a General Population Survey. *Environ. Health Perspect.* **1983**, *48*, 81–86.
Patterson, D. G., Jr.; Needham, L. L.; Pirkle, J. L.; Roberts, D. W.; Bagby, J.; Garrett, W. A.; Andrews, J. S.; Falk, H.; Bernert, J. T., Jr.; Sampson, E. J.; Houk, V. N. Correlation Between Serum and Adipose Tissue Levels of 2,3,7,8-Tetrachlorodibenzo-p-dioxin in 50 Persons from Missouri. *Arch. Environ. Contam. Toxicol.* **1988**, *17*, 139–143.
Savage, E. P.; Keefe, T. J.; Tessari, J. D.; Wheeler, H. W.; Applehans, F. M.; Goes, E. A.; Ford, S. A. National Study of Chlorinated Hydrocarbon Insecticide Residues in Human Milk, USA I. Geographic Distribution of Dieldrin, Heptachlor, Heptachlor Epoxide, Chlordane, Oxychlordane, and Mirex. *Am. J. Epidemiol.* **1981**, *113*, 413–422.
Stemmler, E. A.; Hites, R. A. Methane Enhanced Negative Ion Mass Spectra of Hexachlorocyclopentadiene Derivatives. *Anal. Chem.* **1985**, *57*, 684–692.
Webb, R. G.; McCall, A. C. Quantitative PCB Standards for Electron Capture Gas Chromatography. *J. Chromatogr. Sci.* **1973**, *11*, 366–373.

Received for review April 22, 1988. Accepted October 3, 1988. Use of trade names is for identification only and does not constitute endorsement by the Public Health Service or the U.S. Department of Health and Human Services. Partial funding for this work was provided by the Agency for Toxic Substances and Disease Registry (ATSDR), U.S. Public Health Service, from the Comprehensive Environmental Response, Compensation, and Liability Act trust fund (Superfund), through an interagency agreement with the Environmental Protection Agency (EPA).

Secondary Metabolites of *Fusarium* Species: Apotrichothecene Derivatives[1,2]

Roy Greenhalgh,* David A. Fielder, Lisa A. Morrison, Jean-Pierre Charland, Barbara A. Blackwell, Marc E. Savard, and John W. ApSimon

Two epimers (3α-OH, 3β-OH) of 3,13-dihydroxy-11-epiapotrichothec-9-ene have been isolated from liquid cultures of *Fusarium* species. These epimers are common minor metabolites of *Fusarium crookwellense*, *Fusarium culmorum*, *Fusarium graminearum*, and *Fusarium sporotrichioides*, the ratio of the epimers being species dependent. The absolute configuration of the 3α-OH epimer was determined by X-ray crystallography and shown to have a trans A/B ring system with H-11 in the β-configuration as opposed to the cis A/B ring system of the trichothecenes. Both epimers readily undergo oxidation. The 3α-OH epimer also is involved in a unique rearrangement to form a ketal, 3,11-epoxy-13-hydroxyapotrichothec-9-ene, a compound also detected in the crude extracts of *F. culmorum* and *F. sporotrichioides* fermentations. The mass and NMR spectral data of these compounds and their epoxy derivatives are discussed. Speculations are made on the biosynthesis of the apotrichothecenes and sambucinol, another minor metabolite ubiquitous to the *Fusarium* species.

The impact of *Fusarium* mycotoxins on animal and human health and on the economy has resulted in an increase of interest in the trichothecenes (Ueno, 1983; Foster et al., 1986), which are the major toxic metabolites. The trichothecenes are tricyclic sesquiterpenes having in common a 9,10 double bond and a 12,13-epoxide moiety but varying in the degree of oxidation and acylation in rings A and C. Oxidation of the trichothecene ring appears to be species dependent. The stereochemistry of the oxygen moieties at any specific carbon atom is well-defined. This together with the degree of substitution appears to be related to the relative toxicity, with T-2 toxin being the most toxic.

In addition to the trichothecenes, all the *Fusarium* species studied by us to date, i.e. *Fusarium crookwellense*, *Fusarium culmorum*, *Fusarium graminearum*, and *Fusarium sporotrichioides*, also produce a variety of minor metabolites in liquid culture. Some of these compounds are common to all four species, e.g. sambucinol (IX), first isolated by Mohr et al. (1984), and 3,13-dihydroxy-11-epiapotrichothec-9-ene (I) (Greenhalgh et al., 1986; Zamir et al., 1987). This latter compound possesses two asymmetric methine centers (C-11 and C-3), but to date only isomers at C-3 have been reported (Lauren et al., 1987). Other compounds including sambucoin (X) (Mohr et al., 1984) and its 8-hydroxy analogues (Corley et al., 1987b) have been reported to be formed by specific *Fusarium* species, the latter on a solid medium. These minor metabolites differ from the trichothecenes in the size and stereochemistry of the ring system.

Although apotrichothecenes appear to be noncytotoxic to animal cells (Grove and Mortimer, 1969), they are phytotoxic (Wang and Miller, 1988). In addition, the biosynthetic origins of these compounds are of interest since they appear to arise from trichodiene, in common with the trichothecenes but by a different cyclization mechanism. This paper describes the resoluton of the two epimers of I, the absolute configuration of the 3α-hydroxy isomer IA, and the characterization of some rearranged products.

EXPERIMENTAL SECTION

Apparatus. Gas chromatography/mass spectrometry (GC/MS) analysis was performed on a Finnigan Model 4500 system using a DB-5 capillary column (15 m × 0.32 mm (i.d.), 0.25-μm film), which was temperature programmed from 140 to 260 °C at 15 °C/min. The helium carrier gas was set at 10 psi. Some MS data were generated also by a Finnigan ion trap detector (IT). High-performance liquid chromatography (HPLC) was carried out with a Varian Model 5500 system equipped with a UV-200 variable-wavelength detector set at 205 nm. Separations were

Plant Research Centre, Agriculture Canada, Ottawa, Ontario K1A 0C6, Canada (R.G., B.A.B., M.E.S.), Ottawa-Carleton Chemistry Institute, Carleton University, Ottawa, Ontario K1S 5B6, Canada (D.A.F., L.M., J.W.A.), and Chemistry Division, National Research Council, Ottawa, Ontario K1A 0R9, Canada (J.-P.C.).

[1] PRC Contribution No. 1150.

[2] NRC Contribution No. 29516.

0021-8561/89/1437-0699$01.50/0 Published 1989 by the American Chemical Society

Table I. ^{1}H NMR of 3,13-Dihydroxy-11-epiapotrichothecene and Its Derivatives

carbon atom	3α-OH-APO IA		3β-OH-APO IB		epoxy-α-OH-APO VIIIA		epoxy-β-OH-APO VIIIB	
2α	1.74	12.2 (AB)	2.23	14.9 (AB)	1.75	12.2 (AB)	1.77	14.8 (AB)
2β	2.60	2.2 (2α,3)	2.10	4.1 (2β,3)	2.64	1.6 (2α,4α)	2.39	1.6 (2α,4)
		1.7 (2β,4β)		1.3 (2β,4)		6.3 (2β,3)		6.3 (2α,3)
		6.2 (2β,3)		6.3 (2α,3)				2.7 (2β,3)
3	4.28	2.2 (3,2α)	4.51 (m)	6.3 (3,2α)	4.31	6.2 (3,2β)	4.51	3.8 (3,2α)
		6.2 (3,2β)				7.5 (3,4α)		4.8 (3,4α)
		1.3 (3,4α)				10.2 (3,4β)		
		10.2 (3,4β)						
4α	2.13	12.9 (AB)	2.45	14.6 (AB)	1.63	18.0 (AB)		
4β	1.67	1.3 (4β,3)	1.38	1.3 (4β,2β)	2.04	1.6 (4α,2α)		
		10.1 (4α,3)		5.8 (4β,3)		7.4 (4α,3)		
		1.7 (4β,2β)		6.4 (4α,3)		10.3 (4β,3)		
7	1.37	13.1 (AB)	1.36 (m)	13.5 (AB)	1.17			
	1.54	2.7 (7α,8α)	1.57		1.30			
		5.6 (7α,8β)						
		9.8 (7β,8α)						
8	1.93	2.8 (8α,7α)	1.99 (m)	1.8	1.98		2.21	
	2.03	7.3 (8β,7α)		5.4				
10	5.52	2.5 (10,11)	5.50	1.6 (10,16)	3.10		3.05	
		1.3 (10,16)						
11	4.15	2.5 (11,10)	4.11		3.4		3.44	
		2.3						
13	3.18	11.4 (AB)	3.56	10.9 (AB)	3.21	11.6 (AB)	3.63	11.1 (AB)
	3.73		3.78		3.71		3.78	
14	0.92		1.07		0.84		1.04	
15	0.99		0.52		0.95		0.84	
16	1.63	1.3 (16,10)	1.63	1.6 (16,10)	1.33	1.1 (16,10)	1.37	
		2.3 (16,11)						

carbon atom	II		III		V		VI
2α	2.13	11.5 (AB)	1.21	11.4 (AB)			
2β	1.44	2.0 (2α,4α)	2.43		2.34		2.24
		3.8 (2β,3)					
3	4.51	1.8 (3,2α)	4.53 (m)		4.54 (m)		4.44 (t)
		3.4 (3,2β)					
		1.8 (3,4β)					
4α	2.46	13.5 (AB)	1.49	11.5 (AB)			1.4
4β	1.16	2.0 (4α,2α)	2.20	3.8 (4α,3)	2.12		2.82
		3.4 (4α,3)		5.2 (4β,3)			
7	1.34	12.9 (AB)	1.11	13.3 (AB)			1.9–2.1
	1.86	1.6 (7α,8α)	1.41	1.4 (7α,8α)			
		6.6 (7α,8β)		7.3 (7α,8β)			
		5.6 (7β,8α)		6.5 (7β,8α)			
		4.4 (7β,8β)		5.6 (7β,8β)			
		1.0 (7β,15)		0.8 (7β,15)			
8	1.80	7.2 (AB)	1.78	15.1 (AB)	5.30		13–1.6
	2.03		1.95	1.4			
10	5.35	1.6 (10,16)	2.53		3.92		(9) 2.52
11							4.81
13	3.66	12.1 (AB)	3.68	12.4 (AB)	3.77	12.5 (AB)	3.76
	3.79		3.73		3.83		3.89
14	1.04		1.02		1.06		0.92
15	0.90	0.8 (15,7β)	0.94	0.7 (15,7β)	0.99		1.25
16	1.67	1.6 (16,10)	1.34		1.75		1.04

achieved on a CSC-S nitrile column (28 × 0.94 cm) using a 6% 2-propanol/hexane mobile phase with a flow rate of 4 mL/min. ^{1}H and ^{13}C NMR spectra were determined in $CDCl_3$ with a Bruker WM-250 spectrometer. For the purpose of comparison, the ^{1}H NMR data of all the compounds isolated are presented in Table I. Structural characterization was facilitated with COSY-45 and NOE difference spectra.

X-ray Crystallography. A single crystal of IA was analyzed on an automated Picker four-circle diffractometer. Accurate cell parameters and intensity data were obtained at room temperature with use of graphite-monochromatized Cu Kα radiation. The data were corrected for the Lorentz effect and measured direct-beam polarization (Le Page et al., 1979) but not for absorption. The cell parameters were obtained by least-squares refinement of the setting angles of 64 reflections ($100 < 2, 120$) [λ(Cu Kα_1) = 1.54056 Å]. Other specific details are given in Table II.

Space group symbol $P2_12_12_1$ was uniquely defined from the systematic absences noted in the full data set. The structure of IA was solved by direct methods using MULTAN (Germain et al., 1971). All non-hydrogen atoms were refined anisotropically. The hydrogen atoms were located on the subsequent difference map and refined isotropically. Full-matrix least-squares refinements were carried out with weights based on counting statistics $(1/\sigma(F_o)^2)$. The residuals are quoted in Table II. The final difference Fourier map was featureless with a general background lower than ±0.14 e/A^3.

The absolute configuration determination was carried out by measuring the intensities of selected pairs of Friedel reflections and their symmetry-related reflections, which showed the highest difference between calculated (+) and (−) structure factors. All computations were performed with the NRCVAX system of programs (Gabe et al., 1985). Neutral-atom scattering curves were from standard sources (Birmingham, 1974).

Further details of the crystallographic analysis of IA, including the refined atomic coordinates, bond lengths and angles, and torsion angles for the non-hydrogen atoms of the structure and

Table II. Crystallographic Data for 3α,13-Dihydroxy-11-epiapotrichothec-9-ene

formula	$C_{15}H_{24}O_3$
fw	252.3
cryst syst	orthorhombic
space group	$P2_12_12_1$
a, Å	6.9401 (2)
b, Å	11.1071 (8)
c, Å	17.8608 (5)
V, Å^3	1376.8
Z	4
d_{calcd}, g/cm^3	1.218
cryst dimens, mm	0.1 × 0.2 × 0.4
radiation	Cu Kα_1 (1.54056 Å)
octant measd	hkl
max 2θ, deg	120
no. of unique reflcns	1212
no. of obsd reflcns ($I_{net}/\sigma^*(I_{net}) \geq 2.0$)	1062
av std fluctns, %	0.8
abs coeff, mm^{-1}	0.68
R_F	0.031
R_w	0.023
R_F (all reflcns)	0.038
R_w (all reflcns)	0.023
S (GOF)	2.276
sec ext coeff (×10^{-6} m)	0.37 (2)

structure factor amplitudes have been submitted as supplementary data.

MATERIALS AND METHODS

The crude extract from liquid cultures of *Fusarium roseum* ATCC 28114 (*F. graminearum*) was chromatographed on Florisil as previously described (Greenhalgh et al., 1984) to give fraction A. Removal of 3-acetyldeoxynivalenol (ADON) left an oil (4.08 g), which was purified by medium-pressure liquid chromatography on a LiChroprep silica gel column (2.5 cm × 1 m, 220 g). The column was eluted with (a) 10% ethyl acetate/hexane (1.3 L, fractions 1–69), (b) 20% ethyl acetate/hexane (1.3 L, fractions 70–142), (c) 30% ethyl acetate/hexane (5.8 L, fractions 143–460), and (d) ethyl acetate (3 L, fractions 461–650). The fractions were analyzed by TLC, and those that were similar combined to give a total of 50 fractions.

Isolation of IA and IB. Fraction 48 (43.9 mg) was an oil. GC/MS analysis on a DB-5 capillary column showed only a single sharp peak, but on a DX-4 column the sample was resolved into two peaks (GC: R_t = 7.32 and 7.47) in the ratio 1:10, respectively. Two compounds were isolated by HPLC. The major one (28.2 mg) was recrystallized from 2-propanol/hexane. IA: R_t 20.1 min; mp 139–141 °C; $[\alpha]_D$ −33.2° (EtOH); $C_{15}H_{24}O_3$, calcd m/z 252.173, found 252.173; MS, m/z 107 (100), 91 (70), 139 (64), 81 (62), 55 (56), 77 (50), 67 (47), 252 (m$^+$, 38), 97 (34), 224 (28), 124 (27), 237 (26), 119 (22). The minor one was an oil. IB: R_t 13.5 min; $C_{15}H_{24}O_3$, calcd m/z 252.173, found 252.174; MS, m/z 107 (100), 124 (66), 81 (64), 91 (64), 55 (46), 67 (40), 237 (28), 139 (26), 224 (25), 252 (m$^+$, 16). Both compounds showed only UV end absorption; however, their mass spectra and ^{1}H NMR (Table I) were similar, indicating them to be isomers.

Rearrangement of IA. This compound (5 mg) in ethyl acetate (0.5 mL) rearranged ($t_{1/2} \simeq$ 8.5 days) at room temperature and in the light to give five products. The major product (2.1 mg) isolated by preparative HPLC was recrystallized from acetone/hexane. II: GC, R_t 2.37 min; mp 90–92 °C; $[\alpha]_D$ −50.0° (EtOH); $C_{15}H_{22}O_3$, calcd m/z 250.159, found 250.157; MS, m/z 235 (100), 161 (75), 250 (m$^+$, 42), 124 (40), 82 (40), 55 (38), 109 (26), 67 (20), 95 (20). Two minor products were isolated as oils. III: 8%; GC, R_t 3.07 min; $C_{15}H_{22}O_4$, calcd m/z 266.152, found 266.152; MS, m/z 122 (100), 177 (34), 55 (27), 251 (91), 81 (20), 112 (20), 266 (m$^+$). IV: 22%; GC, R_t 3.27 min; $C_{15}H_{22}O_4$, calcd m/z 266.152, found 266.156; MS, m/z 122 (100), 55 (53), 95 (38), 177 (36), 81 (34), 109 (32), 67 (31), 193 (28), 137 (20), 251 (20), 266 (m$^+$). Compounds III and IV appeared to be isomers from their mass spectra and did not absorb in the UV (<200 nm). Both compounds gave monoacetyl derivatives (m$^+$, 308), indicating the presence of one hydroxyl group. Compound IV rearranged in methylene chloride to give isomeric compound V, which showed some end UV absorption: GC, R_t 3.48 min; $C_{15}H_{22}O_4$, calcd m/z 266.152, found 266.149; MS, m/z 159 (100), 233 (95), 122 (78), 248 (67), 55 (41), 109 (36), 95 (30), 217 (27), 79 (27), 67 (22), 91 (22), 175 (20), 266 (m$^+$).

Rearrangement of IB. The compound (2 mg), when dissolved in ethyl acetate (0.2 mL), slowly rearranged ($t_{1/2} \simeq$ 28 days) at room temperature and in the light to give 12 products. Only one product was isolated as an oil. VI: 21%; GC, R_t 4.51 min; $C_{15}H_{22}O_4$, calcd m/z 266.152, found 266.150; MS, m/z 111 (100), 95 (72), 55 (63), 123 (58), 200 (55), 71 (50), 253 (45), 81 (34), 139 (28), 266 (m$^+$). Another product present in smaller amounts (8%) was not isolated but had a similar mass spectrum, suggesting that it was an isomer. VII: GC, R_t 4.39 min; $C_{15}H_{22}O_4$, calcd m/z 266.152, found 266.146; MS, m/z 111 (100), 55 (95), 253 (91), 95 (90), 69 (68), 123 (66), 200 (65), 81 (54), 139 (42), 235 (22), 152 (21), 165 (20), 266 (m$^+$).

Epoxidation of IA. Compound IA (2 mg) was added to methylene chloride (2 mL) containing *m*-chloroperoxybenzoic acid (MCPBA; 2.5 mg) at room temperature. The reaction gave a single product, an oil. VIIIA: 91%; GC, R_t 4.45 min; $C_{15}H_{24}O_4$ (m − 15), calcd m/z 253.144, found 253.147; MS (IT), m/z 123 (100), 95 (40), 211 (34), 251 (26), 107 (20), 269 (m + 1).

Epoxidation of IB. The compound (3.5 mg) was oxidized as above with MCPBA (5.8 mg) and gave only one product, an oil. VIIIB: 93%; GC, R_t 4.38 min; $C_{15}H_{24}O_4$ (m − 15), calcd m/z 253.144, found 253.145; MS (IT), m/z 123 (100), 251 (90), 95 (72), 109 (67), 233 (46), 149 (28), 203 (22), 219 (21), 269 (m + 1).

Epoxidation of II. A 2-mg portion was oxidized as above with MCPBA (2.2 mg) and gave a single product (yield 83%), which had the same GC R_t (3.27 min) and mass spectrum (m + 1, 267) as IV.

RESULTS AND DISCUSSION

In an acidic medium, 12,13-epoxytrichothec-9-enes rearrange to form 10,13-cyclotrichothecanes or apotrichothec-9-enes (Grove, 1986). The latter products have a cis-fused A/B ring system and are substituted at the C-2 position. The dihydroxyapotrichothecenes (IA and IB) isolated from liquid cultures of *Fusarium* species have a trans-fused A/B ring system with a coupling constant of $J_{10,11}$ = 2.5 Hz as compared to $J_{10,11}$ = 5.2 Hz for the cis-fused system and also lack any substitution at C-2. These data indicate that IA and IB are in fact metabolites and do not stem from the rearrangement of trichothecenes.

GC/MS analysis of crude fungal extracts of *F. roseum* ATCC 28114 showed that the isomers IA and IB were present in the ratio 10:1. This ratio appears to be species dependent since it was 2:1 for *F culmorum* HLX 1503 and 3:1 for *F sporotrichioides* DAOM 165006, with IA (3α-OH isomer) predominating in all cases.

The X-ray crystallographic data of IA using graphite-monochromatized Cu Kα radiation provided the absolute configuration of the molecule, showing a half-chair apotrichothecene ring system with H-11 in the β-configuration and hydroxyl groups located at C-3(α) and C-13. An ORTEPII (Johnson, 1976) view of the molecule is shown in

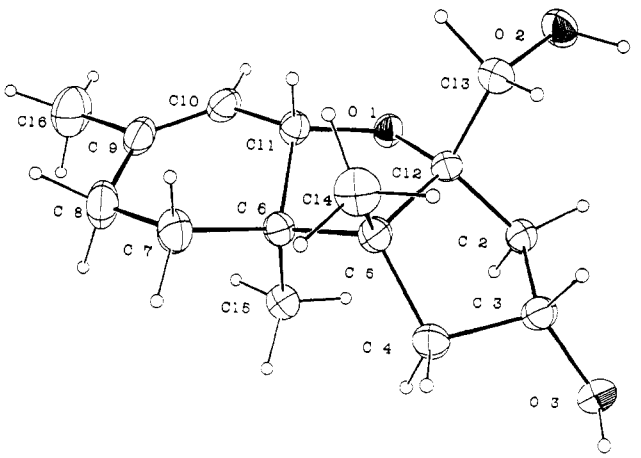

Figure 1. ORTEPII drawing of IA showing the ring-numbering scheme.

Figure 1. The NOE difference spectra of IA and IB indicated that they had similar conformations. Irradiation of H-14 in both IA and IB gave strong NOEs to H-11, H-13, and one H-4 showing that these protons also have the β-configuration. Irradiation of H-15 gave small NOEs for H-4α, H-2α, and H-3 only in the case of IB. The 3β-OH configuration in IB was shown by irradiation of H-3, which gave an NOE to H-15.

The EI/MS of IA and IB differed only in the intensity of the common ions, m/z 91, 124, 224, 237, and 252 (m^+). The ^{1}H NMR spectra of the epimers differ principally in the resonances of the CH_3-14 and CH_3-15 methyls, the C-13 AB system, and the H-2, H-3, and H-4 protons (Table I). Both isomers IA and IB gave monoacetyl (m^+ 294) and diacetyl (m^+ 336) derivatives, confirming the presence of two hydroxyl groups in each isomer.

The formation of IA and IB can be considered to occur by nonspecific oxidation, in view of the ratios isolated. Similarly, the isolation of the epimers of 8-hydroxysambucoin is another example of this occurring in *F. sporotrichioides* (Corley et al., 1987b).

Isomer IA in ethyl acetate rearranged more readily in light than IB. Preliminary experiments showed that the reaction is not influenced by the presence of acetic acid nor does it proceed in the dark. IA gave five products, of which only three were isolated, II (m^+ 250), which predominated, and the two isomers III and IV (m^+ 266). All three compounds gave monoacetyl derivatives (m^+ 292, 308, and 308, respectively), indicating the presence of an hydroxyl group in each molecule.

The ^{1}H NMR spectrum of II differed from that of IA in that the resonance assigned to H-11 (4.15 ppm) had disappeared. A COSY spectrum of II showed long-range coupling between H-10 (5.8 ppm) and CH_3-16 and H-8 and a single multiplet (4.55 ppm) coupled to two AB systems, indicating it to be the H-3 proton. In the ^{13}C NMR spectrum of IA, the C-11 chemical shift was 73 ppm, but in II where C-11 is quaternary, the resonance had moved downfield to 108 ppm. By analogy with the C-11 resonance of sambucinol (105 ppm), a ketal function was ascribed to the C-11 of II, which was assigned the structure 3α,11α-epoxy-13-hydroxyapotrichothec-9-ene. The NOE difference spectra of II showed an NOE between H-13 and H-14 and between H-15 and H-4 but not with H-3 as in IA in support of this structure.

The two other minor products, III and IV (m/z 266), obtained from IA showed no UV absorption (>200 nm), indicating the absence of the 9,10 double bond. The ^{1}H NMR spectrum of IV shows a sharp resonance (1.34 ppm, 3 H) which was assigned to the CH_3-16 protons. The shift of this resonance upfield, together with the absence of any $J_{16,10}$ and $J_{16,8}$ long-range coupling, corroborates the absence of the 9,10 double bond. The H-10 resonance (5.4 ppm) found in II was absent from the spectrum of IV, and a new resonance (2.80 ppm) was assigned to H-10. Decoupling experiments showed that ring C remained unchanged.

The elucidation of the structure of IV was derived from oxidation experiments with IA and II. Peroxidation of IA gave VIIIA (m^+ 268), which had no UV absorption. Its ^{1}H NMR spectrum showed a sharp resonance (1.37 ppm, 3 H), which was attributed to the CH_3-16 protons, and two singlets (3.09 and 3.46 ppm), corresponding to one proton each assigned to H-10 and H-11, respectively. The small $J_{10,11}$ coupling (<1 Hz) is consistent with a bond angle $\Phi_{10,11}$ = 105° resulting from a β-9,10-epoxide with ring A in a half-chair conformation. Corroboration of the β-9,10-configuration was obtained from the NOE difference spectra where irradiation of CH_3-15 protons enhanced the NOE of H-10 and CH_3-14 that of H-11. The epoxide derivative VIIIB from IB showed similar chemical shifts for the CH_3-16, H-10, and H-11 protons as in VIIIA, indicating that the epoxide also had the β-configuration.

m-Chloroperbenzoic acid oxidation of the macrocyclic (Jarvis et al., 1980) and type A trichothecenes (Kaneko et al., 1982) yielded β-9,10-epoxides. Similarly, the 9,10-epoxide moiety in the naturally occurring baccharinoids B9, 10, 17, and 20 all have a β-configuration (Jarvis et al., 1980). Typical $J_{10,11}$ values found for the β-9,10-epoxy protons are in the range 5–6 Hz, whereas those for the synthetic α-9,10-epoxy isomers are 2 Hz (Jarvis et al., 1984). Recently, Burrows and Szafraniec (1987) obtained an epoxy derivative from the hypochlorite oxidation of deoxynivalenol and assigned it an α-configuration on the basis of its J value (<1 Hz). These data do not invalidate our assignment in view of the different configuration at H-11 for III and IV and the NOE difference spectra of VIIIA.

Peroxidation of II gave only one product with mass and NMR spectra identical with that of IV. Since the H-10 and H-11 resonances in VIII and H-10 in IV are all singlets, with similar chemical shifts and $J_{10,11}$ values, IV must also have a β-9,10-epoxy moiety. Thus, the structure of IV is 3α,11α;9β,10-diepoxy-13-hydroxyapotrichothecane. In methylene chloride a sample of IV readily rearranged to V (m^+ 266). Its ^{1}H NMR showed a chemical shift for the CH_3-16 resonance of 1.72 ppm, indicating its location on a double bond but not 9,10 as in II. By analogy with the acid-catalyzed rearrangement of the epoxy moiety of 8-cholesteryl acetate to an unsaturated hydroxy derivative (Windaus et al., 1938), V was assigned the structure of 3α,11α-epoxy-10β-hydroxyapotrichothec-8-ene.

In ethyl acetate, IB gave a number of products of which one was isolated, VI (m^+ 266). Its ^{1}H NMR spectrum showed the CH_3-16 resonance as a doublet (1.04 ppm), indicating a proton at the C-9 position. A singlet at 4.81 ppm was assigned to H-11 and a triplet at 4.4 ppm to H-3. This triplet was coupled with two AB systems (2.49, 2.24 and 1.4, 2.8 ppm) corresponding to H-2 and H-4, indicating that ring C was unchanged. The chemical shifts of H-3 (4.4 ppm) and the AB system of H-13 imply that neither hydroxy moiety was acetylated. The mass and NMR spectra are consistent with the interpretation that VI is the product of the addition of oxygen to the 9,10 double bond, eventually resulting in a carbonyl at C-10. Another compound, VII, which had a similar MS, is thought to represent the other C-9 epimer. The products from the rearrangements of IA and IB are shown in Figure 2. It has been postulated that IA is an end metabolite (Zamir

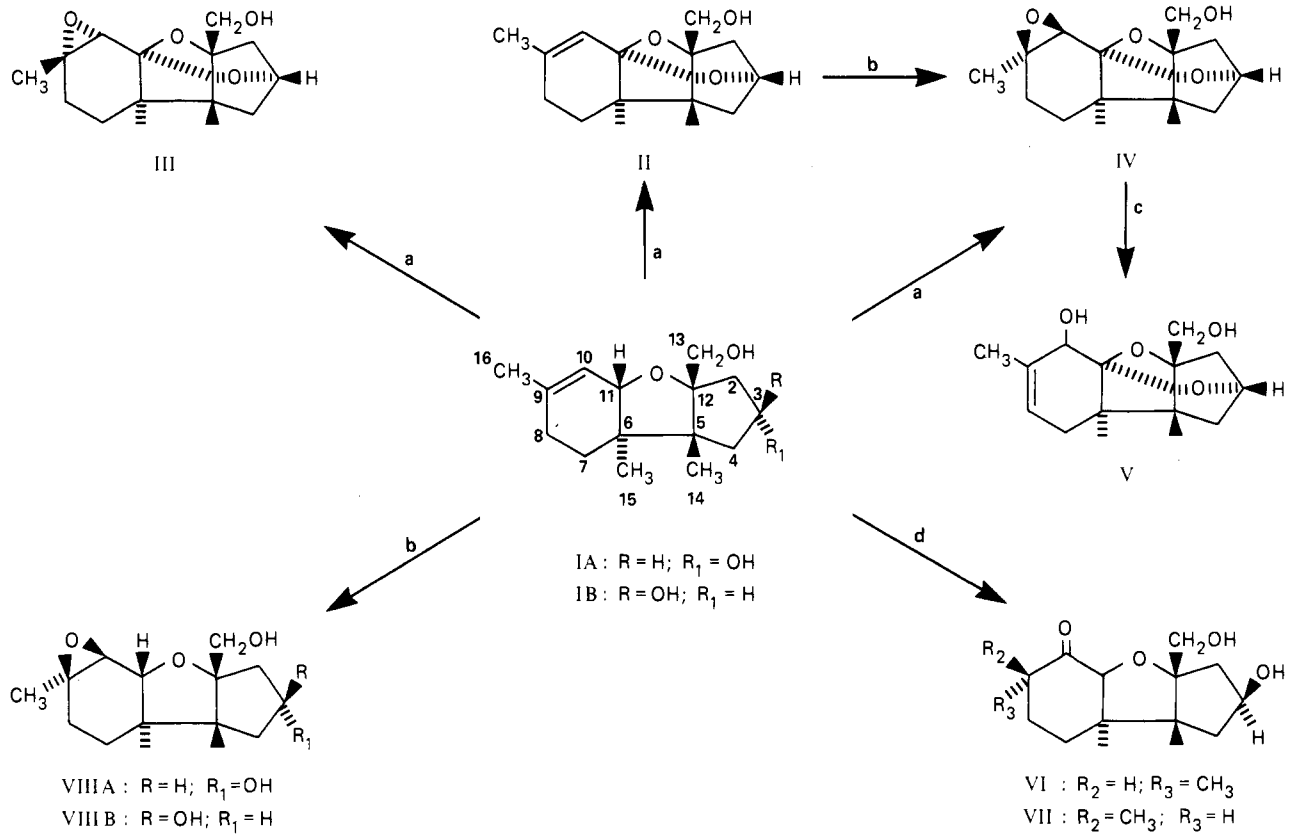

Figure 2. Transformations of the 3,13-dihydroxy-11-epiapotrichothecenes: (a) IA/ethyl acetate/light, (b) MCPBA, (c) methylene chloride, (d) IB/ethyl acetate/light.

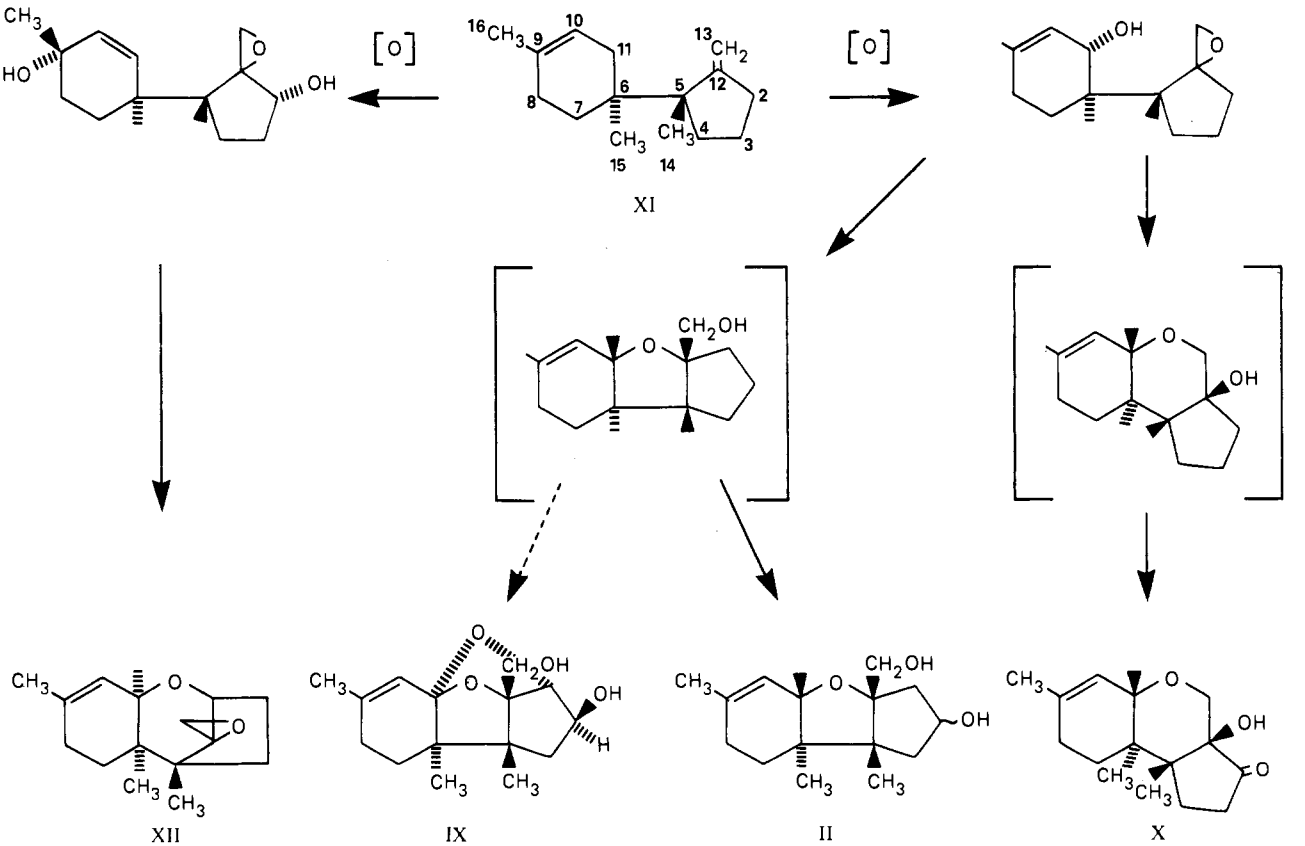

Figure 3. Proposed biooxidation of trichodiene showing the formation of the major metabolites, the trichothecenes, and the minor metabolites 11-epiapotrichothecene (II), sambucinol (IX), and sambucoin (X).

et al., 1987), but the detection of II and oxidation products of IA in the crude fungal extracts of various *Fusarium* species indicates that this is not correct.

The biosynthesis of trichothecenes has been extensively studied (Bamburg, 1976; Tamm, 1977; Cane et al., 1985). *trans,trans*-Farnesyl pyrophosphate cyclizes to the diabolyl

cation, which in turn undergoes cyclization with the simultaneous migration of two methyl groups and hydride ion shifts to yield trichodiene (Cane et al., 1985). Trichodiene must undergo specific oxidation to enable formation of the C-11, C-2 oxygen bridge in trichothecene (XII). Two potential intermediates have been isolated: trichodiol (Nozoe and Machida, 1972) and more recently trichotriol (Corley et al., 1987a). In vitro, the latter compound cyclized to 3-hydroxytrichothecene in $CDCl_3$, with a trace of water present. Both trichodiol and trichotriol have an α-OH at the C-2 position, which is required to produce the cis-fused A/B ring systems of trichothecenes on cyclization, with H-11 having and α-configuration. It is of interest that all the 3-oxygenated trichothecenes have the 3α-configuration, implying some specific biological oxidation.

The hydroxy-11-epiapotrichothecenes IA and IB have trans-fused A/B rings, which implies that a different oxidized derivative of trichodiene is involved in the cyclization. Sambucoin (X) has stereochemistry at C-6 and C-11 similar to that of the hydroxy-11-epiapotrichothecenes, and Mohr et al. (1984) proposed an intermediate that involved allylic oxidation of trichodiene at C-11 to give an α-OH, which could form an oxygen bridge to C-13 and gave the correct stereochemistry for H-11. The apotrichothecenes could also be formed from this intermediate with an oxygen bridge to C-12 (ApSimon et al., 1986). The isolation of metabolites such as FS-1 (Corley et al., 1986) from cultures of *F. sporotrichioides* indicates that oxidation of C-11 in trichodiene to give an α-OH can occur. Mohr et al. (1984) also proposed further oxidation of the C-11 hydroxyl to a ketone could account for the ketal moiety in sambucinol. The proposed biosynthetic pathways are shown in Figure 3.

The suggestion that sambucinol (IX) could arise from trichothecene (XII), however, is known to be invalid since [^{13}C]acetate studies with *F. culmorum* showed that after 72 h the level of IX remained constant, while that of 3-ADON continued to increase. The level of incorporation of the ^{13}C label was also found to be higher in IX (Miller and Blackwell, 1986). A later study has shown that both 3-ADON and sambucinol are mevalonate derived but did not interconvert (Zamir and Devor, 1987).

The fact that the IA epimer cyclizes to form a ketal at C-11 suggests an alternative pathway for the formation of sambucinol. In this case it would involve 2,3-dihydroxy-11-epiapotrichothecene as a precursor.

In summary, the trichothecene-producing *Fusarium* species also form several minor metabolites, e.g. 11-epiapotrichothecenes, sambucinol, and sambucoins, in addition to the trichothecenes. Formation of sambucinol and the epimers of 3-hydroxy-11-epiapotrichothecene is common to the *Fusarium* species, but the ratio of the latter compounds is species dependent. By X-ray crystallography the absolute configuration of 3α,13-dihydroxy-11-epiapotrichothec-9-ene showed a trans-fused ring system. This indicates that they arise from a different oxidized derivative of the common precursor trichodiene than that for the trichothecenes. Cyclization of the 3α-OH isomer of hydroxy-11-epiapotrichothecene to form a compound having a ketal moiety suggests an alternative mechanism for the formation of sambucinol.

ACKNOWLEDGMENT

We thank Dr. A. Taylor and Dr. J. David Miller for providing the crude fungal extract, P. Lafontaine for the mass spectral data, and G. Montgomery and W. Adams for technical assistance in the isolation of the metabolites.

Registry No. IA, 104148-45-0; IB, 120052-56-4; II, 119971-19-6; III, 119971-20-9; IV, 120052-55-3; V, 119971-21-0; VI, 119996-92-8; VII, 120056-09-9; VIIIA, 119971-22-1; VIIIB, 120142-36-1.

Supplementary Material Available: Listings of atomic parameters x, y, z and B_{ISO}; bond lengths, angles, and torsion angles for the non-hydrogen atoms; and temperature factors (U_{ij} on $U \times 100$); and ORTEPII stereodrawing of IA (4 pages); calculated observed and unobserved structure factor amplitudes (9 pages). Ordering information is given on any current masthead page.

LITERATURE CITED

ApSimon, J. W.; Blackwell, B. A.; Greenhalgh, R.; Meier, R.-M.; Miller, J. D.; Pare, J. R. J.; Taylor, A. Secondary metabolites produced by some Fusarium species. In *Proceedings of the Sixth IUPAC International Symposium on Mycotoxins and Phycotoxins*; Steyn, P. S., Ed.; Elsevier Press: Amsterdam, Holland, 1986; pp 125–136.

Bamburg, J. R. Chemical and Biochemical Studies of the Trichothecene Mycotoxins. In *Mycotoxins and Other Fungal Related Food Problems*; Rodricks, J. V., Ed.; Advances in Chemistry 149; American Chemical Society: Washington, DC, 1976; pp 144–162.

Birmingham, P. *International Tables for X-Ray Crystallography*; Kluwer Academic: Dordrecht, Denmark, 1974; Vol. 4.

Burrows, E. P.; Szafraniec, L. L. Hypochlorite-Promoted Transformation of Trichothecenes, 3-Deoxynivalenol. *J. Nat. Prod.* **1987**, *50*, 1108–1112.

Cane, D. E.; Ha, H.-J.; Pargellis, C.; Waldmeier, F.; Swanson, S.; Murthy, P. P. N. Trichodiene Biosynthesis and the stereochemistry of the Enzymatic Cyclization of Farnesyl Pyrophosphate. *Bioorg. Chem.* **1985**, *13*, 246–265.

Corley, D. G.; Rottinghaus, G. E.; Tracy, J. K.; Tempesta, M. S. New Trichothecene Mycotoxins of *Fusarium sporotrichioides* (MC-72083). *Tetrahedron Lett.* **1986**, *27*, 4133–4136.

Corley, D. G.; Rottinghaus, G. E.; Tempesta, M. S. Toxic Trichothecenes from *Fusarium sporotrichioides* (MC-72083). *J. Org. Chem.* **1987a**, *52*, 4405–4408.

Corley, D. G.; Rottinghaus, G. E.; Tempesta, M. S. Secondary Metabolites from *Fusarium*. Two New Modified Trichothecenes from *Fusarium sporotrichioides* (MC-72083). *J. Nat. Prod.* **1987b**, *50*, 897–902.

Foster, B. C.; Trenholm, H. L.; Friend, D. W.; Thompson, B. K.; Hartin, K. E. Evaluation of Different Sources of Deoxynivalenol (Vomitoxin) Fed to Swine. *Can. J. Anim. Sci.* **1986**, *66*, 1149–1154.

Gabe, E. J.; Lee, F. L.; Le Page, Y. *Crystallographic Computing 3*; Sheldrick, G., Kruger, C., Gobbard, R., Eds.; Clarendon Press: Oxford, U.K., 1985; pp 167–174.

Germain, G.; Main, P.; Woolfson, M. M. The application of phase relationships to complex structures III, The optimum use of phase relationships. *Acta Crystallogr., Sect. A* **1971**, *27*, 368–374.

Greenhalgh, R.; Meier, R.-M.; Blackwell, B. A.; Miller, J. D.; Taylor, A.; ApSimon, J. W. Minor Metabolites of *Fusarium roseum* (ATCC 28114). *J. Agric. Food Chem.* **1984**, *32*, 1261–1264.

Greenhalgh, R.; Blackwell, B. A.; Pare, J. R. J.; Miller, J. D.; Levandier, D.; Meier, R.-M.; Taylor, A.; ApSimon, J. W. Isolation and characterization by Mass Spectrometry and NMR spectroscopy of secondary metabolites of some *Fusarium* species. In *Mycotoxins and Phycotoxins*; Steyn, P. S., Vleggaar, R., Eds.; Elsevier Science: Amsterdam, The Netherlands, 1986; pp 137–152.

Grove, J. F. Phytotoxic Compounds Produced by *Fusarium equiseti*. Part 8. Acid Catalysed Rearrangement of 12,13-Epoxytrichothec-9-enes. *J. Chem. Soc. Perkin Trans. 1* **1986**, 647–654.

Grove, J. F.; Mortimer, P. H. Cytotoxicity of some transformation products of diacetoxyscirpenol. *Biochem. Pharmacol.* **1969**, *18* 1473–1478.

Jarvis, B. B.; Stahly, G. P.; Pavanasasivam, G.; Mazzola, E. P. Antileukemic Compounds Derived from Chemical Modification of Macrocyclic Trichothecenes 1. Derivatives of Verrucarin A. *J. Med. Chem.* **1980**, *23*, 1054–1058.

Jarvis, B. B.; Midiwo, J. O.; Mazzola, E. P. Antileukemic Compounds Derived by Chemical Modification of Macrocyclic

Trichothecenes. 2. Derivatives of Roridins A and H and Verrucarins A and J. *J. Med. Chem.* **1984**, *27*, 239–244.
Johnson, C. K. *ORTEPII*, Report ORNL-5138; Oak Ridge National Laboratory: Oak Ridge, TN, 1976.
Kaneko, T.; Schmitz, H.; Essery, J. M.; Rose, W.; Howell, H. G.; O'Herron, F. A.; Nachfolger, S.; Jutalen, J.; Bradner, W. T.; Partyka, R. A.; Doyle, T. W.; Davies, J.; Cundliffe, E. Structural Modifications of Anguidin and Antitumor Activities of its Analogues. *J. Med. Chem.* **1982**, *25*, 579–589.
Lauren, D. R.; Ashley, A.; Blackwell, B. A.; Greenhalgh, R.; Miller, J. D.; Neish, G. A. Trichothecenes Produced by *Fusarium crookwellense* DAOM 193511. *J. Agric. Food Chem.* **1987**, *25*, 884–889.
Le Page, Y.; Gabe, E. J.; Calvert, L. D. X-ray beam polarization measurements. *J. Appl. Crystallogr.* **1979**, *12*, 25–34.
Miller, J. D.; Blackwell, B. A. Biosynthesis of 3-acetyldeoxynivalenol and other metabolites by *Fusarium culmorum* HLX1503 in a stirred jar fermentor. *Can. J. Bot.* **1986**, *64*, 1–5.
Mohr, P.; Tamm, Ch.; Zurcher, W.; Zehnder, M. Sambucinol and Sambucoin, Two New Metabolites of *Fusarium sambucinum* Possessing Modified Trichothecenes Structures. *Helv. Chim. Acta* **1984**, *67*, 406–412.
Nozoe, S.; Machida, Y. The Structures of Trichodiol and Trichodiene. *Tetrahedron* **1972**, *28*, 5101–5111.
Savard, M. E.; Blackwell, B. A.; Greenhalgh, R. A. ^{1}H Nuclear Magnetic Resonance Study of Derivatives of 3-Hydroxy-12,13-epoxytrichothec-9-enes. *Can. J. Chem.* **1987**, *65*, 2254–2262.
Tamm, C. Chemistry and Biosynthesis of Trichothecenes. In *Mycotoxins in Human and Animal Health*; Rodricks, J. V., Hesseltine, C. W., Mehlman, M. A., Eds.; Pathotox Publishers: Park Forest South, IL, 1977; pp 209–228.
Ueno, Y. Trichothecenes, Chemical, Biological and Toxicological Aspects. In *Developments in Food Science-4*; Elsevier Scientific: New York, 1983; pp 135–149.
Wang, Y. Z.; Miller, J. D. Effects of *Fusarium graminearum* Metabolites on Wheat Tissue in Relation to Fusarium Head Blight Resistance. *J. Phytopathol.* **1988**, *122*, 118–125.
Windaus, A.; Linsert, O.; Eckhardt, H. J. *Ann.* **1938**, *534*, 22–31.
Zamir, L. O.; Devor, K. A. Kinetic Pulse Labelling Study of *Fusarium culmorum*. *J. Biol. Chem.* **1987**, *262*, 15348–15353.
Zamir, L. O.; Devor, K. A.; Nadeau, Y.; Sauriol, F. Structure Determination and Biosynthesis of a Novel Metabolite of *Fusarium culmorum*, Apotrichidiol. *J. Biol. Chem.* **1987**, *262*, 15354–15458.

Received for review June 3, 1988. Accepted October 17, 1988.

Simultaneous Identification and Determination of Tetramine in Marine Snails by Proton Nuclear Magnetic Resonance Spectroscopy

Uffe Anthoni, Carsten Christophersen,* and Per Halfdan Nielsen

Tetramine (tetramethylammonium ion), the toxin from marine gastropod molluscs of the genus *Neptunea*, was determined, qualitatively as well as quantitatively, by ^{1}H NMR spectroscopy. The method was compared to the currently available methods of determination and found superior due to the inherent identification of the molecular species determined.

Poisonous substances in the commericially available carnivorous gastropod molluscs of the genus *Neptunea* recurringly cause serious problems for consumers (Asano, 1952; Asano and Ito, 1959, 1960; Emmelin and Fänge, 1958; Fänge, 1957, 1958, 1960; Anthoni et al., 1989b). Within 30 min after ingestion, severe headache, dizziness, seasickness, nausea, vomiting, and visual disturbances may occur. Current reviews (Baslow, 1969; Hashimoto, 1976; Anthoni et al., 1989a) conclude the active principle to be the tetramethylammonium ion (tetramine) occurring mainly in the salivary glands. However, other substances present in small amounts may have a synergistic action (Asano and Ito, 1959).

Several analytical methods have been proposed to characterize tetramine and to quantitate the content in *Neptunea* species, including thin-layer chromatography (Asano and Ito, 1959), ion chromatography (Saitoh et al., 1983), and bioassay techniques based upon dose–lethal time curves for mice (Saitoh et al., 1983; Kungsuwan et al., 1986) or killifish (Kungsuwan et al., 1986). Estimated amounts in the salivary glands of *Neptunea arthritica* vary from 0.2 to 7.5 mg of tetramine/g of gland. In view of the importance of determining the threshold concentration of tetramine giving rise to poisonings, the agreement in the current available methods leaves much to be desired.

This paper describes a simple, direct method for rapid identification and semiquantitative determination of tetramine in extracts from *Neptunea antiqua* by ^{1}H NMR spectroscopy.

EXPERIMENTAL SECTION

Sample Preparation. The salivary glands (60 g) were carefully excised from *N. antiqua* (30 snails, 1 kg without shells) and freeze-dried to constant weight (19.5 g). Repeated extraction with methanol at room temperature, filtration, and evaporation of the solvent gave a semicrystalline solid (2.7 g). Partitioning between ether and water served to produce a defatted aqueous extract, which after lyophilization left colorless crystals (2.6 g). These crystals were used for the NMR determinations.

The residual animal tissue (940 g) was freeze-dried to constant weight (240 g). Successive extractions with ethyl acetate (three times) and methanol (three times) gave a methanol extract, which after evaporation left a solid. Treatment as described above left 13.8 g of defatted material suitable for NMR analysis.

Measurements. The spectra were recorded at ambient temperature on a JEOL FX-90Q Fourier transform NMR spectrometer or on a Bruker WM-250 FT spectrometer. The experiment was performed on a Bruker AM-500 instrument to determine the effects of the superior resolving power of the higher field. The

Marine Chemistry Section, The H. C. Ørsted Institute, University of Copenhagen, Universitetsparken 5, DK-2100 Copenhagen Ø, Denmark.

0021-8561/89/1437-0705$01.50/0