The extent of chlordiazepoxide absorption was estimated by comparing the area subtended by the chlordiazepoxide plasma levels found after oral administration to the corresponding area seen after intravenous drug administration. This estimate indicated that 81% of the orally administered chlordiazepoxide was absorbed by Subject 6. In addition, it suggests that therapeutic doses of chlordiazepoxide are well absorbed in man.

Plasma levels of desmethylchlordiazepoxide were first measurable 5 hr. after intravenous chlordiazepoxide was given (Fig. 2). At this time, 0.26 mcg./ml. was seen; little decline was evident for the next 19 hr. Measurable levels of demoxepam were found only after 48 and 72 hr. and were close to the sensitivity limit of 0.14 mcg./ml.

Since the oral administration of ^{14}C-chlordiazepoxide to two human subjects resulted in the urinary excretion of only negligible amounts of intact drug (2), it is evident that chlordiazepoxide in man is eliminated almost entirely by biotransformation. The appearance of plasma desmethylchlordiazepoxide and the absence of plasma demoxepam in the present study support the contention (3) that desmethylchlordiazepoxide is an intermediate in the biotransformation of chlordiazepoxide to demoxepam. This pathway was recently demonstrated in the dog by Kaplan *et al.* (7). Their pharmacokinetic evaluation of chlordiazepoxide disposition revealed that the drug was eliminated by essentially quantitative biotransformation to desmethylchlordiazepoxide, which was then itself extensively biotransformed with approximately one-half going to demoxepam.

The accumulation of plasma levels of desmethylchlordiazepoxide and demoxepam in a human subject administered chlordiazepoxide chronically was previously reported (3). This finding is also supported by those presented here which showed that chlordiazepoxide administration led to persistent plasma levels of desmethylchlordiazepoxide and that demoxepam was eliminated at a considerably slower rate than was chlordiazepoxide. Since demoxepam was reported (10) to have antianxiety activity in man, this metabolite may play a significant role in contributing to the activity of chlordiazepoxide.

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Comparison of Pigments in Carpophores and Saprophytic Cultures of *Paxillus panuoides* and *Paxillus atrotomentosus*

M. C. GAYLORD and L. R. BRADY

Abstract □ Chromatographic studies, using several solvent systems with thin-layer polyamide and silica gel G plates, indicated that *Paxillus panuoides* (Fr.) Fr. and *P. atrotomentosus* (Batsch) Fr. contain several common pigments in both carpophores and surface cultures. Atromentin was isolated and identified from the carpophores of *P. panuoides*, and atromentic and xerocomic acids were recovered and identified from the cultures of this species. These pigments were reported previously from the corresponding growth forms of *P. atrotomentosus*. Identification of the pigments was based on comparisons of chromatographic properties and spectral data (IR, UV, and high-resolution mass spectra) with authentic materials. Preliminary interpretation of the complex mass spectrum of atromentin suggested the involvement of at least three basic fragmentation pathways. The pKa values and fluorescent spectra (before and after exposure to radiant energy) of atromentic, pulvinic, and xerocomic acids were determined, and the response of xerocomic acid to thermal energy during sublimation was clarified.

Keyphrases □ *Paxillus atrotomentosus*, *P. panuoides*—pigment comparison □ Atromentin, *Paxillus* carpophores—isolation, identification □ Atromentic acid, *Paxillus* cultures—isolation, identification □ Xerocomic acid, *Paxillus* cultures—isolation, identification □ Tetronic acids—*P. atrotomentosus*, *P. panuoides* □ TLC—isolation, tetronic acids

Paxillus panuoides (Fr.) Fr. and *P. atrotomentosus* (Batsch) Fr. are lignicolous mushrooms, frequently found around old conifer stumps (1). In addition, the smaller, sessile *P. panuoides* may be found on sawdust piles or on timbers of mines, cellars, and similar places where it is a active wood destroyer. In the latter areas, carpophore formation can occur in the semidarkness after the soft yellow mycelium causes extensive decay and a vivid yellow discoloration of the wood (2). Similarly diffusible yellow pigments, which accumulate in surface cultures of *P. atrotomentosus*, were shown to include atromentic and xerocomic acids (3), diphenyl-substituted tetronic acid derivatives. Knowledge of pigments in carpophores of *P. atrotomentosus* and *P. panuoides* is restricted to the isolation of atromentin from the former species (4).

The desirability of comparative studies of *P. atrotomentosus* and *P. panuoides* was suggested by the distinctive metabolic capabilities noted previously for carpophores and vegetative mycelium of *P. atrotomen-*

Table I—Pigments Detected by TLC Examination of Extracts of Carpophores and Culture Mixtures of *P. atrotomentosus* and *P. panuoides*

Pigment	*P. atrotomentosus*				*P. panuoides*			
	Carpophore		Culture		Carpophore		Culture	
	A[a]	B	A	C	A	B	A	C
Atromentin	+	+	−	−	+	+	−	−
Atromentic acid	−	−	+	+	−	−	+	+
Thelephoric acid	+	+	−	−	−	−	−	−
Xerocomic acid	−	−	+	+	−	−	+	+
Unidentified purple pigment	R_f 0.10[b]	R_f 0.71	−	−	R_f 0.10[b]	R_f 0.71	−	−
Unidentified red pigment	−	−	R_f 0.25	R_f 0.37	−	−	R_f 0.25	R_f 0.37

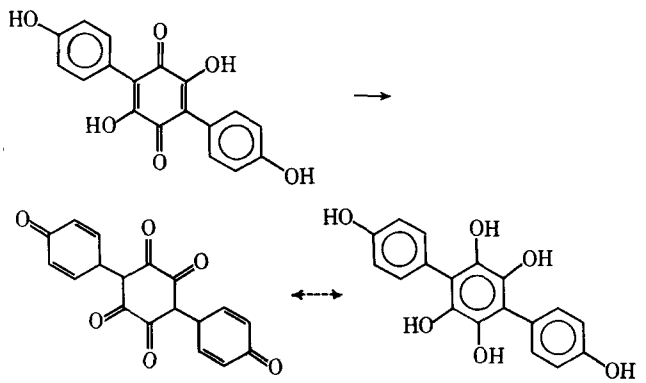
[a] System A: silica gel G thin layer, ether saturated with concentrated HCl; System B: polyamide thin layer, methanol–chloroform–H_2O–glacial acetic acid (54:36:6:1); System C: silica gel G thin layer, methyl ethyl ketone–H_2O–formic acid (250:25:1). [b] Evanescent.

tosus and the common lignicolous habitat and other gross properties shared by the two species. Terphenylquinone and tetronic acid derivatives, presumably metabolically related fungal constituents, were selected for the initial comparative studies to make the best utilization of existing knowledge. Studies were also undertaken to determine various properties of selected tetronic acids and to develop methods for their manipulation. The latter studies focused on the properties and manipulations that could have application in subsequent biologic studies and included a quantitative spectrophotometric procedure, pKa values, and UV and fluorescent spectra of the compounds at different stages of ionic dissociation.

EXPERIMENTAL

Source of Carpophores and Cultures—The carpophores of *P. panuoides* used were collected near McCleary, Washington; the fruiting bodies of *P. atrotomentosus* were obtained from Bainbridge Island, Washington. Both collections[1] were made in the autumn of 1968. The material was dried at 48° in a forced-air oven for 72 hr. The cultures[2] of *P. panuoides* and *P. atrotomentosus* were maintained routinely on cherry and 2% malt extract agar media.

Media and Cultural Conditions—Two media were used for surface cultivation of the *Paxillus* species. The cherry medium was prepared as previously described (3), and the 2% malt extract was acidified with HCl to pH 3.2 (2). Roux bottles (500 ml.), containing 75 ml. of the appropriate nutrient medium, were inoculated from a homogenate prepared from 14-day-old agar slant cultures of either *P. atrotomentosus* or *P. panuoides*. Cherry medium was inoculated with mycelium grown on cherry agar slants; growth from malt agar slants was used to inoculate the acidified malt extract medium.



Scheme I—Presumed heat and/or electron-bombardment-induced oxidation–reductions in the inlet system

The cultures were incubated at 20° for 50–60 days. Since deterioration of cultures was recognized as a potential problem in cultures grown in continuous or intense light, the cultures were incubated routinely in the dark. The aerial hyphae of *P. panuoides*, which had developed on both nutrient media at the time of harvest, were much flatter and thicker than the fluffy, droplet-covered aerial hyphae noted in the cultures of *P. atrotomentosus*. In all cases, the nutrient broth was yellow at this stage of development.

Chromatographic Studies—Several TLC procedures, previously used for separation and chromatographic monitoring of fungal terphenylquinones and pulvinic acid derivatives (3, 5), were selected for the initial evaluation of the extracts. Diethyl ether was used to extract the pigments from defatted carpophores, absolute methanol was employed with the freeze-dried culture mixtures, and authentic reference materials were used for chromatographic comparison. Table I summarizes the significant observations revealed by chromatographic examination of extracts of the carpophores and cultures of the two *Paxillus* species.

No chromatographic indication was observed for the presence of atromentin in extracts of the culture mixtures or for the occurrence of tetronic acids in the carpophores. The TLC system, utilizing silica gel G and HCl-saturated ether, was the best all-purpose system for the pigments in the extracts; it was used (preparative adsorbent layer) for separation of atromentic and xerocomic acids preparatory to quantitative determinations.

Extraction and Isolation of Atromentin—Samples (100 g.) of the dried mushrooms were reduced to a 40-mesh powder in a mill (Wiley). The powders were defatted with petroleum ether using a soxhlet apparatus. This procedure was followed by exhaustive extraction with diethyl ether, and these extracts were concentrated to dryness under reduced pressure at 35°. The samples of *P. atrotomentosus* and *P. panuoides* yielded 5.1 and 4.9 g., respectively, of diethyl ether-soluble extractives. A pigment with chromatographic properties corresponding to those of atromentin was obtained from the extracts by fractional crystallization using methanol and dioxane. The material was recrystallized three times from methanol–dioxane, and the respective yields of chromatographically pure pigment from the two species were 2.0 and 1.2 g.

Identification of Atromentin—The IR, UV, and mass spectra[3] of the pigment samples from both species were identical. The UV spectra ($\lambda_{max.}^{ethanol}$ 360 and 278 nm., $\lambda_{max.}^{dioxane}$ 385 and 268 nm., and $\lambda_{max.}^{pyridine}$ 550 and 360 nm.) agreed with those of authentic atromentin. IR spectra (KBr pellets) of the isolated samples and reference atromentin were indistinguishable, with absorption peaks at 3340, 2870, 1650, 1600, 1525, 1435, 1310, 1235, 1180, 1000, and 835 $cm.^{-1}$. Melting points of all atromentin samples were >300°.

High-resolution mass spectra of known atromentin and the samples isolated from the two *Paxillus* species exhibited base and parent ion peaks at *m/e* 324.0630 (calc. $C_{18}H_{12}O_6$, 324.0633). The mass spectrum of atromentin was observed to be complex; the fragments

[1] Identification was provided by Dr. D. E. Stuntz, Department of Botany, University of Washington, Seattle, Wash.

[2] Centraalbureau voor Schimmelcultures, Baarn, The Netherlands.

[3] IR and UV spectra were obtained with a Beckman IR spectrophotometer, model IR5A, and a Beckman UV spectrophotometer, model DB, Beckman Instruments, Inc., Fullerton, Calif. Mass spectra were determined with a Picker-AEI MS9 mass spectrometer, Picker Nuclear Division, White Plains, N. Y.; the mass spectrometer was used in conjunction with a Programmed Data Processor, model DPD-12, Digital Equipment Corp., Maynard, Mass., for studies of the fragmentation of atromentin.

Table II—Major Peaks in the High-Resolution Spectrum of Atromentin

Observed *m/e*	Calculated *m/e*	Formula	Relative Intensity, %
326.0790	326.0789	$C_{18}H_{14}O_6$	27
325.0710	325.0711	$C_{18}H_{13}O_6$	76
324.0630	324.0633	$C_{18}H_{12}O_6$	100
323.0470	323.0545	$C_{18}H_{11}O_6$	19
322.0430	322.0477	$C_{18}H_{10}O_6$	10
307.0590	307.0606	$C_{18}H_{11}O_5$	15
306.0540	306.0528	$C_{18}H_{10}O_5$	14
297.0730	297.0762	$C_{17}H_{13}O_5$	27
296.0700	296.0684	$C_{17}H_{12}O_5$	43
295.0610	295.0606	$C_{17}H_{11}O_5$	21
279.0630	279.0657	$C_{17}H_{11}O_4$	17
278.0540	278.0578	$C_{17}H_{10}O_4$	15
268.0710	268.0735	$C_{16}H_{12}O_4$	12
267.0640	267.0657	$C_{16}H_{11}O_4$	13
251.0660	251.0708	$C_{16}H_{11}O_3$	13
250.0590	250.0630	$C_{16}H_{10}O_3$	16
240.0730	240.0786	$C_{15}H_{12}O_3$	14
239.0690	239.0708	$C_{15}H_{11}O_3$	27
230.0190	230.0214	$C_{12}H_6O_5$	13
224.0800	224.0837	$C_{15}H_{12}O_3$	10
223.0770	223.0758	$C_{15}H_{11}O_2$	34
222.0680	222.0680	$C_{15}H_{10}O_2$	17
221.0620	221.0602	$C_{15}H_9O_2$	18
211.0760	211.0758	$C_{14}H_{11}O_2$	12
210.0670	210.0682	$C_{14}H_{10}O_2$	11
202.0260	202.0054	$C_{11}H_6O_4$	13
194.0720	194.0731	$C_{14}H_{10}O$	11
190.0210	190.0418	$C_{10}H_6O_4$	16
189.0180	189.0340	$C_{10}H_5O_4$	41
174.0310	174.0315	$C_{10}H_6O_3$	15
163.0380	163.0394	$C_9H_7O_3$	21
162.0290	162.0315	$C_9H_6O_3$	25
161.0330	161.0338	$C_9H_5O_3$	27
147.0080	147.0151	$C_8H_3O_3$	17
146.0090	146.0073	$C_8H_2O_3$	21
145.0000	144.9995	C_8HO_3	38
139.0330	139.0394	$C_7H_7O_3$	11
135.0450	135.0444	$C_8H_7O_2$	29
134.0380	134.0366	$C_8H_6O_2$	43
133.0290	133.0288	$C_8H_5O_2$	28
125.0320	125.0237	$C_6H_5O_3$	14
121.0280	121.0288	$C_7H_5O_2$	27
118.0400	118.0417	C_8H_6O	21
117.0320	117.0439	C_8H_5O	10
107.0480	107.0495	C_7H_7O	31
106.0410	106.0417	C_7H_6O	26
105.0330	105.0339	C_7H_5O	36
97.0361	97.0388	$C_5H_5O_2$	13
94.0408	94.0417	C_6H_6O	18
89.0424	89.0390	C_7H_5	15
82.0360	82.0317	C_5H_6O	10
78.0510	78.0468	C_6H_6	17
77.0432	77.0390	C_6H_5	24
76.0337	76.0372	C_6H_4	12

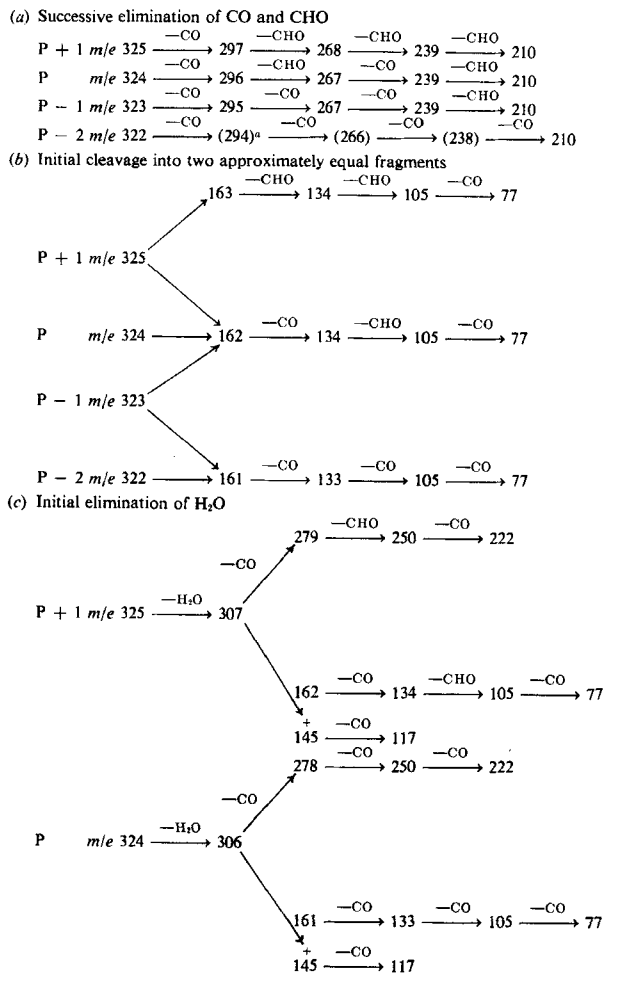
with a relative intensity >10% of base are listed in Table II. Possible fragmentation patterns are best visualized by assuming the initial reactions, when the sample is placed in the source at the high temperature (240°) required to obtain an adequate vapor pressure, involve heat and/or electron-bombardment-induced oxidation–reductions to give a series of compounds (Scheme I). Comparatively strong P + 1 and P + 2 ion peaks were observed, a phenomenon typical of *p*-benzoquinones (6). Correlation of the probable initial molecular forms and the observed major fragmentation peaks (Table II) suggests the involvement of at least three basic fragmentation pathways: (*a*) successive elimination of CO and CHO fragments, (*b*) cleavage into two equal or nearly equal fragments and then successive elimination of CO and CHO fragments, and (*c*) elimination of water and then successive elimination of CO and CHO fragments or cleavage into two large fragments prior to elimination of CO and CHO fragments. Table III summarizes some suggested major fragmentation pathways. It may be particularly significant that the pathways originating with the P − 2 ion peak parallel those reported for atromentic acid lactone (7).

Isolation and Identification of Thelephoric Acid—A small quantity of a black pigment was observed on the walls of the flask used for the diethyl ether extraction of carpophores of *P. atrotomentosus*, and 7 mg. of black crystalline material was obtained. The melting point was >300°, and the chromatographic properties of the pigment corresponded to those of thelephoric acid. The UV spectra ($\lambda_{max.}^{pyridine}$ 311 and 495 nm. and $\lambda_{max.}^{pyridine\text{-}H_2O}$ 338 and 540 nm.) agreed with those previously reported for thelephoric acid (5). The IR spectra of the black pigment and authentic thelephoric acid were identical, with absorption peaks at 3378, 2941, 1634, 1520, 1471, 1429, 1265, 1036, 863, 800, and 698 $cm.^{-1}$.

Isolation and Purification of Pigments from Cultures—Mixtures of the mycelium and nutrient broth from the two *Paxillus* species were freeze dried, and the dried samples were extracted exhaustively by repeated blending with cold absolute methanol. The alcoholic extracts were concentrated to dryness under reduced pressure at 35°, and the pigments were separated using a previously developed (3) dry-column chromatographic procedure with a silica gel adsorbent and ether saturated with concentrated HCl as the solvent system. The pigment bands corresponding to atromentic and xerocomic acids were eluted from the adsorbent with the acidified ether mixture, the solvent was removed, and the purified material was used for confirmation of identity.

Atromentic and xerocomic acids were recovered from culture mixtures of *P. atrotomentosus* and *P. panuoides* grown on both cherry and malt extract media. Xerocomic acid was the major pigment in all cases; tentative indications suggested that cultures of *P. panuoides* contained lower concentrations of the two tetronic acids, an indication that was confirmed by subsequent quantitative

Table III—Apparent Major Mass Spectral Fragmentation Pathways of Atromentin



[a] The relative intensities of fragments in parentheses were < 10% of base.

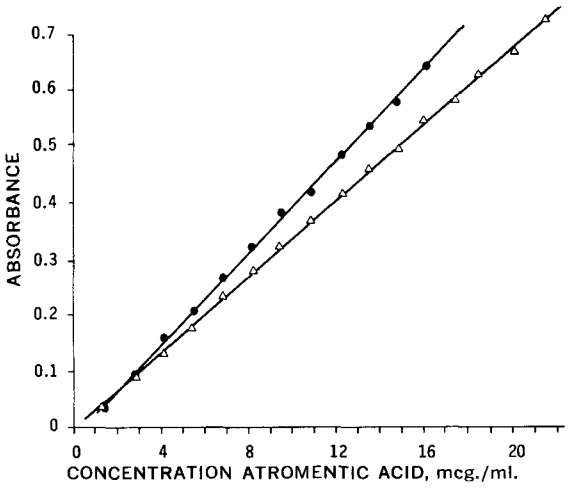


Figure 1—*Quantitation of atromentic acid. Key:* ●, *0.01* M $NaHCO_3$, *364 nm.; and* △, *distilled water, 378 nm.*

determinations. The unidentified red pigment remained at or near the origin in the dry-column procedure, and methanol was required to elute it from the silica gel.

Identification of Atromentic and Xerocomic Acids—IR, UV, and mass spectra were used to establish the identity of the isolated pigment samples with chromatographic properties corresponding to xerocomic acid. The limited availability of material corresponding to atromentic acid necessitated the use of only UV and mass spectra for identification of this pigment. The UV spectra [$\lambda_{max.}^{ethanol}$ 408 and 260 nm., $\lambda_{max.}^{water}$ 378 and 256 nm., and $\lambda_{max.}^{0.01\ M\ NaHCO_3}$ 620, 362, 320 (sh.), and 230 nm.] were identical for the xerocomic acid samples from both *Paxillus* species and were in agreement with those previously published for this tetronic acid (3). The atromentic acid samples from all of the cultures had identical UV spectra [$\lambda_{max.}^{ethanol}$ 390 and 257 nm., $\lambda_{max.}^{water}$ 375 and 258 nm., and $\lambda_{max.}^{0.01\ M\ NaHCO_3}$ 360 (sh.), 320, and 238 nm.], and these spectra were consistent with those known for atromentic acid (3). The IR spectra (KBr pellets) of authentic xerocomic acid and samples obtained from the cultures were indistinguishable, with absorption peaks at 3190, 2882, 2558, 1739, 1675, 1600, and 1513 $cm.^{-1}$.

Xerocomic acid samples from the cultures were observed to give high-resolution mass spectral parent ion peaks at *m/e* 338.0430, as anticipated for xerocomic acid lactone (calc. $C_{18}H_{10}O_7$, 338.0426); the fragmentation pattern, with ion peaks at *m/e* 310, 282, 226, 177, 161, 149, 133, 121, and 105, further confirmed the identity of these samples (3, 7). The corresponding data with the atromentic acid samples were parent ion peaks at *m/e* 322.0470 (calc. $C_{18}H_{10}O_6$, 322.0477) and fragmentation ions at *m/e* 294, 266, 238, 210, 161, 133, and 105, as anticipated (3, 7).

Effect of Heat on Xerocomic Acid—Xerocomic acid was observed to sublime at 242° to give dark-red needles. Agarwal and Seshadri

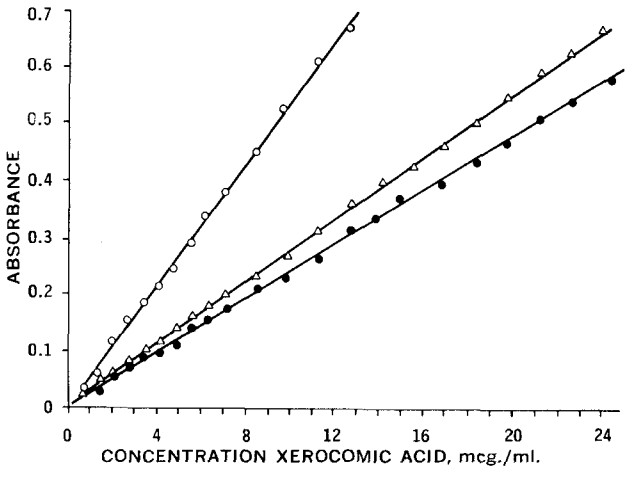


Figure 2—*Quantitation of xerocomic acid. Key:* ○, *0.01* M $NaHCO_3$, *364 nm.;* △, *distilled water, 378 nm.; and* ●, *0.01* M $NaHCO_3$, *600 nm.*

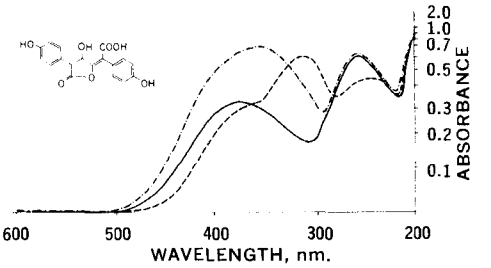


Figure 3—*Atromentic acid (9.08 mcg./ml.) spectra for pH 2.1 through 5.4* (—), *pH 8.5 through 9.3* (- - -), *and pH 10.8 through 11.9* (·-·); *analytical wavelength 316 nm.; pKa 6.39 ± 0.04.*

(8) reported the elimination of methanol and the formation of pulvinic acid lactone upon heating pulvinic acid to 192°. Thus, dehydration of xerocomic acid during sublimation was considered possible, and the properties of the red needles support such a change.

Mass spectral examination of the red needles revealed a high-resolution parent ion peak at *m/e* 338.0470, the parent ion peak of xerocomic acid lactone; this observation precluded the possibility of major pyrolytic fragmentation of the xerocomic acid molecule. The observed UV spectrum ($\lambda_{max.}^{ethanol}$ 438, 320, and 251 nm.) of the red material was distinct from the spectrum of xerocomic acid in ethanol, but it was similar to the UV spectrum ($\lambda_{max.}^{ethanol}$ 410, 312, and 240 nm.) of reference atromentic acid lactone. Absorption peaks in the IR spectrum (KBr pellet) of the red needles at 3448, 3279, 2924, 1815, 1779, 1653, 1600, 1511, 1445, and 1361 $cm.^{-1}$ were nearly identical to those observed with atromentic acid lactone, an observation that suggested similar characteristic functional groups. Also, the red material migrated farther than xerocomic acid in TLC System C (approximately R_f 0.70 and 0.50, respectively), a relationship that is known for other pulvinic acid derivatives and their lactones (3).

Quantitation of Atromentic and Xerocomic Acids—The UV spectra of atromentic and xerocomic acids in water and 0.01 *M* $NaHCO_3$ suggested the possibility of using a spectrophotometric procedure for quantitation of these tetronic acids. Preliminary studies with solutions of pure compounds revealed that satisfactory results could be obtained with both pigments in glass-distilled water measured at 378 nm. and in 0.01 *M* $NaHCO_3$ measured at 364 nm. and with xerocomic acid in the basic solution measured at 600 nm. Standard stock solutions (approximately 0.2 mg./ml.) of reference atromentic and xerocomic acids were prepared in glass-distilled water, and accurately measured volumes of these solutions were transferred to sufficient volumes of either distilled water or 0.01 *M* $NaHCO_3$ solution to give a 3-ml. total volume for each sample. The absorbances of solutions containing a range of approximately 1–28 mcg./ml. of the acids were measured, using water or appropriately diluted $NaHCO_3$ solutions as blanks. Figures 1 and 2 show standard curves for the concentration ranges, permitting feasible quantitative determinations under the various conditions; reproducibility among triplicate determinations at these concentrations was ±3%.

Table IV—Quantitative Data on Atromentic and Xerocomic Acids in 50-Day-Old Cultures of *P. atrotomentosus* and *P. panuoides*

Culture Mixture	Weight of Freeze-Dried Sample[a], g.	Atromentic Acid, mg./g.	Xerocomic Acid, mg./g.
P. atrotomentosus			
Cherry medium	11.37	0.18	39.5
Malt extract medium	8.02	0.01	47.4
P. panuoides			
Cherry medium	12.68	0.30	10.7
Malt extract medium	10.11	0.25	7.0

[a] Total solids representing mycelium and nutrient broth from 10 Roux culture flasks.

Table V—Fluorescent Properties of Atromentic, Pulvinic, and Xerocomic Acids

Tetronic Acid	Methanol: Excitation, nm.	Methanol: Emission, nm.	Methanol: Relative Intensity[a]	0.01 *M* $NaHCO_3$: Excitation, nm.	0.01 *M* $NaHCO_3$: Emission, nm.	0.01 *M* $NaHCO_3$: Relative Intensity
Nonirradiated						
Pulvinic acid	450	535	+	425	530	++++
Atromentic acid	480	550	+	450	535	++
Xerocomic acid	450	575	+	400	470	++
Irradiated						
Pulvinic acid	450	535	+++	425	530	++++
Atromentic acid	400	470	+++	400	470	++
		535	++		530	++
Xerocomic acid	350	470	++++	400	470	+
	425	510	++			

[a] Key to relative intensities: +, fluorescence ratio (ratio of intensity of emission peak to intensity of excitation peak) approximately 0.01; ++, fluorescence ratio >0.01 and <0.5; +++, fluorescence ratio >0.5 and <1.0; and ++++, fluorescence ratio >1.0.

Atromentic and xerocomic acids were separated, using either preparative TLC or the dry-column procedure, from known aliquots of extracts of the various culture mixtures, and the concentrations of the two acids were determined spectrophotometrically. Table IV lists the quantitative observations obtained with cultures of the two *Paxillus* species harvested after a 50-day incubation period.

The distinctive blue chromophore, formed in base by tetronic acids with a catechol function (6, 7), also suggested the possibility for quantitative determinations of mixtures of atromentic and xerocomic acids by making measurements at two wavelengths. Experimental observations revealed that such quantitative determinations were feasible in solution mixtures containing a total concentration of the two acids between 5 and 20 mcg./ml. and a minimum xerocomic acid concentration of 2.5 mcg./ml. The xerocomic acid in the mixture is determined from the absorbance in base at 600 nm. Absorbance of the same solution or a comparable solution in distilled water is measured at 364 or 378 nm., allowance is made for the amount of absorbance that can be attributed to the previously determined concentration of xerocomic acid, and the balance of the absorbance is assumed to represent the atromentic acid in the mixture. Ideally, it is preferable to make the measurements at both wavelengths with the same basic solution, but the steep slope of the standard curve for xerocomic acid in base at 364 nm. introduces practical limitations in mixtures containing a large xerocomic acid component.

Ionization Constants of Atromentic, Pulvinic, and Xerocomic Acids—Spectrophotometric procedures can be utilized to determine ionization constants for compounds characterized by ionic species with distinctive UV and visible spectra. The UV spectra observed for atromentic and xerocomic acids in water and $NaHCO_3$ solutions suggested the possibility of using this procedure with the pulvinic acid derivatives. A series of 0.01 *M* buffers (pH 2.1–11.9) was prepared in carbonate-free, glass-distilled water, according to the procedure of Albert and Serjeant (9); stock solutions of atromentic and xerocomic acids were prepared in the glass-distilled water, and a stock solution of pulvinic acid was prepared in absolute ethanol. Small volumes (0.15 ml.) of the stock solutions were added to sufficient volumes of buffer to give 3 ml. of buffer solutions, which contained approximately 10 mcg./ml. of a tetronic acid. The spectra were obtained at 24° with a recording spectrophotometer, and analytical wavelengths were selected for each compound. Figures 3–5 illustrate the representative spectra. After the analytical wavelength and the approximate pKa value were determined, spectra with buffer systems at increments of 0.2 pH unit were used to calculate the exact pKa value. The experimentally determined pKa values were 6.39 ± 0.04 (316 nm.) for atromentic acid, 6.86 ± 0.05 (296 nm.) for pulvinic acid, and 6.25 ± 0.05 (316 nm.) for xerocomic acid.

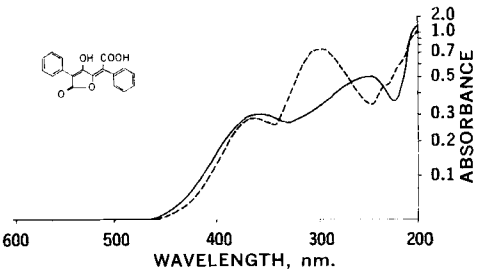


Figure 4—*Pulvinic acid (9.43 mcg./ml.) spectra for pH 2.1 through 6.0 (—) and pH 8.5 through 11.9 (- - -); analytical wavelength 296 nm.; pKa 6.86 ± 0.05.*

The spectra (Figs. 3 and 5) suggested that it should be possible to determine the ionization constants for the monohydroxyphenyl portions of both atromentic and xerocomic acids at analytical wavelengths in the 355–380-nm. region with mildly alkaline buffers (approximately pH 9). However, presumably the measurement of more than two ionic species under these conditions interfered with the determination of precise pKa values. The blue ionic species with xerocomic acid, which was attributed to the dihydroxyphenyl portion of the molecule and which could be measured at 600 nm. over the buffer range pH 6.3–11.9, faded rapidly above pH 8.5; thus, pKa values were unobtainable.

Fluorescent Properties of Atromentic, Pulvinic, and Xerocomic Acids—Light-induced degradative changes were noted previously with tetronic acid-containing cultures of *P. atrotomentosus* (3), and radiant energy was known to produce fluorescent photoproducts with atromentin (5). Studies were undertaken to determine the fluorescent properties of the tetronic acids at different stages of ionic dissociation, both before and after exposure to shortwave UV light. Stock solutions in spectrograde methanol were prepared, containing 1 mg./50 ml. and 10 mg./50 ml. of atromentic, pulvinic, and xerocomic acids. A portion of each solution was placed in a quartz cell and irradiated[4] for 18 hr. The fluorescent properties[5] of the dilute methanol solutions and of $NaHCO_3$ solutions (prepared by adding 0.3 ml. of the concentrated methanolic solutions to 2.7 ml. of 0.01 *M* $NaHCO_3$) are recorded in Table V.

CONCLUSIONS

The pattern of pigment distribution in carpophores and cultures of *P. atrotomentosus* and *P. panuoides* was observed to be identical, except for the presence of thelephoric acid in carpophores of *P. atrotomentosus*. Atromentin was isolated and identified from the

[4] Exposed at approximately 20 cm. to a Hanovia high pressure quartz mercury vapor lamp, model 30600, with a complete spectrum from 1849 to 4000 Å; Hanovia Chemical and Manufacturing Co., Newark, N. J.

[5] Baird Atomic Fluorispec, model SF-1, Baird Atomic Inc., Cambridge, Mass.

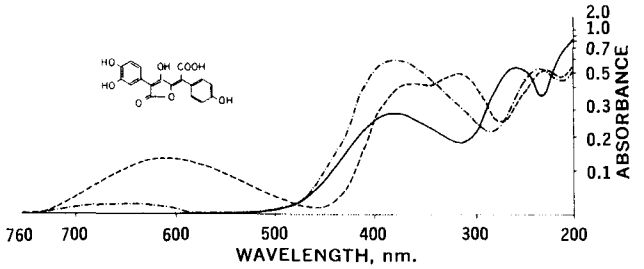


Figure 5—*Xerocomic acid (10.17 mcg./ml.) spectra for pH 2.1 through 5.2 (—), pH 8.5 through 9.3 (- - -), and pH 11.9 (·-·). Analytical wavelength 316 nm.; pKa 6.25 ± 0.05.*

carpophores of both species, and atromentic and xerocomic acids were isolated and identified from the culture mixtures. The tetronic acids were produced equally well when the fungi were grown on a cherry medium and an acidified malt extract medium; the utility of the latter medium was particularly significant for the fermentative production of these compounds, since it offered the advantage of circumventing the isolation problems caused by the anthocyanin pigments in the cherry decoction. Xerocomic acid was the major tetronic acid found in the cultures of both species; the ratios of xerocomic acid to atromentic acid were greater than 200 and approximately 36 for cultures of *P. atrotomentosus* and *P. panuoides*, respectively. An unidentified purple pigment was present in carpophores of both species, and the culture mixtures contained an unidentified red pigment. The observed distribution of pigments in the two *Paxillus* species further confirmed the distinctive metabolic capabilities of carpophores and vegetative mycelium which had been detected initially with *P. atrotomentosus* (3), but any contribution of the two unidentified pigments toward clarifying these metabolic capabilities must await further characterization of the pigments.

The quantitative spectrophotometric studies revealed feasible procedures for determination of atromentic and xerocomic acids in low concentrations (2–24 mcg./ml.) and in mixtures of certain compositions. Application of such quantitative methods, in conjunction with observations on UV and fluorescent properties and on molecular response to electron bombardment, may be useful in future studies to clarify the biologic involvement of tetronic acids and to establish the interrelationship between terphenylquinones and diphenyl-substituted tetronic acids.

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Drug Transfer across Rat Intestinal Musculature after Edetic Acid Treatment

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Abstract □ The columnar epithelium of the *in vitro* rat intestine was separated from the intestinal musculature by treatment with ice-cold edetic acid solution. The *in vitro* absorption kinetics through the denuded intestinal musculature for salicylate ion and acetanilide were followed in pH 7.4 sodium phosphate and potassium phosphate buffers, as well as in an isotonic sodium chloride solution where the effect of rinsing with Mg^{+2} and Ca^{+2} was also studied. Differences in directional transfer rates, as noted in previous studies with the untreated intestinal membrane, were not found. The transfer rates through the muscle layer were found to be at least twice as high as those found in the untreated membrane. As noted previously, nonionized acetanilide transfer rates were still approximately twice those seen for salicylate ion.

Keyphrases □ Drug-transfer rates, *in vitro*—effect of intestinal musculature, rat □ Membrane permeability coefficients—effect of edetic acid treatment □ Absorption kinetics, *in vitro*—salicylate and acetanilide through denuded intestinal musculature, rat □ Edetic acid—treatment of rat intestinal musculature, effect on drug transfer

Recent studies in this laboratory pointed out that drug ions exhibit directional permeability coefficients for passage across the rat intestine *in vitro* (1, 2), with mucosal to serosal transfer occurring at faster rates than serosal to mucosal transfer. It was proposed that the difference in directional permeability coefficients