Occurrence of Atromentin and Thelephoric Acid in Cultures of *Clitocybe subilludens*

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Abstract □ Atromentin was found to be present in both 30- and 45-day-old cultures of *Clitocybe subilludens* Murr., whereas thelephoric acid was present in the 30-, 45-, and 60-day-old cultures. These cultures were grown on 6% malt extract agar under continuous light conditions at 29° for 15, 30, 45, and 60 days. Cultures grown in the absence of light failed to produce detectable quantities of either terphenylquinone. Examination of all cultures for diphenyl-substituted tetronic acids failed to demonstrate detectable amounts. It is postulated from these observations that atromentin may act as a precursor for thelephoric acid, since the disappearance of the former coincides with the appearance of the latter terphenylquinone.

Keyphrases □ *Clitocybe subilludens*—occurrence of atromentin and thelephoric acid in cultures □ Atromentin—occurrence in 30- and 45-day-old *Clitocybe subilludens* cultures □ Thelephoric acid—occurrence in 30-, 45-, and 60-day-old *Clitocybe subilludens* cultures

The production of terphenylquinones and tetronic acids by saprophytic cultures of fungi has been established. Divekar *et al.* (1) reported the isolation of volucrisporin from cultures of the hyphomycete *Volucrispora aurantiaca* Haskins. Phlebiarubrone, an orthoquinone produced by *Phlebia strigosozonata* (Schw.) Lloyd, also was detected in cultures of this basidiomycete (2). Gaylord *et al.* (3) were unable to detect atromentin in cultures of *Paxillus atrotomentosus* (Batsch) Fr., an agaric which produces this terphenylquinone under natural growing conditions. However, two diphenyl-substituted tetronic acids, xerocomic and atromentic acids, were isolated from the vegetative mycelia by these investigators.

Since carpophores of *Clitocybe subilludens* Murr. are known to contain atromentin (4), it appeared desirable to examine surface cultures of this basidiomycete for the presence of terphenylquinones and diphenyl-substituted tetronic acids.

EXPERIMENTAL

Origin and Maintenance of Culture—Carpophores of *C. subilludens*[1] were collected in Austin, Tex., in the autumn of 1967. Isolates[2] were obtained from these mushrooms and maintained on 6% malt extract agar slants in a humidified incubator at 29°.

Incubation of Surface Cultures—Sixteen 2800-ml. Fernbach flasks, each containing 200 ml. of sterile 6% malt extract agar, were aseptically inoculated with equal aliquots of a homogeneous suspension of *C. subilludens*. The suspension was prepared from one of the stock slants, sterile water, and a sterilized microblender. One group (Group A) of eight flasks was incubated at 29° under continuous light exposure. The remaining eight flasks (Group B) were incubated at 29° in continuous darkness. At incubation periods of 15, 30, 45, and 60 days, respectively, the mycelium of one flask from Group A and one flask from Group B was harvested and extracted according to Procedure I. Concurrently, the mycelium of a second flask from each of Groups A and B was harvested and extracted according to Procedure II.

Table I—Dry Weights of Harvested Mycelial Mats

Incubation Period, Days	Dry Weight, g. Group A	Dry Weight, g. Group B
15	0.6793	0.4618
30	0.7430	0.4981
45	1.6050	0.4098
60	3.9400	0.5242

Extraction Procedure I—Each harvested mycelial mat was dried at 45° for 48 hr., ground to a 20-mesh powder with a Wiley mill, weighed, placed in a soxhlet apparatus, and sequentially extracted with *n*-hexane (48 hr.), ether (24 hr.), chloroform (24 hr.), a benzene–methanol azeotrope[3] (24 hr.), methanol (2 hr.), acetone (36 hr.), and ethanol (48 hr.). Each extract was evaporated to a dry residue, weighed, and analyzed by TLC.

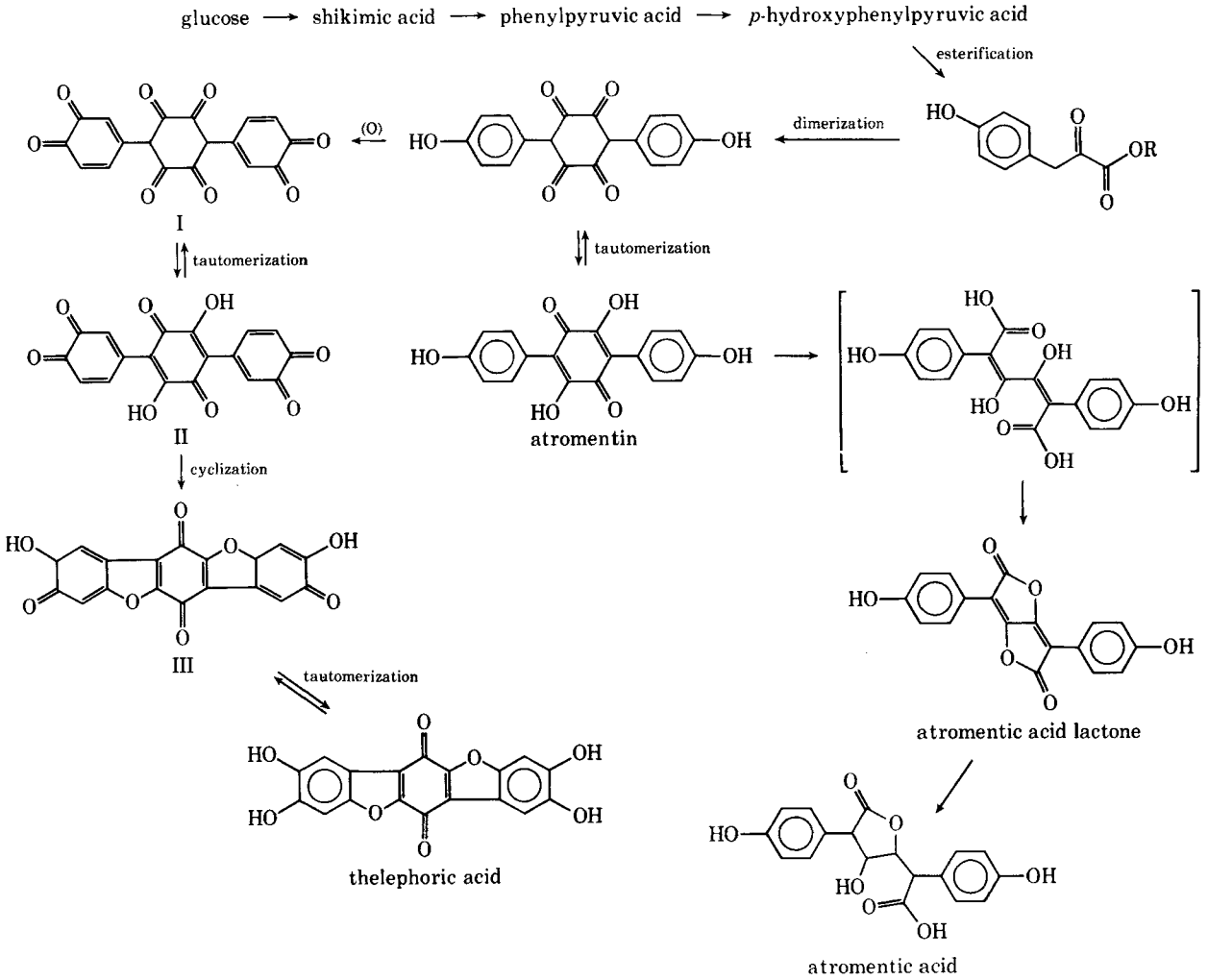
Extraction Procedure II—This procedure was carried out under conditions of subdued light to minimize possible photodegradation of tetronic acids. An aqueous slurry of each harvested mycelial mat was prepared with a microblender, placed into a 250-ml. separator, and extracted with successive 100-ml. portions of ether and ethyl acetate. Each organic extract was concentrated and analyzed by TLC. No evidence for the presence of tetronic acid derivatives was noted.

Chromatographic Analysis—Silica gel G[4] plates, prepared according to Stahl (5), were employed for each TLC system. Longwave UV light and iodine vapors were used for visualization. Authentic samples[5] of tetronic acids (atromentic and pulvinic), atromentin, and thelephoric acid were used as reference markers on each TLC plate. These reference compounds were found to have R_f values of 0.87, 0.86, 0.70, and 0.44, respectively, when methanol–0.30 molal oxalic acid dihydrate in methanol–formic acid (100:2:0.1) was used as the mobile phase. When benzene–ethyl formate–formic acid (13:5:4) was employed as the mobile phase, R_f values of 0.50, 0.78, 0.45, and 0.00, respectively, were obtained.

Isolation and Identification of Atromentin—The residue obtained from the ether extract (Procedure I) of the 30-day-old culture grown in continuous light was selected, since TLC analysis indicated the presence of a significant amount of material whose TLC characteristics were identical with authentic atromentin. The residue was shaken with ether, filtered, evaporated to a dry residue, and shaken with warm chloroform. The resulting suspension was filtered, and the residue was washed with ethanol and filtered. Evaporation of the ethanolic filtrate to dryness produced a brownish solid which was washed with water and dried. The dried brownish solid was subjected to a pyridine–water color reaction test (6); it was observed to sublime and decompose without melting above 280°; $\nu_{max.}^{KBr}$ 3448, 3350, 1650, 1515, 1450, 1110, 999, and 834 cm.$^{-1}$; $\lambda_{max.}^{dioxane}$ 268 and 385 nm.; $\lambda_{max.}^{MeOH}$ 276 and 360 nm. All data obtained by these analyses were identical to those of reference atromentin.

Isolation and Identification of Thelephoric Acid—TLC analysis indicated that a significant amount of material of R_f value identical to that of reference thelephoric acid was present in the acetone fraction (Procedure I) of the 60-day-old culture grown in continuous light. This solid residue was washed twice with ether and twice with methanol. Black crystals were obtained after filtration. The black crystalline compound gave a positive pyridine–water color reaction

[1] Previously identified by Dr. H. E. Bigelow, Department of Botany, University of Massachusetts.
[2] Voucher specimen, American Type Culture Collection No. 22516.
[3] Benzene–methanol (60.5:39.5).
[4] Brinkmann Instruments, Inc., Westbury, N. Y.
[5] Obtained from Dr. L. R. Brady, College of Pharmacy, University of Washington.



Scheme I—Biogenetic Scheme

test; it did not melt below 350°; $\nu_{max.}^{KBr}$ 3200, 1645, 1525, 1273, 1038, and 802 cm.$^{-1}$; $\lambda_{max.}^{EtOH}$ 264, 305, and 485 nm.; $\lambda_{max.}^{dioxane}$ 263, 305, and 450 nm. All these data were identical to those of reference thelephoric acid. In addition, the tetraacetate derivative of the isolated thelephoric acid was prepared according to the method described by Lounasmaa (7). This derivative exhibited m.p. 330–335°; $\nu_{max.}^{KBr}$ 1780, 1680, 1530, 1366, 1262, 1200, 1154, 1124, 1033, and 1015 cm.$^{-1}$; $\lambda_{max.}^{dioxane}$ 263, 283, and 360 nm. These data were identical to those reported in the literature for thelephoric acid tetraacetate (8).

RESULTS AND DISCUSSION

The weights of the dried mycelial mats obtained after 15-, 30-, 45-, and 60-day-incubation periods under conditions of continuous light exposure (Group A) and continuous dark exposure (Group B) are shown in Table I.

Chromatographic analysis of all residues obtained by soxhlet extraction of the *C. subilludens* mycelial mats from 15-, 30-, 45-, and 60-day-incubation periods, under continuous exposure to light, indicated atromentin to be present only in the ether fractions from the 30- and 45-day-old cultures. Thelephoric acid was initially detected in the acetone and ethanol fractions from the 30-day-old cultures. All remaining soxhlet fractions (except hexane) from the 45- and 60-day-old cultures were found to contain increasing amounts of thelephoric acid. Therefore, the concentration of thelephoric acid was noted to increase with time; the greatest amount of this terphenylquinone was detected in the ethanol fraction of the 60-day-old culture. Tetronic acids were not detected in any fraction.

No terphenylquinones were detected in any extract obtained from cultures grown in the absence of light (Group B). In addition, no tetronic acids were detected in any extract obtained from cultures from either Group A or B.

It is apparent from these results that light plays a major role in both continued mycelial growth and the production of terphenylquinones in cultures of *C. subilludens*.

Read *et al.* (9) demonstrated the incorporation of ^{14}C-labeled shikimic acid, phenylalanine, phenyllactic acid, and *m*-tyrosine into the terphenylquinone pigment, volucrisporin. From these observations, it was postulated that atromentin, leucomelone, polyporic acid, and probably thelephoric acid would be derived from appropriately substituted phenylpropanoid compounds by a similar pathway. The incorporation of labeled phenylalanine into the terphenyl ring system of phlebiarubrone (2) further substantiated the existence of this biogenetic pathway. Tracer experiments with phenylalanine, phenyllactic acid, and polyporic acid, which were readily incorporated into pulvinic acid lactone and calycin in the lichen *Pseudocyphellaric croccata* (10), established a definite relationship between terphenylquinones and diphenyl-substituted tetronic acids. A particularly interesting observation was made by Gaylord *et al.* (3). These investigators found that cultures of *P. atrotomentosus* produced detectable amounts of xerocomic and atromentic acids, whereas the mature carpophore yielded only atromentin.

The occurrence of atromentin and thelephoric acid and the apparent absence of tetronic acid derivatives in cultures of *C. subilludens* do not contradict the possible interrelationships of these compounds, since the biogenetic control mechanism may be inoperable or nonexistent in cultures of this basidiomycete. The simultaneous disappearance of atromentin with the appearance of thelephoric acid suggests that atromentin may act as a precursor to thelephoric acid.

Scheme I represents a possible biosynthetic relationship which

may exist between terphenylquinones and diphenyl-substituted tetronic acid. Although Read *et al.* (9) suggested that hydroxylation of the phenylpropanoid compound occurs prior to the formation of the terphenyl skeleton, it appears reasonable that hydroxylation could occur after dimerization. If atromentin is a precursor to thelephoric acid, this reaction must take place after terphenyl formation. It appears that Compounds I, II, and III are plausible biosynthetic intermediates in the conversion of atromentin to thelephoric acid. Tracer studies are indicated to establish the biogenetic relationship between atromentin and thelephoric acid as they exist in cultures of *C. subilludens*.

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Isolation and Identification of Constituents from *Cudrania javanensis*

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Abstract □ Osajaxanthone, vanillic acid, monomethyl fumarate, *p*-hydroxybenzoic acid, and (−)-(*S*)-stachydrine were isolated from extracts of the bark of the tropical shrub *Cudrania javanensis* Trécul (Moraceae).

Keyphrases □ *Cudrania javanensis*—isolation and identification of osajaxanthone, vanillic acid, monomethyl fumarate, *p*-hydroxybenzoic acid, (−)-(*S*)-stachydrine □ Column chromatography—separation of *Cudrania javanensis* constituents

Cudrania javanensis Trécul (Moraceae) is a climbing shrub or small tree of tropical Asia, Australia, and Polynesia (1). Extracts of the plant have been used to dye cotton yellow (2). The plant is commonly called cockspur thorn in Australia, where it has been suspected of poisoning stock (3). The absence of knowledge of the constituents of *Cudrania* species prompted us to undertake a phytochemical investigation of this plant. The isolation and identification of osajaxanthone (I), vanillic acid, monomethyl fumarate, *p*-hydroxybenzoic acid, and (−)-(*S*)-stachydrine (II) are reported here.

The plant material was moistened with dilute ammonium hydroxide and extracted by percolation to exhaustion with ethanol. The extract residue was partitioned between dilute hydrochloric acid and ether. The ether solution was extracted with dilute potassium hydroxide, washed with water, and set aside. The alkaline solution was acidified and reextracted with ether. Chromatography of this ether extract over silicic acid–Celite (4:1) afforded osajaxanthone (I), vanillic acid, monomethyl fumarate, and *p*-hydroxybenzoic acid. Osajaxanthone was first isolated from the root bark of the Osage Orange [*Maclura pomifera* (Moraceae)] (4) and subsequently from the trunk wood of Brazilian *Kielmeyera corymbosa* (Guttiferae) (5) and the heartwood of Malaysian *Calophyllum scriblitifolium* (Guttiferae) (6). Closely related xanthones from *Maclura* species have been reported to be toxic to goldfish and mosquito larvae (4). The presence of this xanthone may account for the use of *Cudrania* extracts as yellow dyes.

The dilute hydrochloric acid extract was basified with ammonium hydroxide and extracted successively with ether and chloroform. The organic extracts were combined and set aside. The remaining alkaline solution was acidified, and a quaternary alkaloid fraction was collected by precipitation with ammonium reineckate. Treatment of the reineckate complex with an anion-exchange resin in the chloride form afforded a quaternary alkaloid chloride fraction which, upon chromatography over neutral alumina, yielded (−)-(*S*)-stachydrine (II), characterized as the hydrochloride salt.

EXPERIMENTAL[1]

Plant Material—*C. javanensis* (Moraceae) bark[2] was received from Australia and identified at the point of collection. The bark was air-dried and ground to a No. 4 mesh powder.

[1] Melting points were taken on a Thomas-Hoover apparatus and are uncorrected. The UV spectra were obtained on a Perkin-Elmer model 202 recording spectrophotometer, and the IR spectra were determined on a Perkin-Elmer model 257 spectrometer or a Beckman model IR-8 spectrometer in KBr pellets. NMR spectra were recorded in $CDCl_3$ solution on a Bruker Scientific, Inc., model B-90 instrument equipped with a time-averaging computer, with $(CH_3)_4Si$ as the internal standard and chemical shifts reported in δ (p.p.m.) units. Mass spectra were taken with a LKB-9000 mass spectrometer. Optical rotations were measured in a Rudolph polarimeter. All reagents were analytical reagent grade, unless otherwise noted.

[2] Obtained from the Meer Corp., New York, N. Y. (Lot. No. MS 1871).