and 23.0; for Jan 1970, 13.4 and 22.7. For the Valencia samples the values are as follows: for Feb 1970, 12.8 and 10.8; for April 1970, 13.5 and 15.0; for June 1970, 10.9 and 10.9. Insufficient samples of Hamlin and Pineapple orange puree were available to establish a similar seasonal trend.

The present study confirms earlier studies (Kefford and Chandler, 1977) that comminuted orange bases contain about the same amount of vitamin C as orange juice and undergo the same degree of degradation at ambient storage temperatures. Since whole orange purees are diluted 10 to 1 for the preparation of citrus-flavored beverages (Lime and Cruse, 1972), such beverages are not nearly as useful a source of natural vitamin C as is orange juice.

Registry No. Vitamin C, 50-81-7.

LITERATURE CITED

Association of Official Analytical Chemists "Official Methods of Analysis", 12th ed.; Association of Official Analytical Chemists: Washington, DC, 1975; 830–831.
Cruse, R. R.; Lime, B. J. *Food Eng.* **1970**, *42* (6), 109–116.
Deutsch, M. J.; Weeks, C. E. *J. Assoc. Off. Anal. Chem.* **1965**, *48*, 1248–1256.
Kefford, J. F. *World Rev. Nutr. Diet.* **1973**, *18*, 60–120.
Kefford, J. F.; Chandler, B. V. In "Citrus Science and Technology"; Nagy, S.; Shaw, P. E.; Veldhuis, M. K., Eds.; Avi Publishing Co.: Westport, CT, 1977; Vol. 2, Chapter 7.
Lime, B. J.; Cruse, R. R. *J. Food Sci.* **1972**, *37*, 250–252.
Lime, B. J.; Cruse, R. R. U.S. Patent 3 745 020, 1973.
Nagy, S. *J. Agric. Food Chem.* **1980**, *28*, 8–18.

Roger F. Albach*
Amelia T. Murray

Agricultural Products Quality Research
Agricultural Research Service
Science and Education
U.S. Department of Agriculture
Weslaco, Texas 78596

Received for review November 12, 1981. Revised manuscript received July 15, 1982. Accepted January 7, 1983. Names of companies or commerical products are given solely for the purpose of providing specific information; their mention does not imply recommendation or endosement by the U.S. Department of Agriculture over others not mentioned.

Two Classes of Alkaloid Mycotoxins Produced by *Penicillium crustosum* Thom Isolated from Contaminated Beer

An apparent natural human intoxication resulted from consumption of beer contaminated with *Penicillium crustosum*. Under laboratory culture, the *P. crustosum* isolate produced two classes of toxic alkaloids consisting of roquefortine [10β-(1,1-dimethyl-2-propenyl)-3-(imidazol-4-ylmethylene)-5α,10β,11,11α-tetrahydro-2*H*-pyrazino[1′,2′:1,5]pyrrolo[2,3-*b*]indole-1,4(3*H*,6*H*)-dione], roquefortine A (isofumigaclavine A) (9α-acetoxy-6,8β-dimethylergoline), roquefortine B (isofumigaclavine B) (6,8β-dimethylergolin-9α-ol), and festuclavine (6,8β-dimethylergoline). Samples of the beer were not available for analysis.

This study was prompted by a clinical case that apparently resulted from a natural intoxication of a 44-year-old Caucasian male who consumed some commercial beer that was contaminated with a large mycelial mass of the fungus identified as *Penicillium crustosum* (Figure 1). Approximately 4 h after consuming the contaminated beer (approximately 30 cm^3 consumed), the individual became actuely ill with a throbbing frontal headache, feverish feeling, nausea, vomiting, diplopia, weakness, and bloody diarrhea. After 12 h, handwriting was illegible due to tremor. The symptoms prevented eating and other activities for approximately 30 h. After this time all symptoms disappeared and no apparent residual effects were noted. Five other family members and five visitors shared the evening meal but did not consume any beer and had no symptoms.

When the container was opened, there was no prior indication of contamination of the beer since the can of beer appeared to be properly filled and normally carbonated. Microbiological analysis of the pellicle-like mycelial mass showed a *Penicillium* sp. and a *Rhizopus* sp. to be present. Although accurate quantitation of the two isolates present was not possible, it appeared that the *Penicillium* sp. rather than the *Rhizopus* sp. mainly contributed to the mass of material. The *Penicillium* sp. was identified by Dr. Kenneth B. Raper as *Penicillium crustosum* Thom. However, the isolate did not form nearly as heavy crusts of conidia on agar plates typical of most isolates of this species.

This paper evaluated the toxin-producing potential of the isolate identified as *P. crustosum* when grown on two different media.

MATERIALS AND METHODS

The isolate of *P. crustosum* was cultured at 25–27 °C for 2 weeks in Fernbach flasks (2.8 L), each containing 100 g of shredded wheat and 200 mL of mycological broth supplemented with 2% yeast extract and 15% sucrose or 200 mL of a commercial brand of beer. The toxigenicity of the *P. crustosum* isolate and purification of the toxins were done by using the bioassay method of Kirksey and Cole (1974) with 1-day-old chickens dosed orally via crop intubation.

Mass production of the toxin was done in Fernbach flasks containing the shredded wheat medium under the cultural conditions described above.

The cultures were extracted with chloroform by homogenization with an Ultra-Turrax homogenizer (Tekmar Co.,

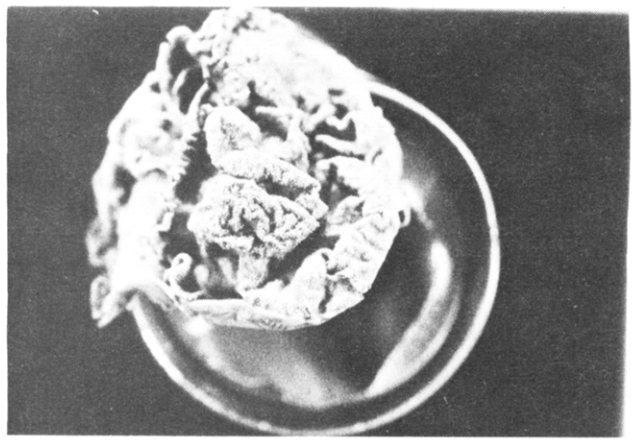

Figure 1. Large mycelial mass of *P. crustosum* from commercial beer.

Cincinnati, OH). The extract was filtered through two layers of cheesecloth, dried over anhydrous sodium sulfate, and concentrated under vacuum at 60 °C with a rotary evaporator.

The toxic crude extract was transferred to a separatory funnel in ethyl acetate solution and partitioned twice against HCl solution (pH 2). The solution was made basic with sodium carbonate (pH 10) and partitioned twice against chloroform. Portions of both extracts were bioassayed with 1-day-old chicks.

The chloroform extract partitioned from basic solution was concentrated, dissolved in a minimum of benzene, and applied to a column (3.5 × 15 cm) containing Woelm neutral activity grade IV alumina packed as a slurry in benzene. The column was eluted with a linear gradient from benzene to ethyl ether (107 × 17-mL fractions collected). Following completion of the linear gradient, the column was eluted with 750 mL each of ethyl acetate, acetone, and methanol. Three alkaloids eluted together in tubes 43–59 and two more alkaloids eluted in the methanol fraction. Tubes 43–59 from the elution gradient were combined, concentrated, dissolved in ethyl acetate, and applied to silica gel column (2.0 × 45 cm) packed in ethyl acetate. The column was eluted with a linear gradient from ethyl acetate to acetone (118 × 17-mL fractions collected). The more nonpolar alkaloid eluted in tubes 34–101. These were combined, concentrated, and designated BMA-1. The column was further eluted with a linear gradient from acetone to methanol (96 × 17-mL fractions collected). Another alkaloid eluted in tubes 143–169 which were combined, concentrated, and designated BMA-2. A third minor alkaloid, BMA-3, eluted in tubes 190–214.

The fractions containing the other two alkaloids were combined, concentrated, and chromatographed on a silica gel column (2.0 × 45 cm) packed in ethyl acetate and eluted with a linear gradient from ethyl acetate to acetone. The major alkaloid, designated RC-1, eluted in tubes 55–106. Tubes 121–163 contained another minor alkaloid which was designated RC-2.

Thin-layer chromatography (TLC) analyses were made with precoated silica gel 60 F-254 plates (5 × 10 cm) (EM Laboratories, Inc., Elmsford, NY) with a developing solvent of chloroform–acetone–methanol, 93:7:5 v/v/v. Toxins were visualized on TLC plates by spraying first with (dimethylamino)benzaldehyde solution (1% in ethanol) followed by 50% ethanolic sulfuric acid and heating for 2 min at 110 °C.

Proton and ^{13}C NMR spectra were obtained on a Varian Associates XL-100-12 NMR spectrometer. Samples of

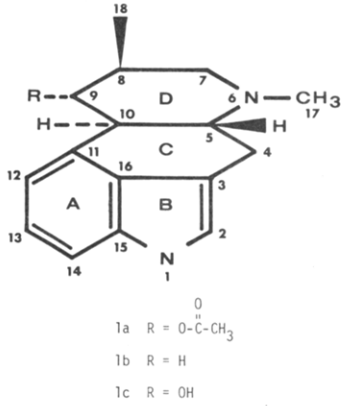

Figure 2. Clavine alkaloids from *P. crustosum*.

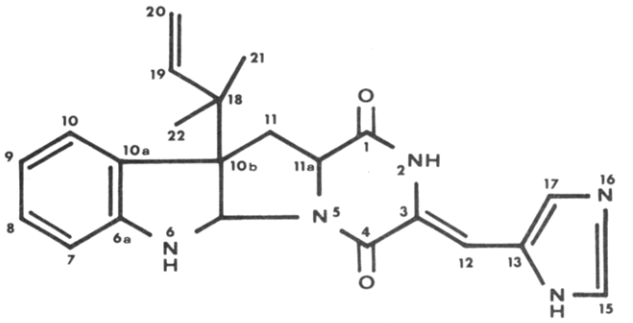

Figure 3. Roquefortine.

BMA-1 were run in $CDCl_3$–Me_2SO-d_6 (50:50) solution and of BMA-2, RC-1, and RC-2 in $CDCl_3$ solution.

Mass spectra were run in the EI mode on a VG Micromass 70-70 spectrometer. Samples were introduced via the direct probe technique.

Ultraviolet spectra in methanol solution were recorded with a Beckman Model DB-G recording spectrophotometer.

Infrared spectra of samples prepared as thin films on KBr windows were obtained with a Perkin-Elmer Model 257 recording spectrophotometer equipped with a 4X beam condenser.

RESULTS AND DISCUSSION

The compound BMA-1 was identified as roquefortine A (isofumigaclavine A) (9α-acetoxy-6,8β-dimethylergoline) (Figure 2, **1a**) based on TLC, UV, IR, and high-resolution mass spectral analysis and ^1H and ^{13}C NMR spectral analyses (Cole and Cox, 1981; Arnoux et al., 1978; Scott et al., 1976; Ohmomo et al., 1975a,b). On the basis of the same analytical criteria, BMA-2 was identified as festuclavine (6,8β-dimethylergoline) (Figure 2, **1b**) (Bach et al., 1974). The amount of BMA-3 was insufficient for a confirmed identification; however, based on chromatographic characteristics, it was tentatively identified as roquefortine B (isofumigaclavine B) (6,8β-dimethylergolin-9α-o1) (Figure 2, 1c) (Spilsbury and Wilkinson, 1961).

The alkaloid RC-1 showed a molecular ion peak at 389.1840 ($C_{22}H_{23}O_2N$, calculated for $C_{22}H_{23}O_2N$ = 389.1851) and λ_{max}^{MeOH} 209 (29 600), 240 (16 200), and 328 nm (27 100). With a comparison of these data and ^{13}C and ^1H NMR spectra and TLC data, RC-1 was conclusively identified as roquefortine [10β-(1,1-dimethyl-2-propenyl)-3-(imidazol-4-ylmethylene)-5α,10β,11,11α-tetrahydro-2*H*-pyrazino[1′,2′:1,5]pyrrolo[2,3-*b*]indole-1,4-(3*H*,6*H*)-dione) (Figure 3) (Cole and Cox, 1981; Ohmomo et al., 1975b; Scott et al., 1976, 1979). The identity of RC-2 was not determined except that on the basis of a comparison of its UV spectrum with roquefortine it showed

that their UV chromophores were very similar.

It has been previously shown that roquefortine (Figure 3, roquefortine A (Figure 2, **1a**), and roquefortine B (Figure 2, **1c**) were produced by certain strains of *Penicillium roqueforti* and have been found naturally occurring in varieties of blue cheese (Scott et al., 1976).

The alkaloids fumigaclavine A and B produced by *Aspergillus fumigatus* Fres. were shown to be stereoisomers of roquefortines A and B, respectively. These alkaloids differ only in the stereochemistry at positions 8 and 9 (Arnoux et al., 1978).

The alkaloids were produced on both the solid and the liquid (beer) media; however, yields on the solid media were observed to be higher under the cultural conditions imposed. The physical environmental conditions under which the *P. crustosum* grew in the can of beer are not known and, therefore, could not be reproduced to see what toxic secondary metabolites, if any, were produced under these conditions. Since samples of the contaminated beer were not available for analysis, it is not known if these alkaloids were present in the beer and involved in the apparent intoxication. However, it has been demonstrated that this *P. crustosum* isolate has the capability of producing two different classes of alkaloids in laboratory culture.

ACKNOWLEDGMENT

We thank G. E. Barden for isolating the fungi from mycelial material and Dr. K. B. Raper for identifying the *P. crustosum* isolate.

Registry No. **1a**, 58800-19-4; **1b**, 569-26-6; **1c**, 58800-20-7; roquefortine, 58735-64-1.

LITERATURE CITED

Arnoux, B.; Merrien, M. A.; Pascard, C.; Polonsky, J.; Scott, P. M. *J. Chem. Res., Synop.* **1978**, 210.

Bach, N. J.; Boaz, H. E.; Kornfeld, E. C.; Chang, C.-J.; Floss, H. G.; Hagaman, E. W.; Wenkert, E. *J. Org. Chem.* **1974**, *39*, 1274.

Cole, R. J.; Cox, R. H. In "Handbook of Toxic Fungal Metabolites"; Academic Press: New York, 1981; Chapter 10, pp 527–568.

Kirksey, J. W.; Cole, R. J. *Mycopathol. Mycol. Appl.* **1974**, *54*, 291.

Ohmomo, S.; Sato, T.; Utagawa, T.; Abe, M. *Agric. Biol. Chem.* **1975a**, *39*, 1333.

Ohmomo, S.; Sato, T.; Utagawa, T.; Abe, M. *J. Agric. Chem. Soc. Jpn.* **1975b**, *49*, 53.

Scott, P.; Merrien, M. A.; Polonsky, J. *Experientia* **1976**, *32*, 140.

Scott, P. M.; Polonsky, J.; Merrien, M. A. *J. Agric. Food Chem.* **1979**, *27*, 201.

Spilsbury, J. F.; Wilkinson, S. *J. Chem. Soc.* **1961**, 2085.

Richard J. Cole*[1]
Joe W. Dorner[1]
Richard H. Cox[2]
Lawrence W. Raymond[3]

[1]U.S. Department of Agriculture
National Peanut Research Laboratory
Dawson, Georgia 31742
[2]Philip Morris USA
Research Center
Richmond, Virginia 23261
[3]Llewellyn Park
West Orange, New Jersey 07052

Received for review September 7, 1982. Accepted December 12, 1982. Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture or Philip Morris USA and does not imply its approval to the exclusion of other products that may also be suitable.

Distribution of Vomitoxin in Dry Milled Fractions of Wheat Infected with *Gibberella zeae*

Two samples of winter wheat, one from Canada and the other from the United States, naturally infected with *Gibberella zeae*, were dry milled and the separate fractions analyzed for the presence of the mycotoxin vomitoxin (3,7,15-trihydroxy-12,13-epoxytrichothec-9-en-8-one). Initial concentrations of vomitoxin in both samples of the whole wheat was 2 μg/g of grain. Vomitoxin was distributed throughout all fractions of the milled U.S. and Canadian grain. After being milled, 65% of the vomitoxin was in straight grade flour fractions and the rest in the bran, red dogs, shorts, and low-grade fractions in the U.S. sample. Although there were differences in the concentration of vomitoxin between the different fractions of the milled U.S. wheat, when the fractions were grouped into (1) bran, red dogs, and shorts, (2) straight-grade flour, and (3) low-grade flour, there did not appear to be a concentration of vomitoxin in any one fraction. The final concentration of vomitoxin in each of the groupings was 5.2, 4.5, and 4.0 μg/g, respectively. The total amount of vomitoxin extracted from the milled U.S. sample was more than 2 times greater than that predicted from the initial weight of the whole wheat and may have resulted from fungal growth during the week long tempering to raise the moisture content of the wheat.

Vomitoxin (3,7,15-trihydroxy-12,13-epoxytrichothec-9-en-8-one), is a naturally occurring cytotoxic trichothecene responsible for emesis and feed refusal in swine (Mirocha et al., 1976; Forsyth et al., 1977; Vesonder et al., 1973) and is commonly associated with cereal grains infected by *Fusarium roseum* f. sp. *graminearum* (Schw.) Synder and Hansen (conidial state of *Gibberella zeae* Schwabe).

Vomitoxin is not known to be mutagenic or carcinogenic. Its LD_{50} of 40–70 mg/kg of body weight in rats is higher than other trichothecenes such as T-2, HT-2, or diacetoxyscirpenol (Yoshizawa and Morooka, 1977; Sato and Ueno, 1977). Because vomitoxin is difficult to produce in large quantities there have been no studies on the long-term effect of feeding sublethal doses to animals. However, sublethal doses of T-2 and diacetoxyscirpenol have been shown to suppress the immune response of test animals (Rosenstein et al., 1979, 1981). In 1980, because of the high incidence of vomitoxin in Canadian wheat, the Canadian Government suggested a tolerance level of 300 ng of vomitoxin/g of wheat intended for human consumption and recommended a zero tolerance for wheat intended for use in infant food. Concentrations of vomitoxin in the Can-

0021-8561/83/1431-0657$01.50/0