^{13}C Nuclear Magnetic Resonance Spectra of Fungal Melanins

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The ^{13}C resonance spectra of fungal melanins from *Aspergillus niger*, *Eurotium echinolatum*, and *Stachybotrys chartarum* are reported. The spectra were taken in 5% W/W solution of the substances in 0.1 N NaOD in D_2O using the Fourier-transform-technique. The spectra and possible assignments of the bands are discussed. The similarities of these spectra to those of soil humic acids are unexpectedly small.

Introduction

Plant and fungal melanins usually have structures based on catechol as opposed to the indole melanins of animal origin. However, it appears that some fungal melanins such as those from *Aspergillus niger*, *Stachybotrys chartarum* and *Eurotium echinulatum* do not belong to these two types. These pigments are complex quinonoid polymers formed by oxidative polymerization and linked to cell wall materials.

Information about the chemical structure of fungal melanins has been obtained so far by chemical degradation techniques [1–3], which yield limited information only or tend in some cases to produce artifacts. On the other hand, spectrometric methods have failed to provide detailed information on the structure of these biopolymers [4, 5].

Fairly well resolved ^{13}C resonance spectra have been obtained for humic acids [6]. In the present paper, the first application of ^{13}C NMR to fungal melanins is given.

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Material and Methods

The fungi *Aspergillus niger* and *Eurotium echinulatum* were isolated from a Spanish vertisol. Methods of cultivation and isolation of melanin, as well as some of its physical and chemical characteristics were reported previously [7, 8]. The pigments of *Aspergillus niger* were extracted from spores and the pigments of *Eurotium echinulatum* from mycelium and also from a culture medium. Mycelial *Stachybotrys chartarum* melanin was provided by Dr. K. Haider, Braunschweig, Germany. Elementary compositions of the melanins are given in Table I. The ^{13}C spectra were taken of 5% W/W solutions of melanins in 0.1 N NaOD as described for soil humic substances [6]. A Varian XL-100-15 spectrometer operating at 25.2 MHz was used to obtain the spectra by the Fourier-transform-technique. The protons are noise decoupled. 250 blocks of 1000 transients were accumulated in the long-term-averaging-mode. The resolutions of the spectra was ± 10 Hz (approximately 0.5 ppm). The spectrometer was locked to an external Fluorine-19-lock. Shift values quoted are given in the TMS scale, referenced to a coaxial internal neat TMS capillary. The position of the TMS signal was obtained in a separate run with accumulation of 1000 transients. No attempts to correct for magnetic susceptibility effects were made.

Table I. Relative contents of carbon, hydrogen and nitrogen in percent by weight (Oxigen has assumed to comprise the remainder) and the percentage of ash yielded from fungal melanins.

	C	H	N	O	Ash
A. niger (spores)	45.2	5.4	2.7	46.7	1.2
S. chartarum (mycelium)	56.0	5.9	4.9	33.2	1.5
E. echinulatum (medium)	54.2	4.1	4.2	37.5	2.2
E. echinulatum (mycelium)	55.2	3.8	4.7	36.3	1.8

Results

Fig. 1 shows the ^{13}C NMR spectra of the fungal melanins. The maximal noise of the baseline is indicated in the figure. For *S. chartarum* a part of the spectrum was recorded with higher amplitudes. Then centers of the signals obtained are listed in

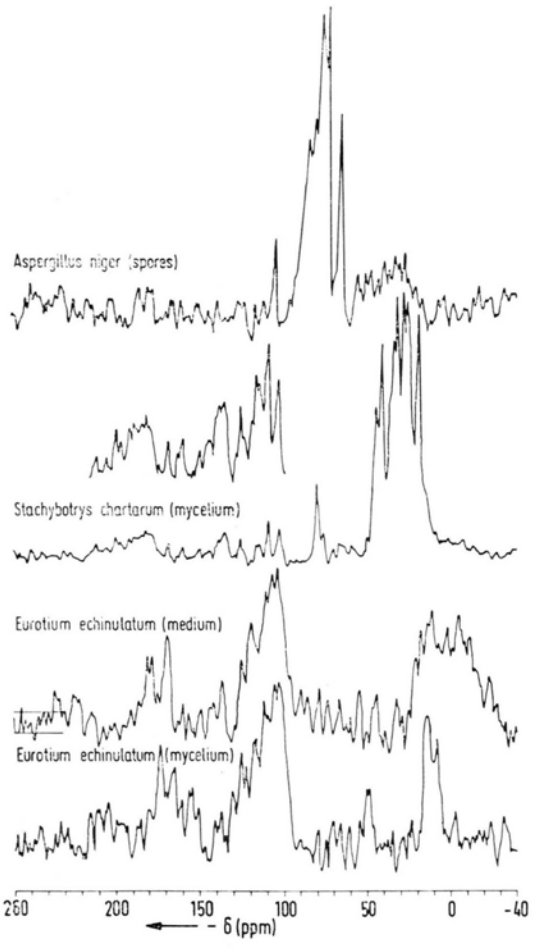

Fig. 1. ^{13}C NMR spectra of fungal melanins. Intensities in arbitrary units. δ is the chemical shift in ppm on the TMS scale. The approximate baseline noise is indicated by two horizontal bars in the second spectrum from the bottom. For *S. chartarum* a part of the spectrum was recorded with higher amplitudes.

Table II. ^{13}C chemical shifts of fungal melanins and plausible assignments.

$-\delta$	*A. niger* (spores)	*S. chart.* (myc.)	*E. ech.* (med.)	*E. ech.* (myc.)	
			230	210	C=O ketones
200		198			
190					quinones COOH
180	180	180	180		
170		168	168	170	
160		160			aromatic and heteroaromatic rings
150					
140		140			
130			136	136	
120		124	120	120	
110		115			
		109			
100	104	102			polysaccharides
90					
80	80	79			
	75		75	75	
70	70				
60	65				
50			54	50	aliphatic carbons $\alpha,\beta,\gamma,-$C (amino acids) OCH_3
40	40	40	45		
30		31	30		
		25			
20		18			
10				10	

Table II. These signals may have more resolution; however, the low signal to noise ratio only allows the safe assignment of the bands listed in Table II. Plausible assignments are also indicated [10, 11]. These assignments could be improved by investigation of a series of model compounds in the same solvent. The absolute chemical shift can be assumed to be accurate to $\pm(5-10)$ ppm because of the high molecular weight, the viscosity of the solution, the neglect of magnetic susceptibility effects and the presence of carbon atoms with unpaired electrons. These unpaired electrons can be deduced from the black colour of the substances and moreover the unstructured signals have been found in preliminary ESR-experiments at g-values typical for carbon radicals. These radicals may be also responsible for the signals in the region of positive chemical shifts. In complex substances like melanins, one has to consider another complication. The molecules might consist of rigid and mobile regions in which only the carbon-13-nuclei of the flexible parts possess sufficiently long transversal relaxation times, T_2, to be observable in a high resolution spectrum. Consequently, it is hazardous to derive absolute concentrations for a certain class of carbon atoms from the intensities of the signals in different samples or to deduce from the absence of a signal the absence of a specific chemical structure. ^{13}C NMR spectra may thus only be used for the positive assignment of certain structural elements in a heterogenous polymer.

The spectrum of *A. niger* pigments (aspergillin) shows prominent signals in the aliphatic zone. These resonance lines probably belong to polysaccharides. *S. chartarum* melanin shows intense signals in the aliphatic region, which are probably due to alkyl

chains. Cultural and mycelial *E. echinulatum* melanins have similar ^{13}C NMR spectra. For the mycelial pigments, the aliphatic lines are more resolved. The aromatic regions in both spectra of *E. echinulatum* are more pronounced than in the spectra of other pigments. This may be due to the phenolic core and the peripherally linked anthraquinones (8).

Discussion

The ^{13}C NMR and also the pyrolysis mass spectra [9] suggest that aspergillin has the highest content of polysaccharides of all the investigated melanins. Nicolaus [5] reported that polysaccharides are bound to this melanin. Also nigeran, a polysaccharide, is produced by *A. niger*. However, other authors [1] have reported a very low amount of aliphatic compounds and a high amount of benzenecarboxylic and phenolic acids. This contradiction and the others reported below may be explained by the variability of different strains from the same fungi.

The *S. chartarum* melanin seems to be constituted by alkylbenzenes and alkylphenols as major components. For a similar melanin, Schnitzer and Neyroud [12] found phenolic, benzenecarboxylic and aliphatic acids as major components from the alkaline cupric oxide oxidation. The IR spectrum of this particular melanin reveals high concentrations of aliphatic and aromatic constituents [14], whereas the ^{13}C spectrum indicates that this melanin has a fairly homogeneous polymeric structure corroborating the pyrolysis mass results by Meuzelaar *et al.* [9].

The *E. echinulatum* pigments show stronger signals in the aromatic region than the other pigments discussed here. These lines appear at a slightly smaller δ value than most C-aromatic compounds. Most probably these resonances can be attributed to phenols and anthraquinones, though other hetero-aromatic compounds cannot be excluded. Phenols and anthraquinones were found in extracts from culture media after 14 – 18 days of incubation. These compounds are incorporated later into the polymer and recovered among the products of chemical degradation [8].

The ^{13}C NMR spectra from fungal melanins can be compared with the spectra from soil humic acids obtained under similar conditions [6]. The similarities of the spectra from both groups of substances are much smaller than would be expected from the similarities of the analytical data reported in the literature, and one should be very cautious in transferring structural information from fungal pigments to humic acids and *vice versa*.

At this stage of the investigation, the signal to noise ratio is poor and the interpretation of the spectra obtained must remain qualitative. Thus it appears unjustified to interprete the broad lines with further information drawn from chemical degradation studies. A better signal to noise ratio may be obtained in the future with apparatus employing higher magnetic fields and/or larger sample volumes.

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