Antimicrobial Activity of Mushroom Metabolites

R. G. BENEDICT and L. R. BRADY▲

Abstract □ Two styrylpyrone, three terphenylquinone, and six diphenyl-substituted tetronic acid derivatives were tested for *in vitro* antimicrobial activity. The best inhibitory responses were seen with hispidin, bisnoryangonin, and vulpinic acid against Gram-positive organisms, including the acid-fast *Mycobacterium smegmatis*, and with atromentin against the acid-fast bacterium, the Gram-negative organisms, and *Candida albicans*. However, all of the observed activities were low relative to those normally associated with clinically useful antibiotics.

Keyphrases □ Antimicrobial activity—mushroom metabolites screened □ Antibiotics—selected mushroom metabolites screened for antimicrobial activity □ Mushroom metabolites—screened for antimicrobial activity □ Basidiomycetes—metabolites screened for antimicrobial activity

Large-scale screening programs of the 1940's for the detection of antibiotic activity included a variety of fleshy basidiomycetes (1–3). A number of more recent reports recorded additional general observations of microbial antagonism with basidiomycetes (4–7). Unfortunately, the identities of the basidiomycete metabolites responsible for the antimicrobial effects are still unknown in most instances.

The polyacetylenes are the most extensively characterized group of antagonistic mushroom constituents. More than 50 of these unsaturated antibiotic substances are known from one or more species of *Aleurodiscus*, *Clitocybe*, *Coprinus*, *Cortinellus*, *Daedalea*, *Marasmius*, *Merulius*, *Pleurotus*, *Polyporus*, *Poria*, *Psathyrella*, and *Tricholoma* (8–10). Other known antagonistic compounds from basidiomycetes include the phenolic metabolites, fomecin A from *Fomes juniperinus* (11) and grifolin from *Grifola confluens* (12); various substituted phenylglyoxylic acids from *Polyporus tumulosus* (13); the purine, nebularine, from *Clitocybe nebularis* (14); such quinones as 5-methoxy-*p*-toluquinone from *Coprinus similis* and *Lentinus degener* (15), 2,5-dimethoxybenzoquinone from *Lenzites thermophila* (16), and 6-methyl-1,4-naphthaquinone from *Marasmius graminum* (17); and the terpenoid derivatives, fomannosin from *Fomes annosus* (*18*), illuden M and S from *Clitocybe illudens* (19), lactaroviolin from *Lactarius deliciosus* (20, 21), and marasmic acid from *Marasmius conigenus* (22).

The authors are interested in the occurrence and properties of phenylpropanoid-derived fungal constituents of the styrylpyrone (23, 24), terphenylquinone (25, 26), and diphenyl-substituted tetronic acid types (27, 28). Examination of the literature revealed only limited information on the antimicrobial properties of such constituents. Hispidin, a styrylpyrone, may be responsible for the antibacterial antagonism noted with extracts of *Polyporus schweinitzii* (29), and 7,8-dihydrokawain, a related kava constituent, exhibited antifungal action against *Aspergillus niger* (30). The broad-spectrum antimicrobial action of the angiosperm constituent, obtusastyrene, and some related cinnamylated phenols (31, 32) may provide a further indirect suggestion of the potential antibiotic capabilities of styryl derivatives. Polyporic acid, a terphenylquinone, allegedly has antibacterial properties (33); and antibacterial antagonism against a few diverse organisms (both Gram negative and Gram positive) has been reported for pulvinic and vulpinic acids (34, 35), diphenyl-substituted tetronic acids occurring in certain lichens.

The accumulation of small quantities of various styrylpyrones, terphenylquinones, and tetronic acids prompted their evaluation for antimicrobial activity.

EXPERIMENTAL

Materials and Methods—The styrylpyrones, terphenylquinones, and diphenyl-substituted tetronic acids were accumulated from various sources during other studies on basidiomycete metabolites. Vulpinic acid was purchased commercially[1]. Atromentin, aurantiacin, and thelephoric acid were isolated from carpophores of members of the Hydnaceae (25, 26); xerocomic acid was obtained from *Paxillus* cultures (27, 28); and hispidin and bisnoryangonin came from *Gymnopilus* species (33). Polyporic acid was synthesized for reference purposes from 2,5-diphenyl-1,4-benzoquinone[2] using an established procedure (36). Known procedures were also used to prepare reference samples of atromentic acid lactone, atromentic acid, pulvinic acid lactone, and pulvinic acid; isolated atromentin and synthetic polyporic acid were oxidized with hydrogen peroxide (37) and lead tetraacetate (36), respectively, to yield the lactones, and alkaline hydrolysis followed by acidification converted the lactones to the corresponding acids.

Bacillus subtilis UWM[3], *Staphylococcus aureus* ATCC 6538-P, *Mycobacterium smegmatis* UWM, *Enterobacter aerogenes* UWM, *Escherichia coli* UWM, *Pseudomonas fluorescens* NRRL B-11, and *Candida albicans* UWM were selected as representative test organisms for determining indications of antimicrobial activity. A serial dilution technique was used for the tests; solubility considerations with some compounds suggested 100 mcg./ml. as the maximum feasible concentration for the studies and necessitated the use of such solubilizing agents as acetone, methanol, and 10% $NaHCO_3$ (Table I). Preliminary control tests revealed no microbial contamination of the compounds to be tested and no inhibitory activity due to the small quantities of carrier solvents, but appropriate controls were routinely included with all studies. Pyridine was the only solvent suitable for dissolving the unusually insoluble thelephoric acid, and the antimicrobial effect of this solvent under the experimental conditions precluded any reliable evaluation of this fungal constituent.

Difco nutrient broth, containing 0.15% of Difco yeast extract, was used as the culture medium; 1.8-ml. volumes of the nutrient broth were transferred to sterile 10 × 100-mm. culture tubes, and 200 mcg. of a test compound dissolved in 0.2 ml. of a carrier solvent (sterile water, filter-sterilized 10% $NaHCO_3$, or an appropriate organic solvent) was added to each tube. The serial dilutions were prepared by transferring successively half of the contents of each tube to a series of tubes containing 1 ml. of sterile nutrient broth. This resulted in broths with 100, 50, 25, 12.5, and 6.25 mcg./ml. of the various compounds.

Inoculum was prepared by growing the organisms in 20 ml. of nutrient medium for 30 hr. at 30° and 200 r.p.m. on a shaker[4];

[1] Carl Roth OHG, Karlsruhe, West Germany.
[2] Aldrich Chemical Co., Inc., Milwaukee, WI 53233
[3] UWM = University of Washington, Department of Microbiology standard strain; ATCC = American Type Culture Collection, Rockville, MD 20852; NRRL = Northern Regional Research Laboratory, U. S. Department of Agriculture, Peoria, IL 61604
[4] New Brunswick Psycrotherm shaker, model G-27.

Table I—Observed Antimicrobial Activity as Minimal Inhibitory Concentrations (mcg./ml.)

	Solubilizing Agent	*B. subtilis*	*S. aureus*	*M. smegmatis*	*E. aerogenes*	*E. coli*	*Ps. fluorescens*	*C. albicans*	Control[a]
Styrylpyrones									
Hispidin	Methanol	50–100	50–100	25–50	>100	>100	>100	>100	—
Bisnoryangonin	Methanol	50–100	25–50	12–25	>100	>100	>100	>100	—
Terphenylquinones									
Atromentin	10% $NaHCO_3$	>100	>100	25–50	50–100	50–100	25–50	25–50	—
Aurantiacin	Acetone	>100	>100	>100	>100	>100	>100	>100	—
Polyporic acid	10% $NaHCO_3$	>100	>100	50–100	>100	>100	50–100	50–100	—
Diphenyl-substituted tetronic acids									
Atromentic acid	Water	>100	>100	>100	>100	>100	50–100	50–100	—
Atromentic acid lactone	Acetone	>100	>100	50–100	>100	>100	50–100	>100	—
Pulvinic acid	10% $NaHCO_3$	>100	>100	>100	>100	>100	50–100	50–100	—
Pulvinic acid lactone	Acetone	>100	>100	50–100	>100	50–100	>100	50–100	—
Vulpinic acid	Acetone	12–25	25–50	25–50	>100	>100	>100	>100	—
Xerocomic acid	Water	>100	>100	>100	>100	>100	50–100	>100	—
Control[b]		+++	+++	++	+++	+++	+++	+++	

[a] 12.5 mcg./ml. of compounds, but no test organism added. [b] No test compounds added; +++ = heavy growth.

1 drop of a 1:200 dilution of the microbial cells, except for *M. smegmatis*, was used to inoculate each tube. Undiluted inoculum was employed for the slower growing *M. smegmatis*. Control tubes containing no test compounds were included to ensure the viability of the test organisms, and uninoculated tubes containing 12.5 mcg./ml. of the test compounds provided a degree of control against microbial contamination. The inoculated cultures and the uninoculated controls were incubated at 32° for 16 hr. and examined visually for growth of microorganisms. Growth, when observed, was judged arbitrarily to be weak (+), moderate (++), or heavy (+++), and the minimal inhibitory concentrations were recorded (Table I). In dilution series where, for example, no growth was observed at 50 mcg./ml. and weak growth was observed at 25 mcg./ml., the minimal inhibitory concentration was recorded as 25–50 mcg./ml.

RESULTS AND DISCUSSION

The best *in vitro* antimicrobial activity (Table I) was seen with hispidin, bisnoryangonin, and vulpinic acid against Gram-positive test organisms, including the acid-fast *M. smegmatis*. Atromentin exhibited some activity against the acid-fast bacterium, the Gram-negative organisms, and *C. albicans*, but no antagonism was detected with aurantiacin (atromentin-2,5-dibenzoate). Only marginal inhibitory effects were noted with atromentic acid, atromentic acid lactone, pulvinic acid, pulvinic acid lactone, polyporic acid, and xerocomic acid. However, all of the observed activities were low relative to the 0.02–2.0-mcg./ml. range of minimal inhibitory concentrations against sensitive organisms which are characteristic of such clinically useful antibiotics as the penicillins and tetracyclines.

The results provide experimental evidence for associating a low level of antibiotic activity with various styrylpyrone, terphenylquinone, and diphenyl-substituted tetronic acid derivatives. This information may provide academic clarification, at least in part, for observations of antimicrobial activity with some fungi. The activity of several compounds against the acid-fast test organism may have some significance, but none of the experimental observations advances strong justification for further study of the antimicrobial properties of these phenylpropanoid-derived fungal constituents.

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Mass Spectrometric Identification of Methylxanthines and Methyluric Acids, the Possible Metabolites of Caffeine

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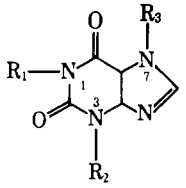
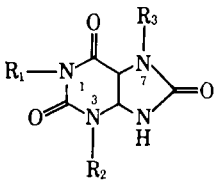
Abstract □ A rapid and direct mass spectrometric method for the identification of caffeine and its 15 possible metabolites formulated as tri-, di-, and monomethyl derivatives of xanthine and uric acid is described. With the exception of uric acid and its 7-methyl derivative, the molecular ion itself was found to be the base peak in the mass spectra of all of the compounds studied. Fragmentation mechanisms, along with the relative intensities of the major fragments and data on metastable ions useful in the identification of these compounds, are reported.

Keyphrases □ Caffeine metabolites, possible—mass spectrometric identification of methylxanthines and methyluric acids □ Methyluric acids—mass spectrometric identification, possible metabolites of caffeine □ Methylxanthines—mass spectrometric identification, possible metabolites of caffeine □ Mass spectroscopy—identification of methylxanthines and methyluric acids as possible metabolites of caffeine

The versatility of mass spectrometric techniques in the identification of trace quantities of drugs and drug metabolites is well recognized (1–4). As a part of studies on caffeine metabolism (5), the mass spectrometric identification of caffeine metabolites was investigated, and this paper describes the procedure for the identification of caffeine and its 15 possible metabolites containing an intact purine ring. Since *N*-dealkylation is a common metabolic reaction, the mass spectrometric behavior was studied of all possible tri-, di-, and monomethyl derivatives of xanthine and uric acid as well as the two parent compounds. Of these 16 compounds, caffeine, theobromine, theophylline, xanthine, and uric acid were previously subjected to mass spectrometric studies (6–9).





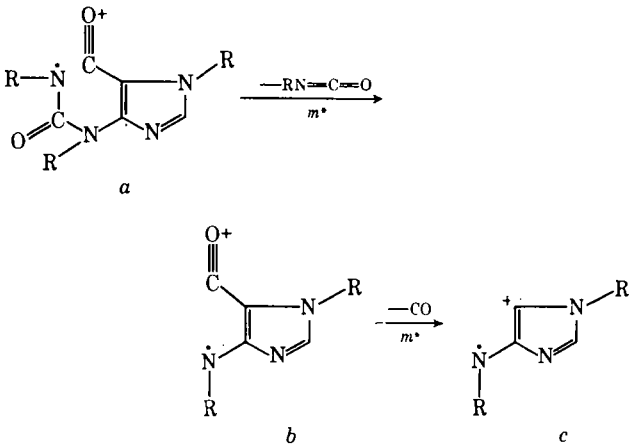
I: $R_1 = R_2 = R_3 = CH_3$
II: $R_1 = H$, $R_2 = R_3 = CH_3$
III: $R_1 = R_3 = CH_3$, $R_2 = H$
IV: $R_1 = R_2 = CH_3$, $R_3 = H$
V: $R_1 = CH_3$, $R_2 = R_3 = H$
VI: $R_1 = R_3 = H$, $R_2 = CH_3$
VII: $R_1 = R_2 = H$, $R_3 = CH_3$
VIII: $R_1 = R_2 = R_3 = H$

IX: $R_1 = R_2 = R_3 = CH_3$
X: $R_1 = R_2 = CH_3$, $R_3 = H$
XI: $R_1 = R_3 = CH_3$, $R_2 = H$
XII: $R_1 = H$, $R_2 = R_3 = CH_3$
XIII: $R_1 = CH_3$, $R_2 = R_3 = H$
XIV: $R_1 = R_3 = H$, $R_2 = CH_3$
XV: $R_1 = R_2 = H$, $R_3 = CH_3$
XVI: $R_1 = R_2 = R_3 = H$

RESULTS AND DISCUSSION

Due to the presence of heteroaromatic rings, purine derivatives generally form highly stabilized molecular ions under electron impact. All the methylxanthines and methyluric acids studied were found to exhibit intense molecular ions in their mass spectra; with the exception of uric acid and its 7-methyl derivative, the molecular ion itself was the base peak in all of the spectra. Tables I and II list the major peaks observed in the mass spectra of the 16 compounds studied. Schemes I–III describe the proposed fragmentation pathways leading to the various peaks seen in the spectra. The suggested fragmentation steps are supported by the presence of requisite metastable ions in the spectra of all the compounds examined and these are listed in Tables III and IV.

The results are in good agreement with the earlier mass spectral studies (6–9) on caffeine, theobromine, theophylline, xanthine, and uric acid derivatives (Schemes I–III). A molecule of either methylisocyanate or isocyanic acid is first expelled from the molecular radical ions (*a* and *d*), depending upon the presence or absence of the



Scheme I