Anthraquinones and Phenols as Intermediates in the Formation of Dark-Colored, Humic Acid-Like Pigments by *Eurotium echinulatum*[1]

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ABSTRACT

Eurotium echinulatum, a fungus isolated from a vertisol in southern Spain, formed humic acid-like pigments during growth in glucose-asparagine or glucose-$NaNO_3$ media. After 6 to 7 days the phenols, orsellinic, *p*-hydroxycinnamic, and *p*-hydroxybenzoic acids, and the anthraquinones, endocrocin, emodin, and physcion were detected in the culture media. Upon further development of the mycelial mats additional aromatic compounds were produced and after 4 to 6 weeks more than 50 different ether-extractable phenols and anthraquinones were detected. With further incubation the amounts and number of these compounds decreased and pigment formation in the media increased. After 2 to 3 months the culture media were dark brown to black and about 1 to 3.5 g/liter of polymer could be recovered upon acidification of the culture medium. Sodium amalgam reduction of this polymer yielded numerous phenols, anthraquinones, anthrones, and possibly anthracene derivatives. Treatment with sodium dithionite yielded largely anthraquinones. The polymer contained 1 to 4.5% N depending upon the N source. From 50 to 60% of this N was released in the form of amino acids upon acid hydrolysis. With both asparagine and $NaNO_3$ as N sources the same amino acids were isolated from 6*N* HCl hydrolysates. Quantitatively, however, there were differences in the percentage distribution of the amino acids.

Additional Index Words: soil fungi; anthraquinone formation by fungi; anthraquinones in fungal polymers; anthraquinones released from fungal polymers; phenols released from fungal polymers; amino acids in fungal polymers.

McGrath (18, 19, 20) extracted several anthraquinones in amounts up to 120 ppm from a variety of Irish and Canadian soils. Chrysotalunin, a hydroxybianthraquinone derived from chrysophanol, was more prominent than monomer anthraquinones such as physcion and chrysophanol. These anthraquinones could be of plant or microbial origin (27). Their occurrence, however, under heath or grass suggests a microbial origin. Numerous soil fungi including *Penicillium*, *Aspergillus*, and *Trichoderma* species synthesize anthraquinones (38) and the possibility that these and other quinones (in addition to simple phenolic compounds) are important constituent units in the formation of soil humus is receiving more consideration. The so-called P-type humic acids, isolated first by Kumada and Sato (14, 15) are associated with perylene quinones, probably of fungal origin (16). Structures related to perylene quinone were also isolated as intermediates in the formation of the dark colored pigment of *Daldinia* and *Aspergillus spp.* and according to Bu Lock (6), they are formed by condensation of 1,8-dinaphthol units.

According to Mathur (24) 2-methyl-1,4-naphthoquinone was obtained in amounts up to 10% from a podzol fulvic acid upon degradation with cultures of *Poria subacida*. These results were confirmed with cell-free preparations. Evidence for the presence of substantial amounts of quinones in soil humic material has also been obtained by chemical analyses of functional groups and by infrared spectroscopy (24, 25, 26, 32). It is possible that the condensed hydrocarbons isolated either directly from the soil (5, 39) or upon zinc dust distillation of humic acids (8, 11, 13) may originate by reduction of more highly condensed quinoid or phenolic structures.

The fringelites of sea sediments discussed by Blumer (4) and by Albrecht and Durisson (1) are dimeric anthraquinones with tetra-hydroxy-naphthodianthrone structures. They are derived from fossil crinoids and undergo a series of hydrations and dehydrations.

For the present paper a study was made of anthraquinones and phenols formed by *Eurotium echinulatum*, a soil fungus isolated from a Vertizol in southern Spain. The linkage of these compounds into dark-colored pigments similar to humic acid by either phenolase or autoxidative polymerization processes in the culture medium and in the cells during and after termination of growth was followed.

METHODS

The methods for cultivation and some properties of the dark-colored pigment have been described (22, 23). The fungus was cultured in Czapek-Dox medium (20% glucose and 0.2% $NaNO_3$) or in glucose-asparagine medium (3% glucose and 0.5% asparagine) as described by Martin et al. (21). Growth and polymer formation was much better in the glucose-asparagine medium, but the Czapek-Dox was preferred for monitoring the formation and transformation of phenols and anthraquinones. The phenols and anthraquinones were isolated from the culture media by acidification to pH 1.0 and extraction with peroxide-free ether. Concentrated ether extracts were spotted on thin layer silica gel (KGF 254, Fa, Merck, Darmstadt) plates and chromatographed in two directions (9, 10, 12) with $CHCl_3$-CH_3COOH (8:2) and dibutylether-CH_3COOH (10:1) or by the method of Leistner (17) with $CHCl_3$-ethylacetate-HCOOH (5:4:1) or benzene-ethylformate-HCOOH (75:24:1) in the first and with dibutylether-CH_3COOH (10:1) in the second direction. The first developing system gave a better separation of the phenols and the second a better separation of the anthraquinones.

Phenol spots were observed under UV-light at 254 nm and after specific color reactions with diazotized nitroaniline or sulfanilic acid. Anthraquinones were detected with UV-light at 366 nm and by spraying with 24% NaOH (2) or with 0.5% Mg-acetate (33). To obtain a better separation of the anthraqui-

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nones from the phenols, the ether extracts were added to a silica gel column (2.5 by 30 cm filled with silica gel, Merck, 30–70 mesh) and eluted with benzene-ethylformate-HCOOH (75:21:1). The colored bands were collected and chromatographed in two directions on thin layer plates as described. Samples of known anthraquinones and phenols were cochromatographed with the unknown compounds. Agreement of Rf-values, color reaction, and spectra in the UV and visible light range of extracted spots with the pure anthraquinones or phenols were used as criteria for identification.

The dark-colored humic acid-type pigments were isolated after 3 months from the $NaNO_3$ medium and after 4 to 6 weeks from the asparagine medium. The pads were removed and the media filtered and centrifuged at 15,000 × *g*. The clear solutions were acidified to pH 1 and the precipitate collected by centrifugation. The precipitate was dissolved in 0.1*N* NaOH and the solution centrifuged at 15,000 × *g*. The clear solution was dialyzed against distilled water, and the humic polymer recovered by precipitation at pH 1.0, washing, and lyophilization.

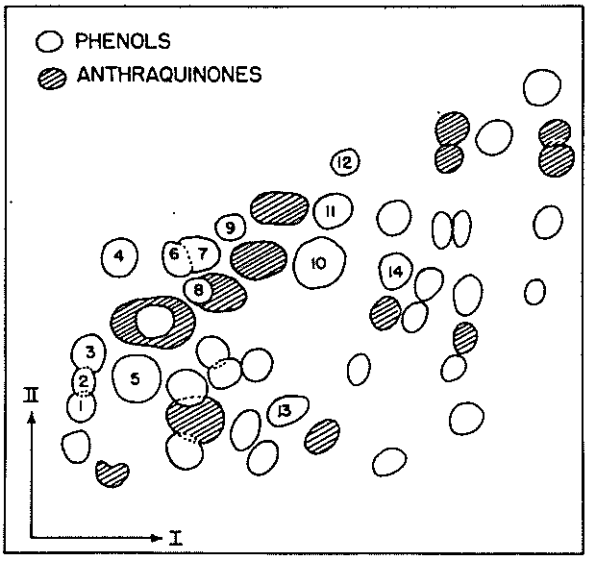
The humic acid-type pigments were degraded by the Na-amalgam method (7, 21, 22, 30, 31). For this purpose 25- to 50-mg portions of the polymers were treated with 40 g of 3% or 25 g of 5% Na-amalgam under N_2. Anthraquinones were specifically released by refluxing 50-mg portions under N_2 with 50 ml of 1% Na-dithionite solution for 10 min. The reaction mixtures were acidified and extracted with peroxide-free ether.

For determining the amino acids in the polymers, 200-mg portions of the pigments were hydrolized with 20 ml of 6*N* HCl for 24 hours at 105C in sealed tubes under N_2. Aliquots of the hydrolysates were spotted on paper and separated in the first direction by high voltage electrophoresis at pH 1.9 (2.2 kV for 60 min) and in the second direction by descending chromatography with sec. butanol-HCOOH-H_2O (75:15:10) as described by Haider and Martin (9). Amino acid spots were noted upon spraying with ninhydrin and their positions compared with known amino acids. For quantitative determination of the amino acids, the hydrolysates were chromatographed on an amino acid analyzer (Biotronik, Model LC 4010). The amounts of the specific amino acids were calculated on the basis of μmole/g of the polymer or in percent of the total amino acids recovered plus the ammonia.

RESULTS

Within 6 to 7 days after inoculation of the culture media with *E. echinulatum* several phenols and anthraquinones could be extracted with ether from the culture solutions. Thin layer chromatography revealed the presence of the anthraquinones, endocrocin, emodin, and physcion, and the phenols, *p*-hydroxycinnamic and *p*-hydroxybenzoic acids. Within 8 to 10 days up to 30 different prominent spots were visible. In addition to those indicated the anthraquinones, dermolutein, questin, questinol, catenarin, erythroglaucin, and dermoglaucin and the phenols orsellinic, protocatechuic, gallic 3,5-dihydroxybenzoic, and 2,4,6-trihydroxybenzoic acids, orcinol, phloroglucinol, acetylphloroglucinol and possibly protecatechualdehyde, *p*-hydroxybenzaldehyde, caffeic acid, and pyrogallol were identified.

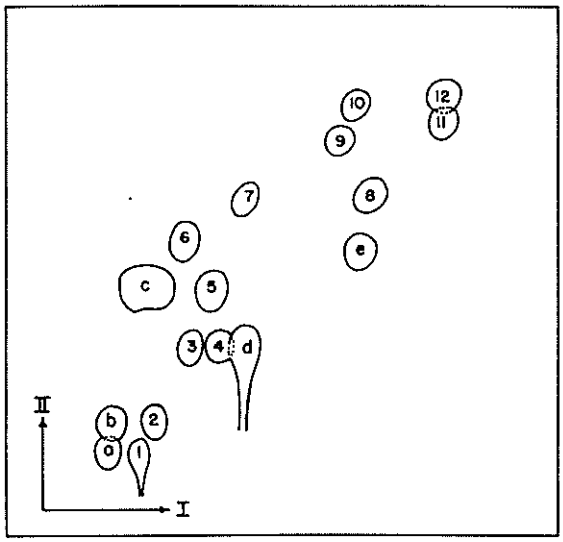
After 14 to 18 days about 50 different spots were visible on the plates. In addition to those previously noted the anthraquinones, fallacinol, fallacinal, parietinic acid, and dermocybin and the phenol, 2,3,5-trihydroxytoluene were identified. The distribution of the phenols and anthraquinones on a thin layer chromatographic plate made from the ether extract of a 14-day-old culture of *E. echinulatum* in Czapek-Dox medium may be noted in Fig. 1. Figure 2



IDENTIFIED PHENOLS

1. 2,4,6-Trihydroxybenzoic acid
2. Gallic acid
3. Phloroglucinol
4. 3,5-Dihydroxybenzoic acid
5. Acetylphloroglucinol
6. Protocatechuic acid
7. Protocatechualdehyde?
8. Caffeic acid
9. Orcinol
10. *p*-Hydroxycinnamic acid
11. *p*-Hydroxybenzoic acid
12. Orsellinic acid
13. 2,3,5-Trihydroxytoluene
14. *p*-Hydroxybenzaldehyde

Fig. 1—Two dimensional thin layer chromatogram showing phenols and anthraquinones extracted with ether from a 14 day Czapek-Dox culture medium of *E. echinulatum*. The developing solution for the first direction (I) was $CHCl_3$-COOH (8:2) and for the second (II) dibutylether-CH_3COOH (10:1).



SPECIFIC ANTHRAQUINONES

1. Dermocybin	0.15/0.11*	11. Physcion	0.79/0.79
2. Questinol	0.19/0.17	12. Erythroglaucin	0.79/0.87
3. Fallacinol	0.26/0.33	a.	0.09/0.11 rose†
4. Questin	0.32/0.33	b.	0.09/0.17 rose (possibly dermoglaucin)
5. Dermolutein	0.31/0.44		
6. Endocrocin	0.25/0.56	c.	0.17/0.67 strong violet
7. Parietinic acid	0.50/0.64	d.	0.37/0.33 strong violet
8. Fallacinol	0.66/0.66	e.	0.61/0.54 rose
9. Emodin	0.57/0.77	* Rf-values I/II	
10. Catenarin	0.61/0.85	† Fluorescens at 366 nm	

Fig. 2—Two-dimensional thin layer chromatogram showing anthraquinones from 14-day Czapek-Dox culture medium of *E. echinulatum*. The developing solution for the first direction (I) was benzene-ethylformate-HCOOH (75:24:1) and for the second (II) dibutylether-CH_3COOH (10:1).

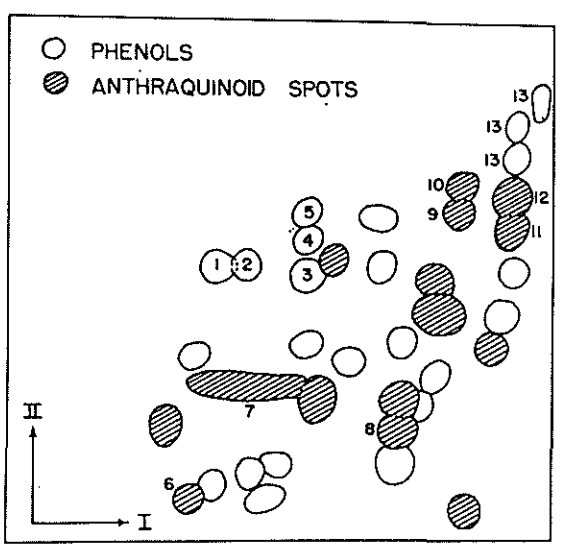
shows the distribution of the anthraquinones only after they were separated from a 14-day culture.

The culture media turned from colorless to a reddish yellow within 10 days after inoculation. After 2 to 3 weeks with the asparagine-glucose medium and 1 to 2 months with the Czapek-Dox medium the culture solutions became deep brown to almost black. The pH of the Czapek-Dox medium decreased from an initial pH of 6 to almost 4 and remained there for 4 months. In the glucose-asparagine medium the pH decreased from 6 to 5 in about 10 days and then increased during autolysis and polymer formation to about 7.8. From 0.8 to 1.0 g/liter of the polymer was recovered from the Czapek-Dox medium and 3.0 to 3.5 g/liter from the glucose-asparagine medium.

Reductive degradation of the polymers with Na-amalgam yielded about 45 to 70% ether-soluble products. The higher amounts were released from the low N polymers obtained from the Czapek-Dox medium. Thin layer chromatography of the ether extracts separated many phenols and anthraquinones (Fig. 3). In addition to anthraquinones, anthrones and anthranols were probably present. These could be formed by the reductive procedure since Na-amalgam degradation of the anthraquinones, emodin, endocrocin, and physcion partially converted the compounds to anthrones, anthranols, and several unidentified substances. Therefore, a conclusive comparison of the spots with known compounds was not possible. Furthermore, a reduction of the anthraquinones to anthracene derivatives may occur. From both pure anthraquinones and the polymer several spots with a pronounced blue fluorescein under UV-light at 254 nm were observed. These were located near the solvent front. With less polar solvents, e.g., cyclohexane-$CHCl_3$ (80:20) or with *n*-heptane, these compounds had similar but not identical R_f-values to anthracene. The spots reacted positively with iodine vapor and with formaldehyde-H_2SO_4 reagent (13).

Treatment of the polymers with Na-dithionite solution released largely anthraquinones. No anthrones or anthranols were noted. The distribution of the spots on thin-layer plates upon chromatography with ethylformate-benzene-HCOOH in the first and with dibutylether-acetic acid in the second direction is shown in Fig. 4.

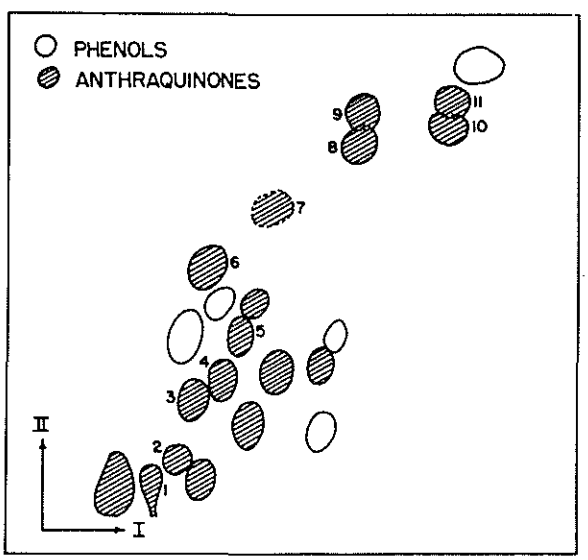
The N content of the polymer ranged from about 1% in the Czapek-Dox medium to 4.5% in the asparagine-glucose medium. About 50% of the total N in the polymers was released by acid hydrolysis. Two dimensional separation of the amino acids in the hydrolysate on paper by electrophoresis and descending chromatography yielded arginine, histidine, glycine, alanine, proline, methionine, tyrosine, valine, phenylalanine, leucine and/or isoleucine, and 3 non-identified amino acids. Hydrolysates from the polymers of both culture media showed the same qualitative distribution of the amino acids. The quantitative amino acid analysis of the hydrolysates is presented in Table 1. The amounts of amino acids in μmole/1 g of polymer and in percent of the individual amino acids from the total amino acids plus ammonia are given. For comparison this table also shows the amino acids from hydrolysates of *Stachybotrys chartarum* polymers isolated from a glucose-asparagine culture medium and from the fungus cells, respectively. The amounts of amino acids from the glucose-asparagine medium varied



IDENTIFIED PHENOLS AND ANTHRAQUINONES

1. Resorcinol
2. Orcinol
3. *p*-Hydroxycinnamic acid
4. *p*-Hydroxybenzoic acid
5. Orsellinic acid
6. Questinol?
7. Endocrocin
8. Physcion anthrone
9. Emodin
10. Catenarin
11. Physcion
12. Erythroglaucin
13. Possibly anthracene derivatives

Fig. 3—Thin layer chromatogram of phenols and anthraquinoid compounds released during Na-amalgam reductive degradation of the humic acid-type polymers of *E. echinulatum*. The developing solution for direction (I) was $CHCl_3$COOH (8:2) and for (II) dibutylether-CH_3COOH (10:1).



IDENTIFIED COMPOUNDS

1. Dermocybin
2. Questinol
3. Fallacinol
4. Questin?
5. Dermolutein
6. Endocrocin
7. Parietinic acid?
8. Emodin
9. Catenarin
10. Physcion
11. Erythroglaucin

Fig. 4—Thin layer chromatogram of anthraquinones released from *E. echinulatum* humic acid-type polymer by treatment with Na-dithionite solution. The developing solution for direction (I) was benzene-ethylformate-HCOOH (75:24:1) and for (II) dibutylether-CH_3COOH (10:1).

Table 1—Distribution of amino acids in 6*N* HCl hydrolysates of humic acid-type polymers from *E. echinulatum* and *Stachybotrys chartarum*

Amino acid	*E. echinulatum* polymer*				*S. chartarum* polymer†			
	Asparagine-glucose medium		$NaNO_3$-glucose medium		From culture medium		From cells	
	μmoles‡	%§	μmoles	%	μmoles	%	μmoles	%
Aspartic acid	141.0	15.5	11.5	9.0	63.1	18.5	148.8	11.3
Threonine	57.5	6.3	8.5	6.7	28.1	8.2	67.9	5.2
Serine	68.0	7.4	16.0	12.5	20.0	5.9	69.6	6.1
Glutamic acid	123.5	13.5	13.7	10.7	28.1	8.2	111.0	8.5
Proline	52.0	5.7	11.1	8.7	16.3	4.8	70.7	5.4
Glycine	89.0	9.7	15.8	12.4	23.1	6.8	109.0	8.3
Alanine	65.0	7.1	11.0	8.6	23.8	7.0	118.1	9.0
Valine	29.5	3.2	3.4	2.7	10.6	3.1	43.8	3.3
Methionine	1.0	0.1	0.2	0.2	1.9	0.6	15.9	1.2
Isoleucine	22.7	2.5	2.9	2.3	8.1	2.4	43.4	3.3
Leucine	38.0	4.2	3.6	2.8	25.0	7.3	96.5	7.3
Tyrosine	25.5	2.8	1.8	1.4	5.6	1.6	31.0	2.4
Phenylalanine	24.5	2.7	3.5	2.7	7.5	2.2	39.4	3.0
Histidine	11.1	1.2	0.8	0.6	5.0	1.5	25.7	2.0
Lysine	31.5	3.4	4.2	3.3	15.2	4.5	17.2	1.3
NH_4^+	136.0	14.9	19.9	15.6	60.4	17.7	296.3	22.5

* The E. echinulatum polymer from the asparagine-glucose medium contained 4.6% N and 49.9% hydrolyzable N. The polymer from the $NaNO_3$-glucose medium contained 1.07% N and 49.8% hydrolyzable N.
† S. chartarum was cultured in asparagine-glucose medium. The polymer from the medium and the cells contained 5.28 and 5.44% N and 50.6 and 57.9% hydrolyzable N, respectively.
‡ μmole amino acid in 1 g of polymer.
§ Percent of total measured amino acids plus ammonia.

from 1 to 141 μmole/g and from Czapek-Dox medium 0.2 to 16 μmole/g of the various amino acids were found. Similar quantities were present in the *S. chartarum* polymers.

DISCUSSION

Eurotium echinulatum cultured on glucose as a carbon source synthesized numerous phenols and anthraquinones. The phenols, *p*-hydroxycinnamic, *p*-hydroxybenzoic, and orsellinic acids and acetyl-phloroglucinol are formed either through the shikimate or the acetate-malonate pathway (3). These are transformed into other phenols by degradation of the C_3-side chain, decarboxylation, oxidation of the methyl to carboxyl groups and by introduction of additional OH-groups into the ring. These transformations follow similar sequences as noted for *Epicoccum nigrum* (9) and *Stachybotrys chartarum* (22).

The anthraquinones, endocrocin and emodin, are also formed through the polyketide pathway from nonaromatic precursors (29). Emodin could either originate from endocrocin by decarboxylation or by a separate biosynthesis directly from polyacetates (36, 37). A possible sequence of the transformations of emodin and endocrocin into other anthraquinones found in the *E. echinulatum* culture media is given in Fig. 5. According to Steglich (36) dermolutein would be derived from endocrocin; however, as indicated by Steglich et al. (35) the other anthraquinones methylated in the 8-position, questin and questinol, are probably also derived from endocrocin. Physcion is formed by methylation of emodin in position 6. This compound can be hydroxylated to form erythroglaucin, dermoglaucin, and dermocybin. Stepwise oxidation of the 3-methyl group of physcion, forms fallacinol, fallacinal, and parietinic acid. During the period of rapid polymer formation the phenols and anthraquinones largely disappeared from the medium. At the termination of incubation only small amounts of physcion, fallacinol, and fallacinal (and possibly questin and questinol) were still present in free forms and the simple phenols had largely disappeared. It is highly probable that these phenols and anthraquinones were linked into the

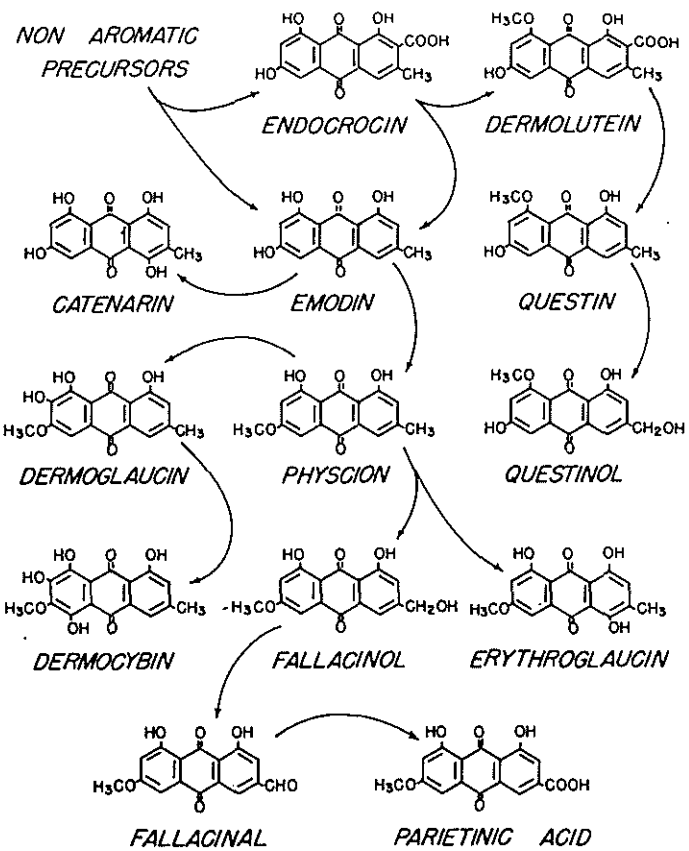


Fig. 5—Possible transformations in the formation of anthraquinones by *E. echinulatum*.

developing polymers. No anthraquinones or phenols could be extracted with ether or alcohol from the polymers even after exhaustive methylation with diazomethane. Reduction of the polymers with Na-amalgam yielded phenols, anthraquinones, anthrones, anthranols, and probably anthracene derivatives. The anthrones, anthranols and the anthracenes, however, appear to be formed by the reductive degradation treatment and are not present as such in the polymers as supported by the release of anthraquinones only upon Na-dithionite treatment. According to Shibata et al. (34) and Ogihara et al. (36) the reductive cleavage with dithionite splits the latter into the single anthraquinones. Piper and Posner (30) using dimer model phenol compounds found that the Na-amalgam reduction reaction splits either linkages with the release of free phenols.

In addition to phenols and anthraquinones, amino acid compounds were important constituents of the pigments as indicated by the release of amino acids upon acid hydrolysis. They represented from 20 to 50% of the hydrolyzable N. The 6*N* HCl hydrolysis solubilizes more organic material and N than can be accounted for as free amino acids and ammonia. It is possible that some fragmentation of the molecules takes place and much of the N not present as free amino groups or ammonia could be in the form of amino acid compounds linked to phenyl rings through the amino group. This linkage is resistant to 6*N* HCl hydrolysis (30, 31).

The N source used in the culture media influenced the content of the polymers, namely, 1% N with $NaNO_3$ and 4.5% N with asparagine. About 50% of the N was released

upon 6*N* HCl hydrolysis of both the high and low N polymers and the same amino acids were released upon hydrolysis of both. Quantitatively, the amounts of amino acids per gram of the polymers were much higher in the polymer from the asparagine than from the $NaNO_3$ medium. When the individual amino acids were calculated in percent of the total measured plus the ammonia some amino acids such as threonine, valine, isoleucine, phenylalanine, and lysine showed similar values while others such as aspartic acid, serine, proline, glycine, and tyrosine were different. This indicates that the N source exerts an important influence on the amino acid composition of the fungal polymers. Therefore, definite conclusions as recently published by Ortiz de Serra et al. (28) based on single fungal polymer preparations prepared with one N source (asparagine) cannot properly be made since the amount and the composition of the hydrolyzable amino acid fraction of fungal humic acids vary greatly and are dependent upon the culture conditions during their formation. Also the fungus species is important as indicated by a different percentage composition of the hydrolyzable amino acid fraction from *S. chartarum* polymers.

The synthesis of humic acid-like polymers by a soil fungus with phenols and anthraquinones as constituent units suggests some new aspects of soil humus formation. Further experiments are needed to explain the mechanism of polymer formation and the significance of these pigments with respect to soil humus formation under natural conditions.

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