SHORT COMMUNICATION

DEGRADATIVE OXIDATION PRODUCTS OF THE MELANIN OF *ULOCLADIUM ATRUM*

G. ALMENDROS

Instituto de Edafología y Biología Vegetal, Serrano 115, 28006 Madrid, Spain

A. T. MARTÍNEZ

Instituto de Microbiología "Jaime Ferrán", Joaquín Costa 32, 28006 Madrid, Spain

F. MARTÍN and F. J. GONZÁLEZ-VILA

Centro de Edafología y Biología Aplicada del Cuarto, Apdo. 1052, Sevilla, Spain

(Accepted 20 February 1985)

It is known that many soil fungi synthesize brown, large aromatic polymers (fungal melanins), chemically related to soil humic acids (Martin and Haider, 1971; Schnitzer and Neyroud, 1975). Since the chemical constituents (or at least, their degradation products) and the physicochemical properties of fungal melanins are comparable to those of humic acids, it is possible that fungal metabolism may represent an important source of constituents for humus formation.

Production of humic acid-like compounds by *Ulocladium* Preuss was reported by Almendros *et al.* (1985). The alkali-soluble melanin of *Ulocladium atrum* Preuss was extracted together with a green pigment having similar properties to the Pg fraction found by Kumada and Sato (1962) in soil humic acids, and of a fungal origin confirmed by Kumada and Hurst (1967). This species also produced a significant proportion of a soluble polysaccharide (Martínez *et al.*, 1984), and is an interesting organism for studies of synthesis and transformation of soil organic matter.

The fungus was grown (30°C; 200 rev min⁻¹) in a medium containing malt extract, 30 g; NaNO₃, 2 g; K₂HPO₄, 1 g; KCl, 0.5 g; MgSO₄, 0.5 g; FeSO₄, 10 mg; water, 1 l (pH-5). After 2 weeks, the freeze-dried fungal biomass was washed with water and ether, the melanin extracted with 0.1 N NaOH under N₂, precipitated with HCl and purified by centrifugation in alkaline pH (20,000 rev min⁻¹), reprecipitation and electrodialysis.

Melanin was degraded by two different methods: persulfate in acidic medium, and alkaline permanganate oxidations, the former reagent being milder than the latter. Persulfate oxidation was carried out according to Martin *et al.* (1981): 200 mg of freeze-dried melanin were oxidized with a saturated K₂S₂O₈ solution in a digestion bomb at 140°C. The same weight of melanin was diazomethane-methylated, in order to protect phenolic groups on aromatic rings (Matsuda and Schnitzer, 1972), and oxidized at 90°C with KMnO₄: 70 ml of 4% KMnO₄ solution were progressively added to the sample in 4-ml aliquots every 15 min. After cooling, the mixture was acidified and the excess of permanganate was destroyed with Na₂S₂O₅.

The digests obtained by both oxidation procedures were extracted with ethyl acetate in a liquid-liquid extractor for 12 h. The organic solvent was dehydrated with anhydrous CaSO₄, evaporated at 40°C, the oxidation products were suspended in methanol and methylated three times with ethereal diazomethane (Schnitzer, 1974).

The degradation compounds were separated and identified using a Hewlett-Packard 5992 B GC-MS com-

puter system equipped with a 25-m cross-linked fused silica capillary column coated with OV-101. Oven temperature was programmed from 100 to 270°C at a heating rate of 6°C min⁻¹. The flow rate of the carrier gas (He) was adjusted to 1 ml min⁻¹, and the ionizing voltage was 70 eV. Chromatographic peaks were identified by searching computer libraries and bibliographic data, and when possible, by comparison with the standard compounds.

Potassium persulfate degraded 40.5% of the initial weight, while no residue was obtained with KMnO₄. Table 1 shows the yields of the oxidation products (Fig. 2) as percentages of the total volatile methylated extract. Quantitative yields respective to the initial weight of the sample were not calculated, following the criteria of Maximov *et al.* (1977).

Permanganate oxidation of *U. atrum* methylated melanin yielded similar compounds to those described for soil humic acids (Matsuda and Schnitzer, 1972), but their quantitative proportions differed greatly. Methyl esters of normal and branched fatty acids were the major peaks (Fig. 1). On the other hand, persulfate oxidation released the most interesting compounds, the major oxidation products being methyl palmitate, fluorene, nonacosane and biphenyl. It is noticeable that there were significant proportions of fluorene (9), naphthalene (1) and a compound having similar mass spectra to a trimethylstyryl naphthalene (31), because it seems that the production of such compounds would be more characteristic of a more drastic degradative method. Similar compounds, when obtained by Zn-dust distillation, are frequently considered as artifacts, but in the present case, the mild character of persulfate oxidation suggest that they were indeed present in the humic-like molecule, though probably loosely linked to the polymer. Biphenyl (6) is not a typical degradation product of soil humic acids, but the presence of dehydrodiveratric acid, a polysubstituted biphenyl, has been frequently reported in these soil components (Schnitzer and Khan, 1972).

The occurrence of some of the above-mentioned compounds could be explained in relation to the biosynthetic pathways of compounds derived from binaphthyl or perylene, whose structure and reactions were studied in *Daldinia concentrica* (Bolt. ex Sr.) Ces. & de Not., a fungus producing pigments similar to the green fraction of *Ulocladium* melanin (Allport and Bu'Lock, 1960). A dihydroxyacetophenone comparable to compound 5 was described in the residue from steam-distillation of chromamone in *D. concentrica*, and has also been found in "fungal humic acids" (Schnitzer and Neyroud, 1975).

Table 1. Relative abundance of the identified compounds

No.	Compound	Degradative methods	
		K ₂ S ₂ O ₈	KMnO ₄
1.	Naphthalene	0.8	0.0
2.	Dimethyl pimelate	0.0	1.1
3.	Trimethyl methoxy-trioxo-hexahydrotriazine	0.6	0.0
4.	Dimethyl suberate	0.0	4.3
5.	Dimethoxy-acetophenone	0.0	1.9
6.	Biphenyl	5.0	0.0
7.	Methyl dodecanoate	0.0	5.3
8.	<i>p</i> -Toluenesulphonic acid methyl ester	0.0	0.4
9.	Fluorene	7.1	0.0
10.	Dichloromethoxy-benzenecarboxylic acid methyl ester	0.0	3.8
11.	C14 branched fatty acid methyl ester	0.5	0.0
12.	Methyl tetradecanoate	2.2	0.9
13.	1,2,3-Benzenetricarboxylic acid trimethyl ester	0.0	0.1
14.	C15 branched fatty acid methyl ester	0.8	0.0
15.	Octadecane	7.0	0.0
16.	Methyl pentadecanoate	1.8	0.7
17.	1,2,4-Benzenetricarboxylic acid trimethyl ester	0.0	0.7
18.	Diisobutyl phthalate	1.2	0.0
19.	C16 branched fatty acid methyl ester	0.0	1.6
20.	Methyl hexadecenoate (palmitoleate?)	2.0	1.0
21.	Methyl hexadecanoate (palmitate)	16.2	35.8
22.	C17 branched fatty acid methyl ester	0.5	0.0
23.	C17 branched fatty acid methyl ester	1.0	14.3
24.	Eicosane	4.5	0.6
25.	Methyl heptadecanoate	1.1	0.5
26.	C21 branched alkane	0.0	1.8
27.	Methyl octadecadienoate (linoleate?)	2.3	0.0
28.	Methyl octadecenoate (oleate)	4.9	0.0
29.	Heneicosane	0.6	0.0
30.	Methyl octadecanoate	7.1	4.9
31.	Trimethylstyryl naphthalene	0.8	0.0
32.	C19 branched fatty acid methyl ester	0.0	1.9
33.	Docosane	0.8	0.5
34.	Methyl nonadecanoate	0.5	0.0
35.	Tricosane	0.7	0.0
36.	Methyl eicosanate	0.6	0.0
37.	Tetracosane	0.7	0.0
38.	Methyl eicosenate	0.7	0.0
39.	Diciclohexyl phthalate	4.0	0.0
40.	Pentacosane	0.6	0.0
41.	Methyl docosanate	0.8	0.0
42.	Hexacosane	0.8	0.0
43.	Methyl tricosanate	0.5	0.0
44.	Heptacosane	4.3	0.0
45.	Methyl tetracosanate	0.4	0.0
46.	Octacosane	0.8	0.0
47.	Nonacosane	7.1	0.0
	Unidentified	8.3	16.2

Benzenecarboxylic acids represented a small proportion of the permanganate digest, and phenolic acids were absent, excepting a chloro-derivative (10). A similar compound, described by Ogner (1973) in a nonhydrolyzable humic residue, was considered as reaction product of the hydrochloric acid. Sulphur-containing aromatic molecules were detected (8) and were also obtained from soil fulvic acids (Griffith and Schnitzer, 1976), though such a compound could also arise from diazald residues in the methylating agent. In the same way, the dialkyl phthalates (18, 39) are frequently considered as impurities, and the small peak (Fig. 1) after compound 9 (persulfate) and after compound 5 (permanganate) is not a degradation product, but the 2,6-di-*t*-butyl 4-methylphenol present as a stabilizer in the ethereal diazomethane.

N-alkanes were abundant in the persulfate digest, mainly the C₁₈, C₂₉, C₁₉ and C₂₇, dominating the hydrocarbons with high molecular weights (>20-C). In the case of fatty acids (as methyl esters), even C numbers dominated, palmitic acid being the most abundant; unsaturated chains (mainly the 18-C ones) were present in significant amounts. A high

proportion of fatty acids was also reported by Martin *et al.* (1983) after persulfate oxidation of four fungal melanins, but *U. atrum* melanin showed a higher diversity of degradation products.

The results obtained by the two oxidative methods suggest that, although alkaline permanganate oxidation showed differences to soil humic acids, this technique is probably too drastic for this immature, humic-like polymer. Aromatic compounds detected after persulfate oxidation were probably destroyed and the chromatogram presented a relatively reduced number of peaks, the majority of them corresponding to compounds without structural significance.

The melanin produced by *U. atrum* after 14 days of incubation could be considered as a polymer with a low condensation and aromaticity degree, rather different to soil humic acids, in agreement with the results of Almendros *et al.* (1985). Probably, the major proportion of aliphatic compounds, the green pigments and the polycyclic aromatic hydrocarbons are not constituents of the polymer, though most of them are not easily separable by physical methods. Nevertheless, the preferential biodegradation of the ali-

Fig. 1. Chemical structure of the compounds obtained.

Fig. 2. Gas chromatographic separation of methylated products obtained by persulfate and permanganate oxidation.

phatic constituents and subsequent rearrangement and condensation of the aromatic network are typical effects of the "maturation" process of soil humus, and should increase the similarities between melanins and humic acids after its incorporation into soil.

REFERENCES

- Allport D. C. and Bu'Lock J. D. (1960) Biosynthetic pathways in *Daldinia concentrica*. *Journal of the Chemical Society*, pp. 654–662.
- Almendros G., Martínez A. T. and Dorado E. (1985) Production of brown and green humic-like substances by *Ulocladium atrum*. *Soil Biology & Biochemistry* **17**, 257–259.
- Griffith S. M. and Schnitzer M. (1976) The alkaline cupric oxidation of humic and fulvic acids extracted from tropical volcanic soils. *Soil Science* **122**, 191–201.
- Kumada K. and Hurst M. (1967) Green humic acids and its possible origin as a fungal metabolite. *Nature* **214**, 631–633.
- Kumada K. and Sato O. (1962) Chromatographic separation of green humic acids from podsol humus. *Soil Science and Plant Nutrition* **8**, 31–33.
- Martín F., Saiz-Jiménez C. and González-Vila F. J. (1981) The persulfate oxidation of a soil humic acid. *Soil Science* **132**, 200–203.
- Martín F., González-Vila F. J. and Martín J. P. (1983) The persulfate oxidation of fungal melanins. *Soil Science Society of America Journal* **47**, 1145–1148.
- Martín J. P. and Haider K. (1971) Microbial activity in relation to soil humus formation. *Soil Science* **111**, 54–63.
- Martínez A. T., Martínez M. J. and Almendros G. (1984) Polisacáridos y pigmentos aromáticos producidos por *Ulocladium atrum*. *Resúmenes de la II Reunión Conjunta de Micología*, Barcelona, p. 91.
- Matsuda K. and Schnitzer M. (1972) The permanganate oxidation of humic acids extracted from acidic soils. *Soil Science* **114**, 185–193.
- Maximov O. B., Shvets T. V. and Elkin Yu. N. (1977) On permanganate oxidation of humic acids. *Geoderma* **19**, 63–78.
- Ogner G. (1973) Oxidation of nonhydrolyzable humic residue and its relation to lignin. *Soil Science* **116**, 93–99.
- Schnitzer M. (1974) The methylation of humic substances. *Soil Science* **117**, 94–102.
- Schnitzer M. and Khan S. U. (1972) *Humic Substances in the Environment*. Dekker, New York.
- Schnitzer M. and Neyroud J. A. (1975) Further investigations on the chemistry of fungal "humic acids". *Soil Biology & Biochemistry* **7**, 365–371.
- Schnitzer M., Ortiz de Serrá M. I. and Ivarson K. (1973) The chemistry of fungal humic acid-like polymers and of soil humic acids. *Soil Science Society of America Proceedings* **37**, 229–236.