Mechanism for Sodium Bicarbonate Inhibition of Trichothecene Biosynthesis in *Fusarium tricinctum*†

Kurt S. Roinestad, Thomas J. Montville, and Joseph D. Rosen*

Department of Food Science, Cook College, Rutgers University, New Brunswick, New Jersey 08903

Treatment of *Fusarium tricinctum* NRRL 13426 cultures with dilute sodium bicarbonate resulted in dramatic reductions in the production of trichothecene mycotoxins, geraniol, and carotenoids. All of these compounds are biosynthesized from mevalonic acid (**I**). In addition, three apparent mevalonic acid metabolites were found in the bicarbonate-treated cultures at concentrations of several parts per million each. Two of these compounds were tentatively identified as 3-methyl-4-oxo-2-pentenoic acid (**IV**) and its rearrangement product, 4,5-dimethyl-5-hydroxy-2(5*H*)-furanone (**VI**). These results suggest a pH-related inhibition of mevalonate kinase or a pH-related activation of an enzyme that is responsible, at least in part, for the conversion of mevalonic acid to the pentenoic acid or its precursor.

Keywords: *Tricothecene biosynthesis; bicarbonate inhibition; mevalonate metabolism*

INTRODUCTION

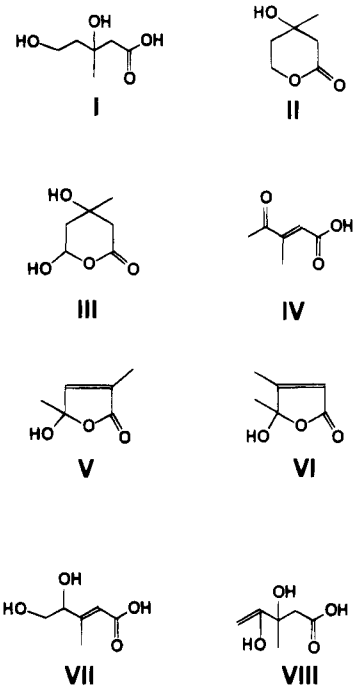
Trichothecenes are toxins biosynthesized by fungi primarily of the genus *Fusarium*. They exert toxicity by inhibiting the synthesis of protein and DNA in eukaryotic cells. Trichothecenes tightly bind to structural elements in the ribosome, thereby inactivating initiation and termination events associated with protein synthesis (Ueno, 1977). In mammals, inhibition of protein and DNA synthesis manifests itself in a wide variety of symptoms. Massive hemorrhage and general tissue necrosis are symptoms typical of trichothecene toxicity. Feed refusal, vomiting, and destruction of white blood cells and bone marrow are also commonly encountered (Joffe, 1971; Ueno, 1977).

Sodium bicarbonate inhibits the formation of aflatoxins in cultures of *Aspergillus parasiticus* (El-Nabarawy *et al.*, 1989) and ochratoxin A in corn (Montville and Shih, 1991). Depasquale *et al.* (1990) demonstrated that dilute solutions of sodium bicarbonate potentiated color and morphology changes when *Fusarium tricinctum* NRRL 13426 was cultured on agar media.

Recently, we determined the effect of 0.11 M sodium bicarbonate on trichothecene formation in both shake and stationary cultures of *F. tricinctum* NRRL 13426. Sodium bicarbonate-treated cultures exhibited a 7–51-fold decrease in the production of diacetoxyscirpenol and T-2 toxin. The production of 15-acetoxyscirpenol, acetyl T-2 toxin, and neosolaniol was completely inhibited (Roinestad *et al.*, 1993). Research presented herein suggests a mechanism by which the inhibition of trichothecene formation by sodium bicarbonate takes place.

EXPERIMENTAL CONDITIONS

Chemicals. Acetone, chloroform, ethyl acetate, hexane (all of HPLC grade), and sodium sulfate were obtained from Fisher Scientific (Springfield, NJ). Sodium bicarbonate was obtained from Church and Dwight (Princeton, NJ). Isobutane, ammonia, and helium were purchased from Matheson (East Rutherford, NJ). Mevalonic acid lactone (**II**) and geraniol were purchased from Sigma Chemical (St. Louis, MO). Mevaldic acid lactone (**III**) was synthesized according to the procedure of Shunk *et al.* (1957). Because of its instability, it was analyzed by GC/MS immediately after synthesis. 3-Methyl-4-oxo-2-pentenoic acid (**IV**), Tween 80, and fluorene were purchased from Aldrich Chemical (Milwaukee, WI).



Inoculation and Incubation. The fungal medium consisted of a peptone (1%) and saccharose (10%) supplemented Czapek–Dox broth (Difco, Detroit, MI). Sodium bicarbonate-treated medium was produced by aseptically adding 0.92 g of sodium bicarbonate to 100 mL of sterile media to obtain a 0.11 M concentration. Spore suspensions of *F. tricinctum* NRRL 13426 were used to inoculate all media in this study. Spore suspensions were prepared by inoculating 100 mL of sterilized potato dextrose agar (PDA, Difco) with *F. tricinctum* NRRL 13426 using a sterile loop. The PDA culture was incubated at 35 °C for 10 days. Twenty milliliters of sterile Tween 80 (0.5% solution) was then aseptically added to the fungal

* Author to whom correspondence should be addressed [telephone (908) 932-9667; fax (908) 932-6776].

† This work was supported with funds obtained from the New Jersey Agricultural Experiment Station, Publication D-10568-2-93.

0021-8561/94/1442-2025$04.50/0

culture; 1 mL of the resulting spore suspension was removed from the PDA flask with a 5-mL serological pipet and released into 100 mL of sterile fungal broth. Broth treatments were incubated as shake cultures (15 days at 28 °C) or stationary cultures (14 days at 19 °C).

Extraction. HPLC grade acetone (100 mL) was added to the culture broth, and the mixture was vigorously shaken and allowed to stand for 12 h before filtration. Acetone was removed from the filtrate using a rotary evaporator. The remaining aqueous portion was adjusted to pH 1.5 with 1 N HCl, transferred to a 500-mL separatory funnel, and extracted three times each with 50 mL of ethyl acetate. The ethyl acetate fractions were combined and dried by passing through a glass funnel containing 50 g of sodium sulfate. The extract was concentrated via a rotary evaporator to approximately 5 mL and then evaporated to dryness under a gentle stream of nitrogen. The extract was redissolved in 1 mL of ethyl acetate and transferred to a vial containing 28 μg of fluorene, which was used as an internal standard. The solution was stored at −15 °C in a 4-mL sample vial fitted with a Teflon-lined cap until analysis or metabolite isolation.

Calibration Curve. Calibration curves for mevalonic acid lactone and geraniol were constructed by preparing eight concentrations (0.5, 1, 2, 5, 10, 20, 30, and 40 ng/μL) of each material in ethyl acetate. Each solution contained 28 ng of the internal standard (fluorene)/μL of ethyl acetate. The standards were analyzed (in triplicate) by gas chromatography/isobutane chemical ionization mass spectrometry. Calibration curves were generated with Finnigan Ion Trap software, revision 3.15.

Isolation of Metabolites from Fungal Extracts. Fungal extracts were filtered with a Gelman (Ann Arbor, MI) 0.2-μm Nylon Acrodisk filter, and the filtrate was passed through a Supelco (Bellefonte, PA) 1-mL volume capacity silica solid-phase extraction cartridge. The eluate was collected, and its components were separated by high-performance liquid chromatography (HPLC) using a Whatman (Hillsboro, OR) Partisil M9 10/50 semipreparative column attached to a Waters (Milford, MA) Prep LC 3000 in the isocratic mode at a flow rate of 3 mL/min. The initial HPLC separation used 100% ethyl acetate as the mobile phase. A Pharmacia (Piscataway, NJ) LKB Redifrac was used to collect fractions at 10-s intervals. Fractions containing the desired metabolites were rechromatographed with the same column using a mobile phase of 50% hexane and 50% ethyl acetate.

Analytical Instrumental Conditions. A Varian (Walnut Creek, CA) Model 3400 gas chromatograph interfaced to a Finnigan 800 ion trap detector (Finnigan MAT, San Jose, CA) and controlled by an IBM PC/AT was used to analyze mevalonic acid lactone, geraniol, and additional fungal metabolites. The analyses and quantifications were performed with Finnigan Ion Trap software, revision 3.15. A splitless on-column injector held at 50 °C was used, and a 2 m × 0.53 (i.d.) deactivated fused silica precolumn was fitted between the injector and capillary column. A 15 m × 0.25 mm (i.d.) J&W (Rancho Cordova, CA) DB-1 fused silica capillary column (1-μm film thickness) was held at 50 °C before being temperature-programmed from 50 to 280 °C at 20 °C/min and then held for 10 min. Carrier gas (He) velocity was 15 cm/s; injection volume was 1 μL. A 15-cm syringe needle was used for the on-column injections. The mass spectrometer was operated in the chemical ionization mode using isobutane reagent gas at a source pressure that gave a 2:1 ratio for m/z 43−57. The filament voltage and current were 70 eV and 80 μA, respectively. Electron multiplier gain was 10^5. Scan range was from 100 to 470 amu at 1 s/scan. Transfer line and manifold temperatures were 250 and 220 °C, respectively.

Magnetic sector EI mass spectra for **IV** and **VI** were obtained on a Finnigan MAT 8230 mass spectrometer.

RESULTS AND DISCUSSION

Previous studies (Roinestad *et al.*, 1993) indicated that 0.11 M sodium bicarbonate greatly inhibited trichothecene formation in cultures of *F. tricinctum* NRRL 13426. Sodium bicarbonate-treated cultures exhibited a 7−51-fold decrease in the production of diacetoxyscirpenol and T-2 toxin, while the production of 15-acetoxyscirpenol, acetyl T-2 toxin, and neosolaniol was completely inhibited. Further analysis of the fungal cultures revealed that trichothecene inhibition was occurring concomitantly with an inhibition of carotenoids and geraniol. The sodium bicarbonate-treated cultures had a bleached, pale white appearance as compared to the bright orange-red control cultures. Carotenoids are formed in toxigenic *Fusaria* (Carlile, 1956; Gribanovski-Sassu and Foppen, 1968) and share much of the same biosynthetic pathway responsible for trichothecene biosynthesis (Robinson, 1975). Geranyl pyrophosphate is an intermediate in the trichothecene and carotenoid biosynthetic pathway (Robinson, 1975; Cane *et al.*, 1981); geraniol is the hydrolysis product of geranyl pyrophosphate. Geraniol was present in fungal control extracts at levels of 3−6 μg/g (cell dry weight) but present in only trace levels (<0.2 μg/g) in the sodium bicarbonate-treated fungal culture extracts. A partial schematic diagram of the trichothecene biosynthetic pathway is presented in Figure 1. Mevalonic acid lactone exists in equilibrium with mevalonic acid, and these materials are intermediates in the formation of geraniol, carotenoids, and trichothecenes (Robinson, 1975; Cane *et al.*, 1981; Goldstein and Brown, 1990). Because we observed changes in mevalonic acid concentration in bicarbonate-treated cultures, our attention became focused on this part of the pathway.

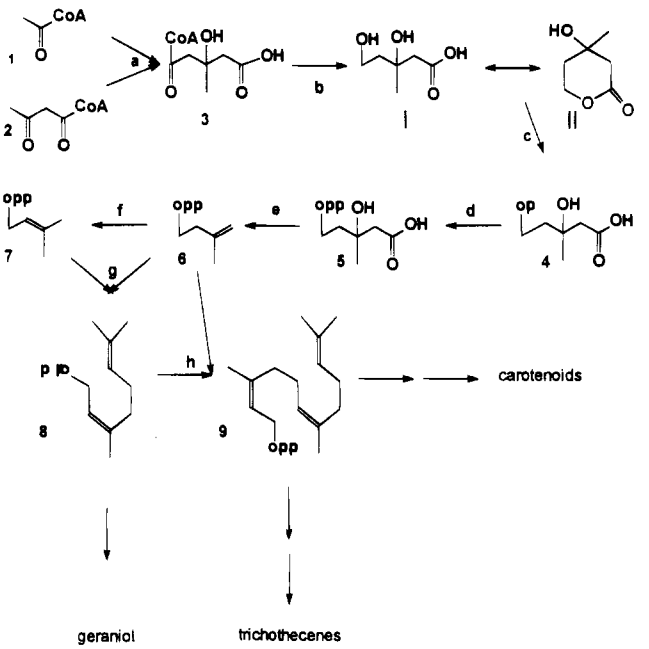


Figure 1. Partial schematic diagram showing the involvement of mevalonate in the biosynthesis of carotenoids, geraniol, and trichothecenes: (**1**) acetyl-CoA; (**2**) acetoacetyl-CoA; (**3**) hydroxymethylglutaryl-CoA; (**I**) mevalonic acid; (**II**) mevalonic acid lactone; (**4**) 5-phosphomevalonate; (**5**) mevalonic acid pyrophosphate; (**6**) isopentyl pyrophosphate; (**7**) dimethylallyl pyrophosphate; (**8**) geranyl pyrophosphate; (**9**) farnesyl pyrophosphate; (**a**) HMG-CoA synthase; (**b**) HMG-CoA reductase; (**c**) mevalonate kinase; (**d**) phosphomevalonate kinase; (**e**) pyrophosphomevalonate decarboxylase; (**f**) isopentyl pyrophosphate isomerase; (**g**) geranyl pyrophosphate synthetase; (**h**) farnesyl pyrophosphate synthetase.

Acidification of the fungal broth extract results in conversion of mevalonic acid to its lactone (Del Puppo *et al.*, 1989), the latter being sufficiently volatile to be analyzed by gas chromatography. Isobutane chemical

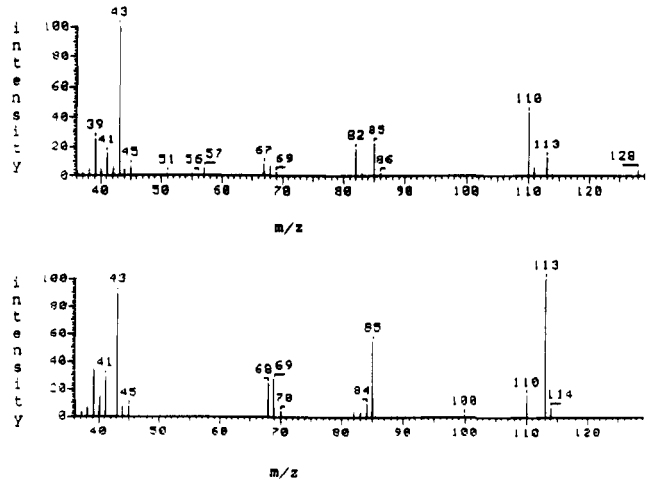


Figure 2. Electron ionization (EI) mass spectra of **IV** (top) and **VI** (bottom).

ionization mass spectrometry (CIMS) of the lactone produced a strong M + 1 ion at *m/z* 131 and a significant ion at *m/z* 113 due to loss of water from the protonated molecular ion. Comparison of the total ion chromatograms from control and sodium bicarbonate-treated extracts showed the presence of significant quantities of three compounds (eluting at 425, 435 and 445 s) in the sodium bicarbonate-treated extracts that were absent or nearly so in control samples. Isobutane CIMS spectra of the materials eluting at 425 and 435 s were similar. Both exhibited M + 1 base peaks at *m/z* 129 and an additional ion at *m/z* 111 (loss of water), suggesting that both compounds were alcohols and had molecular weights of 128. The spectrum of the material eluting at 445 s exhibited an M + 1 peak at *m/z* 147, suggesting a molecular weight of 146. Ammonia CIMS confirmed the molecular weights determined with isobutane. Mevaldic acid lactone (**III**), which also has a molecular weight of 146, had a retention time of 490 s and was not found in the fungal culture extract.

Electron ionization (EI) mass spectrometry of the observed metabolites in the crude fungal extract was not possible due to the complexity of the fungal matrix and the drastically reduced sensitivity of ion trap EI compared to CI. The two materials with molecular weight 128 were therefore isolated by semipreparative high-performance liquid chromatography to obtain sufficient material for EI spectra. The material with molecular weight 146 disappeared during isolation, and attempts to collect it in large quantities were abandoned. After isolation of microgram quantities of the two materials with molecular weight 128, it was observed that the material with GC retention time of 425 s was rearranging to the material with GC retention time of 435 s. This conversion occurred in a variety of solvents: chloroform, ethyl acetate, acetone, and ethyl acetate–hexane (1:1). On the other hand, the material with GC retention time of 435 s was stable in these solvents.

The material with retention time of 425 s had the same retention time and EI mass spectrum (Figure 2, top) as authentic 3-methyl-4-oxo-2-pentenoic acid (**IV**). The authentic material, just like the material that was isolated from the fungal culture, slowly converted to a material that had a retention time of 435 s under our chromatographic conditions. The EI mass spectrum for this compound is shown in Figure 2 (bottom). This spectrum is very similar to the library (*Eight Peak Index of Mass Spectra*, 1983) mass spectrum of 3,5-dimethyl-5-hydroxy-2(5*H*)-furanone (**V**): *m/z* 43 (100), 39 (21), 41 (17), 69 (12), 85 (12), 68 (7), 113 (5), and 110 (4). It is difficult to envision a reasonable mechanism for the conversion of **IV** to **V** in organic solvents at room temperature. On the other hand, conversion of **IV** to 4,5-dimethyl-5-hydroxy-2(5*H*)-furanone (**VI**) represents a simple ring closure.

Table 1. Effect of 0.11 M Sodium Bicarbonate on the Concentration of Mevalonic Acid and Mevalonic Acid Lactone and Their Metabolites in Cultures of *F. tricinctum* NRRL 13426

treatment	no. of days[a]	concn[b] (μmol/g cell dry weight)				
		I/II[c]	**IV**	**VI**	**VII/VIII**	total[d]
		Shake Culture				
control	2	0.028	<0.001	<0.001	<0.001	0.028
$NaHCO_3$	2	0.014	trace	trace	0.012	0.026
control	15	0.014	<0.001	0.008	<0.001	0.022
$NaHCO_3$	15	0.034	0.070	0.063	0.110	0.277
		Stationary Culture				
control	5	0.006	<0.001	<0.001	<0.001	0.006
$NaHCO_3$	5	0.012	0.051	<0.001	0.033	0.096
control	10	0.033	0.001	0.007	<0.001	0.040
$NaHCO_3$	10	0.016	0.038	0.023	0.020	0.097
control	13	0.012	<0.001	<0.001	<0.001	0.012
$NaHCO_3$	13	0.032	0.019	0.019	0.029	0.099
control	18	0.015	<0.001	<0.001	trace	0.015
$NaHCO_3$	18	0.038	0.019	0.023	0.065	0.145

[a] Number of days of incubation. [b] All values are the average of duplicate determinations. [c] **I**, mevalonic acid; **II**, mevalonic acid lactone; **IV**, 3-methyl-4-oxo-2-pentenoic acid; **VI**, 4,5-dimethyl-5-hydroxy-2(5*H*)-furanone; **VII**, 4,5-dihydroxy-3-methyl-2-pentenoic acid; **VIII**, 3,4-dihydroxy-3-methyl-4-pentenoic acid. [d] Total concentration of **I**, **II**, **IV**, **VI**, **VII**, and **VIII**.

Comparison of the two mass spectra lends additional support to the identities of **IV** and **VI**. The former exhibits a small molecular ion at *m/z* 128, loss of methyl radical at *m/z* 113, and loss of water at *m/z* 110. Loss of CO from *m/z* 113 and 110 gives *m/z* 85 and 82, respectively. The base peak at *m/z* 43 is the acetyl ion. While **VI** also has a large *m/z* 43, its base peak is *m/z* 113, formed by loss of the allyl methyl radical. Loss of CO gives *m/z* 85. Since loss of water from the molecular ion of **VI** is not as easy as from **IV**, *m/z* 110 is relatively small and loss of CO to *m/z* 82 is not observed. No molecular ion for **VI** was observed, but its molecular weight of 128 was determined by CI (*vide supra*).

The identification of compound **IV** in the fungal culture together with the accumulation of mevalonate strongly suggests that **IV** is a metabolite of mevolanate and implies that the material with molecular weight 146 is an intermediate between mevalonate and **IV**. Thus, the material with molecular weight 146 may be 4,5-dihydroxy-3-methyl-2-pentenoic acid (**VII**) or 3,4-dihydroxy-3-methyl-4-pentenoic acid (**VIII**). However, more definitive physical evidence would be needed to support either of these assignments.

The results of quantitative analyses for **I/II** (mevalonate), **IV**, **VI**, and for the material we tentatively propose as **VII** or **VIII** are presented in Table 1 for both stationary and shake incubations of *F. tricinctum* 13426. In control cultures, mevalonate and small amounts of **VI** were present, suggesting that conversion of mevalonate to **IV/VI** is a normal, but relatively minor, event. In the presence of sodium bicarbonate (in both stationary and shake cultures) mevalonate accumulates. This results in the greater availability of mevalonate for oxidation and dehydration to **IV** and rearrangement to **VI**. Comparison of the total concentration of mevalonate and its metabolites found in sodium bicarbonate-

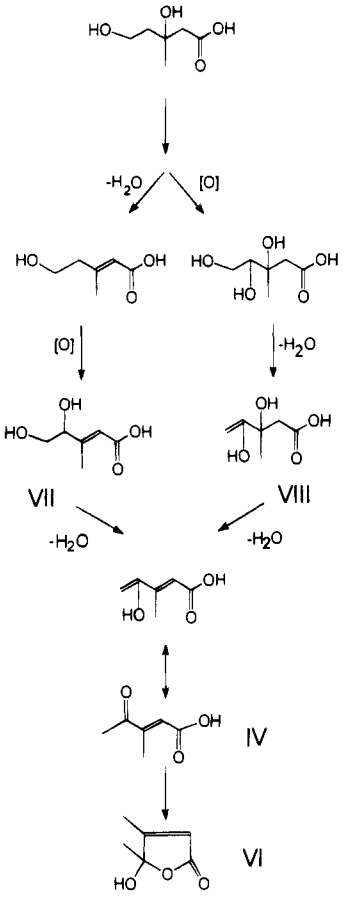


Figure 3. Proposed pathway for the production of **IV** and **VI** in sodium bicarbonate-treated cultures of *F. tricinctum* NRRL 13426.

treated cultures to that found in controls indicates and approximate 1 order of magnitude increase in the treated cultures. A reasonable pathway to explain these results is summarized in Figure 3. Mevalonate kinase, which normally converts mevalonic acid to 5-phosphomevalonate, is inhibited at high pH. This results in the accumulation of mevalonic acid, which is then converted to **VII** by dehydration followed by oxidation (or converted to **VIII** by oxidation followed by dehydration). Further dehydration of **VII** or oxidation of **VIII** results in the formation of **IV**, which rearranges to **VI**. Alternatively, the results may be explained by a pH-dependent activation of the enzyme system responsible for converting mevalonate to **IV** or a precursor of **IV**. In any event, the inability of mevalonate to be converted to 5-phosphomevalonate results in the failure of the fungus to produce the carotenoids, geraniol, and trichothecenes as well as other secondary metabolites.

LITERATURE CITED

Cane, D. E.; Swanson, S.; Murthy, P. Trichodiene Biosynthesis and the Enzymatic Cyclization of Farnesyl Pyrophosphate. *J. Am. Chem. Soc.* **1981**, *103*, 2136–2138.

Carlile, M. J. A Study of the Factors Influencing Non-genetic Variation in a Strain of *Fusarium oxysporum*. *J. Genet. Microbiol.* **1956**, *14*, 643–654.

Del Puppo, M.; Cighetti, G.; Kienle, M. G.; De Angelis, L. Measurement of Mevalonate in Human Plasma and Urine by Multiple Selected Ion Monitoring. *Biomed. Environ. Mass Spectrom.* **1989**, *18*, 174–176.

DePasquale, D. A.; El-Nabarawy, A.; Rosen, J. D.; Montville, T. J. Ammonium Bicarbonate Inhibition of Mycotoxigenic Fungi and Spoilage Yeasts. *J. Food Prot.* **1990**, *53*, 324–328.

Eight Peak Index of Mass Spectra; Mass Spectrometry Data Centre: Aldermaston, U.K., 1983; Vol. 3.

El-Nabarawy, A.; Hartman, T.; Rosen, J. D.; Montville, T. J. *Aspergillus Parasiticus* Accumulates Averufin and Versicolorin A in the Presence of Bicarbonate. *J. Food Prot.* **1989**, *52*, 493–495.

Goldstein, J. L.;. Brown, M. S. Regulation of the Mevalonate Pathway. *Nature* **1990**, *343*, 425–430.

Gribanovski-Sassu, O.; Foppen, F. H. Lipid Constituents of Some *Fusarium* Species. *Arch. Mikrobiol.* **1968**, *62*, 251–256.

Joffe, A. Z. In *Microbial Toxins*; Kadus, S., Ciegler, A., Ajl, S., Eds.; Academic Press: New York, 1971; Vol. 7, pp 139–189.

Montville, T. F.; Shih, P.-L. Inhibition of Mycotoxigenic Fungi in Corn by Ammonium and Sodium Bicarbonate. *J. Food Prot.* **1991**, *54*, 295–297.

Robinson, T. *The Organic Constituents of Higher Plants*; Cordus Press: N. Amherst, MA, 1975; p 182.

Roinestad, K. S.; Montville, T. J.; Rosen, J. D. Inhibition of Trichothecene Biosynthesis in *Fusarium tricinctum* by Sodium Bicarbonate. *J. Agric. Food Chem.* **1993**, *41*, 2344.

Shunk, C. H.; Linn, B. O.; Huff, J. W.; Gilfillan, J. L.; Skeggs, H. R.; Folkers, K. Resolution of DL-Mevalonic Acid and the Synthesis and Biological Activities of DL-3-Hydroxy-3-methylglutaraldehydic Acid. *J. Am. Chem. Soc.* **1957**, *79*, 3294.

Ueno, Y. Mode of Action of Trichothecenes. *Pure Appl. Chem.* **1977**, *49*, 1737–1745.

Received for review November 4, 1993. Revised manuscript received April 25, 1994. Accepted May 24, 1994.[⊗]

[⊗] Abstract published in *Advance ACS Abstracts,* July 15, 1994.