction of trichothecene phytotoxicity might lead to reduction of disease symptoms in plant−*Fusarium* interactions in which trichothecenes function as virulence factors (*1*). Expression of *TRI101* in tobacco and rice conferred some resistance to phytotoxicity of **11** and **18**, and expression of *TRI101* conferred some protection against infection by *F. graminearum* in wheat but not in barley (*26*−*29*). Furthermore, 3-*O*-glycosylation of **18** has been strongly associated with a quantitative trait locus that confers resistance to the spread of *F. graminearum* in wheat (*30*). In addition, constitutive overexpression of a trichothecene C-3 glucosyltransferase from *A. thaliana* decreased sensitivity of the plant to **18** (*12*).

Expression of trichothecene detoxification genes in cereal crops has the potential to reduce levels of *Fusarium* head blight and to decrease trichothecene contamination of cereal grains. However, our finding of the high phytotoxicity of isotrichodermol **3** and isotrichodermin **4** indicates that early intermediates of the trichothecene biosynthetic pathway are as biologically active as many late pathway intermediates, at least in the dicotyledenous plant *A. thaliana*. The trichothecene early intermediates **1**−**8** remain to be tested for phytotoxicity to wheat and other monocotyledenous cereal plants. A range of studies indicates that modifications of extraskeletal hydroxyl and ester groups are likely to be reversible in vivo, in plants and in animals, and to result in only partial detoxification and incomplete resistance (*31*). For complete detoxification of trichothecenes, discovery of genes that confer the ability to remove the 12,13-epoxide should be a high priority. Such deepoxidation activity has been demonstrated in bacteria but apparently has not yet been successfully transferred to plants (*31*).

In summary, we have shown that *A. thaliana* provides a reproducible and quantitative assay for the characterization of trichothecene structure−activity relationships. The phytotoxicity

assay could be completed within 1 week, and the detached leaves remained healthy for the duration of the experiment. *A. thaliana* also provides a genetically tractable plant system for gene discovery and a large collection of ecotypes, some of which have already been shown to differ in susceptibility to *F. graminearum* and to fungal toxins in detached leaf assays (*9*, *10*). Thus, *A. thaliana* provides a good model system for the identification of novel plant traits and genes for trichothecene detoxification and resistance.

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