Target-Oriented Inhibitors of the Late Stages of Trichothecene Biosynthesis. 2. In Vivo Inhibitors and Chick Embryotoxicity Bioassay†

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On the basis of the knowledge of the biosynthetic pathway of trichothecenes (Zamir, L. O. *Tetrahedron* **1989**, *45*, 2277–2305; Zamir, L. O., et al. *J. Chem. Soc., Chem. Commun.* **1989**, 598–600) many possible inhibitors were synthesized (Zamir, L. O., et al. *J. Agric. Food Chem.* **1992**, preceding paper in this issue). These compounds were tested in vivo with *Fusarium culmorum* cultures. The inhibition of [2-^{14}C]mevalonate incorporation into trichothecenes in whole cells of *F. culmorum* by these new compounds was analyzed by HPLC with UV and Berthold radioactivity detectors. For comparison and evaluation purposes the cytochrome P450 inhibitors ancymidol and menadione were also tested. There were nine synthetic compounds that inhibited appreciably 3-acetyldeoxynivalenol biosynthesis. The most effective inhibitors, which *also* suppressed the production of all other trichothecenes, were 15-fluoroepoxytrichothecene (**5**), 15-fluorotrichotheca-9,12-diene (**8**), and trichothec-9,12-diene (**6**). The degree of toxicity of these nine synthetic compounds was evaluated using the chick embryotoxicity bioassay. All of the compounds showed a very low to moderate toxicity with the exception of 15-fluoroisotrichodermin (**4**). The toxicity of the most effective inhibitors (**5**, **6**, and **8**) was very low and not related to dose (highest dose was 20 mg/mL). Therefore, there is a very good inverse correlation between in vivo inhibition and chick embryotoxicity.

INTRODUCTION

Trichothecene mycotoxins such as deoxynivalenol (DON) (Figure 1) are commonly found in *Fusarium*-contaminated grains (Foster et al., 1986). Our approach was to design compounds that could mimic the natural substrate and bind to the same enzymatic site, thereby inhibiting the biosynthesis of this toxin. The synthetic compounds would differ from natural substrates by the introduction of a fluorine at a strategic site. In the literature we can see the inhibition of cholesterol biosynthesis with fluorinated mevalonate analogues (Reardon and Abeles, 1987). The new syntheses of nine of these putative inhibitors are described in the preceding paper (Zamir et al., 1992).

In this paper, we tested these synthetic putative inhibitors in vivo in *Fusarium culmorum* cultures which were already producing trichothecenes. To obtain accurate results, we calculated the inhibition of incorporation of the radiolabeled natural precursor [2-^{14}C]mevalonate into 3-acetyldeoxynivalenol. For comparison purposes, we also tested known cytochrome P450 inhibitors such as ancymidol and menadione in *F. culmorum* cultures. Early work (Van Middlesworth et al., 1986; Desjardins et al., 1987) showed that the addition of ancymidol to *F. culmorum* cultures induced the accumulation of the bicyclic precursor trichodiene (Figure 2). We found several inhibitors to trichothecene biosynthesis in vivo. The most promising were 15-fluoro-12,13-epoxytrichothec-9-ene (**5**, Figure 2), 15-fluoro-12,13-deepoxytrichothec-9-ene (**8**, Figure 2), and trichothec-9,12-diene (**6**, Figure 2). The chick embryotoxicity screening test (CHEST) had been previously found

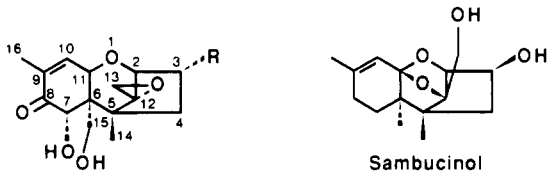

Figure 1. Major metabolites of *F. culmorum*.

to be very reliable in evaluating the relative toxicities of a wide range of mycotoxins (Prelusky et al., 1987, 1989). In addition, a reasonable correlation of lethality between the CHEST and published mouse data was reported (Prelusky et al., 1989). In the current paper, the CHEST assay was used to evaluate the toxicity of several synthetic inhibitors.

MATERIALS AND METHODS

In Vivo Inhibition Experiments. *F. culmorum* strain HLX 1503 was inoculated from a malt extract agar slant as previously described (Zamir and Devor, 1987), except that 5 mL of inoculum was used for each 50-mL seed culture which was grown for 67 h. Duplicate samples of each putative inhibitor (final concentration 0.25 or 0.50 mM) were used to check the reproducibility of the results. The experiments proceeded in the following manner. The compounds were dissolved in ethyl acetate or methanol added to sterile 125-mL Erlenmeyer flasks, and the solvents were evaporated under N_2 to dryness. To each flask were added 1 mL of 5% Brij 35 and 0.25 mL of (3*R*)-[2-^{14}C]-mevalonate (Amersham) (2.7×10^6 dpm) (2.3 GBq/mmol). The flasks were shaken, and a 55-h-old production culture (Zamir and Devor, 1987) was added to each flask. Three controls containing only Brij 35, (3*R*)-[2-^{14}C]mevalonate, and production cultures but *no* putative inhibitors were also used. All of the flasks (with and without inhibitors) were incubated for 20 h at 25 °C and shaken at 220 rpm in the dark. Each culture was then filtered and extracted on two 1020 Chemelute tubes (Analytichem International, Harbor City, CA) with 8 × 12 mL of ethyl

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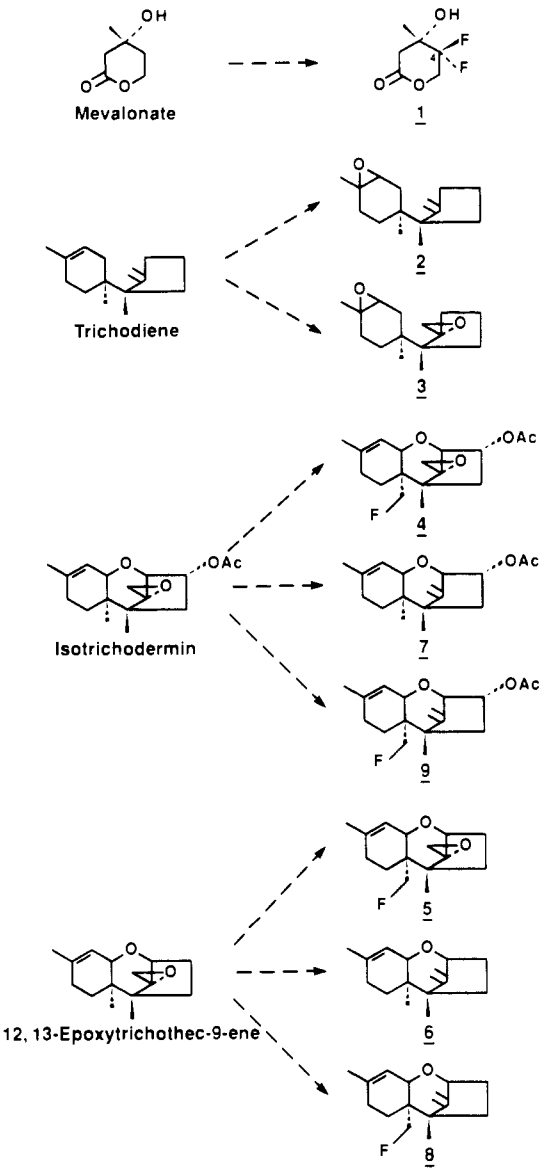

Figure 2. Natural precursors of trichothecenes (mevalonate, trichodiene, isotrichodermin, 12,13-epoxytrichothec-9-ene) and designed inhibitors (1–9).

acetate. The extracts were evaporated to dryness in vacuo and dissolved in methanol for fractionation by HPLC.

Separation of ^{14}C-Labeled Metabolites. The chromatographic and detection system employed consisted of a Series 3B liquid chromatograph and LC-75 ultraviolet detector (Perkin-Elmer) and a Berthold LB505 radioactivity detector equipped with a GT650U4 cell (Zamir et al., 1990). The columns used were two ODS-2 reversed-phase analytical columns connected in series (4.6 × 500 mm) (Whatman). The spectrophotometric (at wavelength 204 nm) and radioactive elution profiles were monitored with a dual-pen recorder. Metabolites were eluted using a linear gradient of increasing concentrations of methanol in water at 1 mL/min, 15–75% in 50 min, and then the methanol concentration was held at 75% for 30 min and then again increased linearly to 99.9% in 10 min and then held at 99.9% for 15 min. Thirteen fractions were collected as follows: fraction 1, 43.2–48.5 min; fraction 2, 48.5–53.4 min; fraction 3, 53.4–56.7 min; fraction 4, 56.7–58.7 min; fraction 5, 58.7–61.5 min; fraction 6, 61.5–64.5 min; fraction 7, 64.5–68.9 min; fraction 8, 68.9–78.0 min; fraction 9, 78.0–87.0 min; fraction 10, 87.0–92.5 min; fraction 11, 92.5–100 min; fraction 12, 100–110 min; fraction 13, 110–118 min. Aliquots (0.6 mL) of each fraction were counted in the scintillation counter, and the total counts of each fraction were calculated. Standards of known trichothecenes were run under identical conditions.

Chick Embryotoxicity Screening Bioassay. Fertile eggs were collected from White Leghorn hens (ARC strains 8 and 9) and stored for up to 7 days on fiber trays at 10 °C until they were set in the incubator (Model I28). At the time of setting, the eggs were allocated to 54 treatment groups (each of 21 or 14 eggs). All eggs were sequentially numbered and allocated to eight trays (each tray containing 180 eggs). The temperature in the incubator was maintained at 29 °C wet bulb, and eggs were turned through a 90° arc every 4 h. The inhibitors were dissolved in carrier solvent (ethyl acetate), and 5 μL containing 0.01, 0.1, 1.0, 10.0, or 100 μg was injected 1 day after the start of incubation. Deoxynivalenol (DON) dissolved in ethyl acetate was injected into 14 eggs per concentration (3.8, 5.3, 7.2, or 10.0 μg/5 μL). Eggs were candled on days 4, 7, 11, and 18 following injection and incubated until day 22 (hatch). In addition, 128 solvent eggs (injected with 5 μL of ethyl acetate) were randomly distributed in groups of four (4 × 8) on each level of the incubator. Other details concerning the incubation and hatching procedures are included in Prelusky et al. (1989). Results were summarized for 22 days with the percentages related to the number of fertile egg on test (Table II). The mortality of solvent eggs (13.6%) was subtracted from the determined mortality value of each dose. For deoxynivalenol and 15-fluoroisotrichodermin (compound 4, Figure 2) lethal doses were estimated using single line probit analyses (Finney, 1962).

RESULTS AND DISCUSSION

In Vivo Evaluation of the Putative Inhibitors. Ancymidol and menadione are generally known as cytochrome P450 inhibitors (Coolbaugh et al., 1978; Nesnow et al., 1980). Therefore, in addition to the compounds synthesized (Figure 2) ancymidol and menadione were also evaluated as possible inhibitors of trichothecene biosynthesis. Obviously, ancymidol and menadione are *not* specific; in general, they inhibit *all* cytochromes P450 and could, therefore, be very toxic to humans. The values obtained for the inhibition of 3-acetyldeoxynivalenol by ancymidol and menadione are used mostly for comparison with our synthetic inhibitors. The inhibition was monitored using radiolabeled mevalonate to obtain a high degree of sensitivity. The inhibition of [2-^{14}C]mevalonate incorporation into trichothecenes in whole cells of *F. culmorum* by the various compounds was analyzed by HPLC with UV (at 204 nm) and Berthold radioactivity detectors. The compounds were added 55 h after the transfer of the cultures into the production medium, i.e., when they are already actively synthesizing trichothecenes. Two concentrations of putative inhibitors were chosen: 0.25 and 0.5 mM (Table I). We found that 0.25 mM was sufficient for the evaluation of the effective inhibitors. As seen in Table I, at 0.25 mM all of the compounds shown inhibited

Table I. Inhibition of 3-Acetyldeoxynivalenol

inhibitor	% inhibition of 3-ADN biosynthesis	inhibitor	% inhibition of 3-ADN biosynthesis
0.25 mM, 0.50 mM **1**	35, 53	0.25 mM **6**[a]	87.1
0.25 mM, 0.50 mM **2**	82, 92	0.25 mM **7**[a]	86.9
0.25 mM, 0.50 mM **3**	40, 78	0.25 mM **8**[a]	90.3
0.25 mM, 0.50 mM **4**	64, 74	0.25 mM **9**[a]	85.5
0.25 mM, 0.50 mM **5**	89, 96	0.25 mM, 0.50 mM ancymidol	83, 93
		0.25 mM[a] menadione	99

[a] For compounds **6–9** and menadione only one concentration (0.25 mM) was tested.

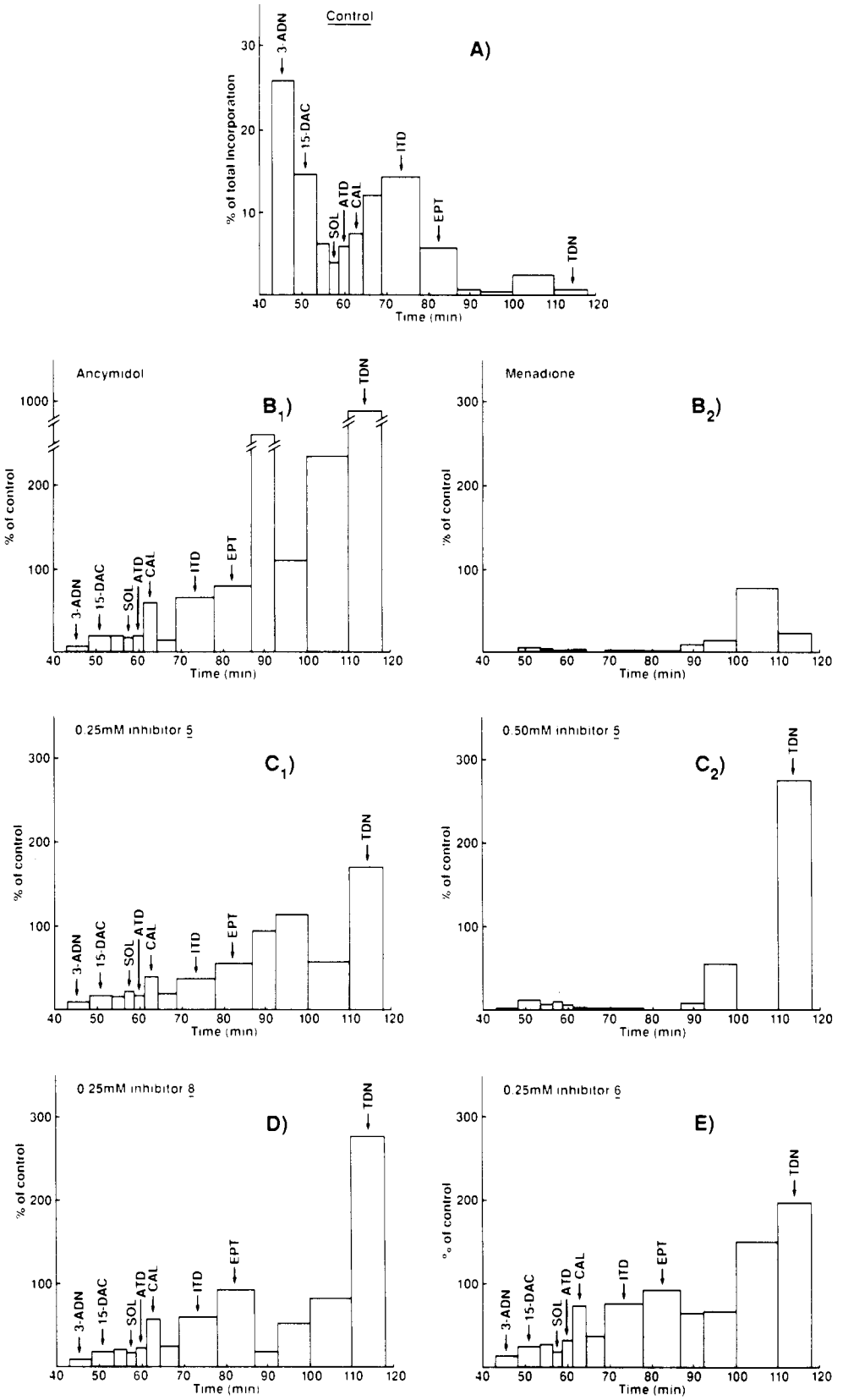

Figure 3. In vivo inhibitors of *F. culmorum* trichothecenes. The upper bar graph (A) represents the control, whereby labeled mevalonate, detergent, and production culture were incubated with *no* inhibitor. The ordinate shows the percentage of total incorporation of labeled mevalonate into the respective trichothecenes. The abscissa shows the retention times on HPLC. The other graphs show the effect of the added inhibitors (B_1, 0.25 mM ancymidol; B_2, 0.25 mM menadione; C_1, 0.25 mM **5**; C_2, 0.50 mM **5**; D, 0.25 mM **8**; E, 0.25 mM **6**). In these last bar graphs the ordinate represents percentage of the control.

the production of 3-acetyldeoxynivalenol (3-ADN) from 35 to 99%. According to Table I, the best inhibitors for 3-ADN biosynthesis are compounds **5–9**. Table I shows only the quenching of 3-ADN biosynthesis. However, *F. culmorum* strain HLX 1503 also produces other trichothecenes, albeit less toxic than 3-ADN. Therefore, to compare inhibitors **5–9**, we should take into account their effects on *all* of the metabolites of *F. culmorum*. We found that the most effective inhibitors of *all* of the trichothecenes produced by *F. culmorum* are 15-fluoro-12,13-ep-

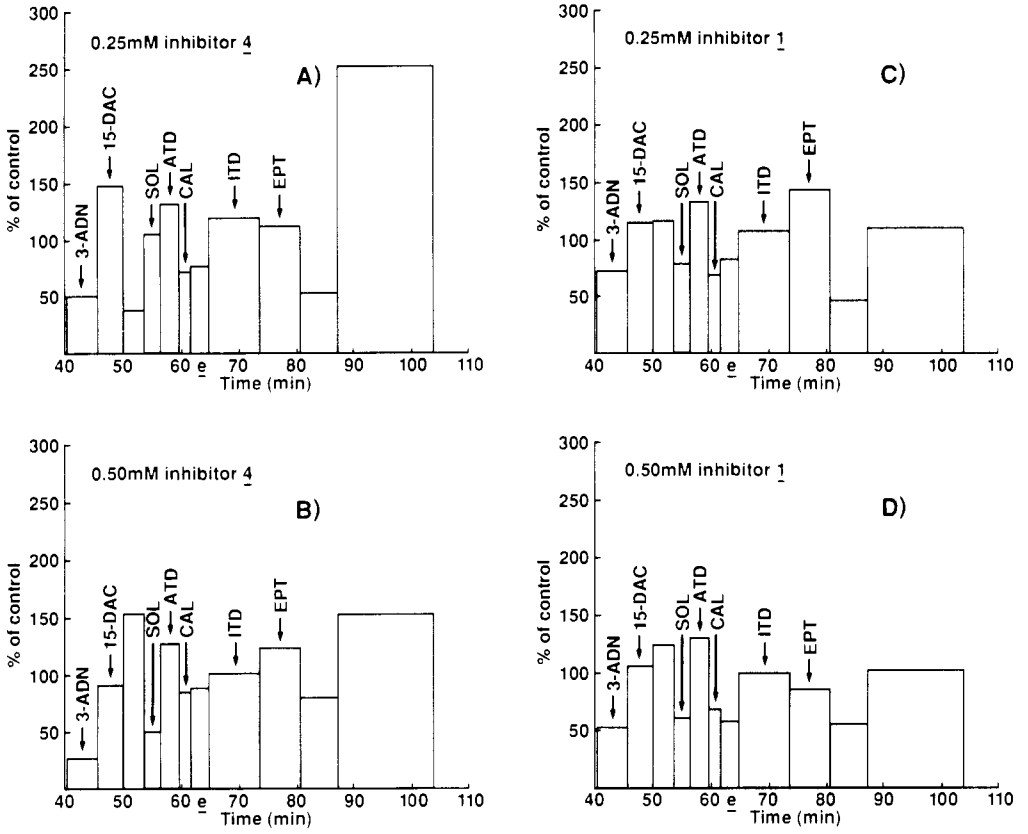

Figure 4. Inhibitors that suppress 3-ADN biosynthesis but not other trichothecenes such as 15-DAC, SOL, ATD, CAL, ITD, and EPT. The effects of two different concentrations of 15-fluoroisotrichodermin (A, 0.25 mM; B, 0.50 mM) and 4,4-difluoromevalonate (C, 0.25 mM; D, 0.50 mM) are shown in this figure.

Table II. Mortality Values[a] (Percent) for Inhibitors 2–9 in Comparison to Deoxynivalenol and 12,13-Epoxytrichothec-9-ene

		concn,[b] μg/egg					
inhibitor	strain	0.01	0.1	1.0	10	100	±SE
2	8	7.8	29.3	29.3	7.8	22.1	5.61
3	8	22.1	29.3	36.4	24.9	0.7	5.79
4[c]	9	0.7	0	9.5	68.2	86.4	
5	8, 9	12.7; 12.7	10.2; 1.4	0.7; 1.4	2.2; 10.2	11.4; 5.4	2.83
6	8, 9	11.4; 3.6[d]	11.4; 5.4	5.4; 10.2	−8.3; 5.4	−4.1; 5.4	2.65
7	8	24.9	15.0	7.8	19.7	17.2	5.67
8	8, 9	5.4; 5.4	1.4; −3.6	10.2; 2.2	−3.1; 0.7	10.2; 11.4	2.67
9	9	9.5	−6.5	0.7	1.8	−6.5	4.13
EPT	9	15.0	1.8	−5.9	−5.9	40.2	5.15
DON[e]	8	22.1 (3.8 μg)	40.2 (5.3 μg)		65.0 (7.2 μg)	79.3 (10.0 μg)	
		24.9	36.4		57.8	71.0	

[a] Solvent (ethyl acetate) mortality (13.6%) was subtracted from each dose. [b] Injection volume was 5 μL. [c] Lethal dose (LD_{50}) was 4.0 μg. [d] Negative value means that mortality on this dose was lower than the solvent mortality. [e] Expected mortality values were 20, 40, 60, and 80% for the respective concentrations.

oxytrichothec-9-ene (**5**, Figure 2), 15-fluorodeepoxytrichothec-9-ene (**8**, Figure 2), and trichothec-9,12-diene (**6**, Figure 2). These three compounds are also the best inhibitors of 3-ADN biosynthesis. This result was determined by measuring the radiolabeled metabolites produced from [2-^{14}C]mevalonate after the addition of the putative inhibitor. In Figure 3A, a bar graph shows the distribution of label from [2-^{14}C]mevalonate into trichothecenes when *no* other compounds were added. As expected, many trichothecenes are found: 3-acetyldeoxynivalenol (3-ADN), 15-deacetylcalonectrin (15-DAC), sambucinol (SOL), apotrichodiol (ATD), calonectrin (CAL), isotrichodermin (ITD), and 12,13-epoxytrichothec-9-ene (EPT). In the presence of ancymidol (0.25 mM, Figure 3B) the production of 3-ADN and SOL was reduced but some trichothecenes (CAL, ITD, and EPT) were still formed (60–78% of the control). In the presence of menadione (0.25 mM) the syntheses of all of the trichothecenes was suppressed (Figure 3B). The only three compounds that diminished the production of all of the trichothecenes are shown Figure 3C–E). The concentration of 0.25 mM was usually sufficient to cause a marked reduction. The best inhibitor, 15-fluoroepoxytrichothecene (15-F-EPT, **5**), showed a marked reduction of trichothecene production (3-ADN, 15-DAC, SOL, ATD, CAL, ITD, and EPT, Figure 3C) at 0.25 mM concentration. With the addition of 15F-EPT at 0.50 mM the trichothecenes are nearly completely eliminated. Two other effective inhibitors of trichothecene biosynthesis are 15-fluorotrichothec-9,12-diene (15F-TTD, **8** in Figures 2 and 3D) and trichothec-9,12-diene (TTD, **6** in Figures 2 and 3E). We can rate all of the inhibitors shown in Figure 2 by their capacity to suppress *all* trichothecenes in the following decreasing effectiveness: 15-fluoroepoxytrichothecene, **5**; 15-fluorotrichothec-9,12-diene, **8**; trichothec-9,12-diene, **6**; 9,10-epoxytrichodiene, **2**; 12,13-deepoxyisotrichodermin, **7**; 15-fluorodeepoxyisotrichodermin, **9**; 15-fluoroisotrichodermin, **4**; 4,4-difluoromevalonate, **1**.

We also note that all of these compounds inhibited appreciably the production of 3-ADN, but in some cases the accumulation of other trichothecenes was even greater than the control. This is the case for compounds **1**, **4**, **7**, and **9**. Figure 4 shows that two concentrations of **1** (Figure 4C,D) and **4** (Figure 4A,B) suppress 3-ADN biosynthesis (Table I) but *increase* the accumulation of other trichothecenes (Figure 4) and are, therefore, not inhibiting general trichothecene production.

Inhibitors Accumulate Trichodiene-Type Compounds and/or Other Trichothecenes. Previous work has shown that addition of cytochrome P450 inhibitors such as ancymidol and xanthothecin (Van Middlesworth et al., 1986; Hesketh et al., 1990; Zamir et al., 1991a,b) cause the accumulation of trichodiene and trichodiene-type compounds. For example, we have shown in this work that at a concentration of 0.25 mM of ancymidol trichodiene was produced at 980% of the control. A similar situation was observed in the presence of our synthetic inhibitors. Trichodiene-type compounds (retention times 90–120 min) (Zamir et al., 1991a,b) accumulated in the presence of our best inhibitors (compounds **5**, **6**, and **8**) as seen in Figure 3 (C_1, C_2, D, and E). A plausible explanation is that enzymatic steps in the later stages of the biosynthesis of trichothecenes are blocked, leading to the accumulation of early biosynthetic precursors.

Some of the least potent synthetic inhibitors (for example, compounds **1** and **4** as seen in Figure 4) suppress 3-ADN biosynthesis but increase the production of other trichothecenes such as ITD, EPT, 15-DAC, CAL, and SOL. These trichothecenes have been shown (Zamir et al., 1990, 1991a,b) to be biosynthetic intermediates to 3-ADN. Therefore, one plausible explanation for this seemingly contradictory result (suppression of 3-ADN biosynthesis but increased formation of other trichothecenes) is that they block the transformation of the last steps such as from 15-deacetylcalonectrin to 3-acetyldeoxynivalenol. Early intermediates such as isotrichodermin will then accumulate. At the moment, the mode of action of these inhibitors is unknown. Our only postulate is that they inhibit late stages in the biosynthesis, since there is always a buildup of trichodiene derivatives.

Chick Embryotoxicity Bioassay. The results of the toxicological evaluation using the chick embryotoxicity screening test are given in Table II. There was no difference between the two strains in response to deoxynivalenol and to the inhibitors **5**, **6**, and **8**; the overall mortality for the last three compounds was very low and not related to the dose. Mortality values for compounds **2**, **3**, and **7** were slightly higher than for inhibitors **5**, **6**, and **8**. However, no relationship was found between mortality and injection dose. 15-Fluoroisotrichodermin (**4**) was found to be more toxic than the other inhibitors with an LD_{50} value of 4.0 μg. The LD_{50} values for deoxynivalenol were also determined as 5.3 and 5.6 μg, similar to reported values (Prelusky et al., 1989).

We had previously determined from in vivo evaluation that 15-fluoroisotrichodermin and 4,4-difluoromevalonate were not effective inhibitors of trichothecenes biosynthesis. 15-Fluoroisotrichodermin was tested with the CHEST assay and found to possess embryotoxic potency similar to that of deoxynivalenol.

It is encouraging that the most effective synthetic inhibitors of trichothecene biosynthesis in *F. culmorum* showed relatively low embryotoxicity compared to that of deoxynivalenol. Work is in progress in our laboratory to understand the detailed mechanism of action of our inhibitors.

ACKNOWLEDGMENT

We thank Dr. H. Locksley Trenholm, Agriculture Canada, for helpful suggestions and Dr. D. B. Prelusky for his advice and technical assistance on CHEST bioassay. This investigation was supported by a strategic grant from the Natural Sciences and Engineering Research Council of Canada (to L.O.Z.) and by the Ontario Pork Producers Marketing Board under the Ontario Pork Industry Improvement Program (to H.L.T.).

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Received for review June 17, 1991. Revised manuscript received December 12, 1991. Accepted January 7, 1992.