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Note

Isolation of a mutagenic fraction from aqueous extracts of the wild edible mushroom *Lactarius necator* (a preliminary note)

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Several members of the mushroom genus *Lactarius* have been found to contain mutagenic compounds in the Ames *Salmonella* assay[1]. In subsequent studies members of other genera of edible mushrooms also gave positive results in microbial mutagenicity tests[2]. Therefore, it was considered necessary to characterize further the mutagenic agents in the mushrooms in order to arrive at a firmer basis of risk estimation. The first mushroom from which the mutagenic agent was isolated was *Lactarius necator*. Extracts of this mushroom gave the strongest mutagenic response in the *Salmonella* assay, although *L. necator* is rated as a first class edible mushroom in some countries. The chromatographic fractionations were monitored with the tester strain TA100, starting with crude aqueous extract and ending up with 400 μg of the pure crystalline compound.

EXPERIMENTAL

High-performance liquid chromatography (HPLC)

The equipment consisted of two M-6000 A pumps, a M-660 gradient programmer, an U6K injector, a μBondapak semiprep column (7.8 × 300 mm), a Radi-pak C_{18} column (8 × 100 mm with 10-μm particles) in a RCM-100 module and a dual-channel M 440 UV-visible detector (all from Waters Assoc., Milford, MA, U.S.A.).

Preparation of mushroom extract

The *Lactarius necator* samples were collected in September 1981 in Southern Finland and stored at −20°C. A crude extract was prepared by boiling 400 g of homogenized mushrooms for 20 min in 1 l of distilled water. The extract was filtered through filter-paper in a Buchner funnel and refiltered through a 0.5-μm HA filter (Millipore, Bedford, MA, U.S.A.).

Fractionations

In order to locate the mutagenic activity, a 1-ml sample of aqueous extract was injected into a liquid chromatograph equipped with a μBondapak column. The chromatography was carried out with a linear gradient from 100% water to 100% methanol, both containing 10 m*M* $NH_4H_2PO_4$ buffer (pH 5.5), for 20 min at a flow-rate of 2 ml/min. Fractions of 2.0 ml were collected in this and all subsequent preparative runs. The μBondapak C_{18} column was replaced by a Radi-pak C_{18} column after it was shown with parallel separations that the latter was more efficient for this separation.

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Extraction of aqueous extract

After thus locating the activity in the less polar part of the chromatogram, 4 l of aqueous extract were extracted overnight in a continuous extractor with freshly distilled diethyl ether. A 100-μl volume of the 400 ml ether extract was dried with a stream of nitrogen, reconstituted with 100 μl methanol, chromatographed and the fractions were tested for mutagenic activity. The rest of the ether extract was concentrated under reduced pressure to 100 ml and treated with 50 ml 1 % $NaHCO_3$. The $NaHCO_3$ solution was acidified with dilute H_3PO_4 to pH 5.5 and extracted twice with 50 ml diethyl ether. The combined ether extracts were concentrated into a 250-μl oily residue and injected into the HPLC instrument. The gradient was run under the conditions above and fractions were collected and tested for mutagenic activity as above. The compound crystallized from the active fraction as bright red crystals during the frozen storage.

Mutagenicity tests

Tests for mutagenicity were performed using the standard Ames procedure[3] with the tester strain TA100 (ref. 4). Metabolic activation was not included. The results were considered positive if the number of induced revertants was at least four times the spontaneous background. The tests were carried out with three duplicates. All the reagents were shown not to be responsible for the mutagenic activity.

Quantitation

The crystallized material was washed twice with 30 μl 50 % methanol–water. After drying in a nitrogen stream the material was found to weigh about 400 μg. It was then dissolved in 50 μl of 0.121 % (w/v) NaOH, as it was shown to be almost insoluble in water but to be capable of forming a water-soluble sodium salt. This solution was centrifuged, a minor insoluble residue was separated and the solution was used as standard for mutagenicity tests and, after acidification, for mass spectrometry.

Mass spectrometry

The mass spectra were obtained with a Jeol JMS-D100 mass spectrometer using electron impact ionization at 70 eV.

RESULTS AND DISCUSSION

The fraction having mutagenic activity had the same retention time in all chromatograms of the various extracts of *Lactarius necator* (Fig. 1). Thus it is unlikely that the substance is an artifact, even taking into consideration the specificity of microbiological tests and the fact that the activity was maintained quantitatively under all these manipulations of the sample.

The mass spectra of the compound (Fig. 2) suggest a molecular weight of 264, in agreement with the amount of NaOH required to dissolve a given amount of it. The microbiological mutagenicity test showed that the activity of the compound is comparable with aflatoxin B_1. The chemical nature of the compound is still uncertain as the much higher activity of the compound than expected led to the need for more of the material. Further data will be reported as soon as more material has been obtained.

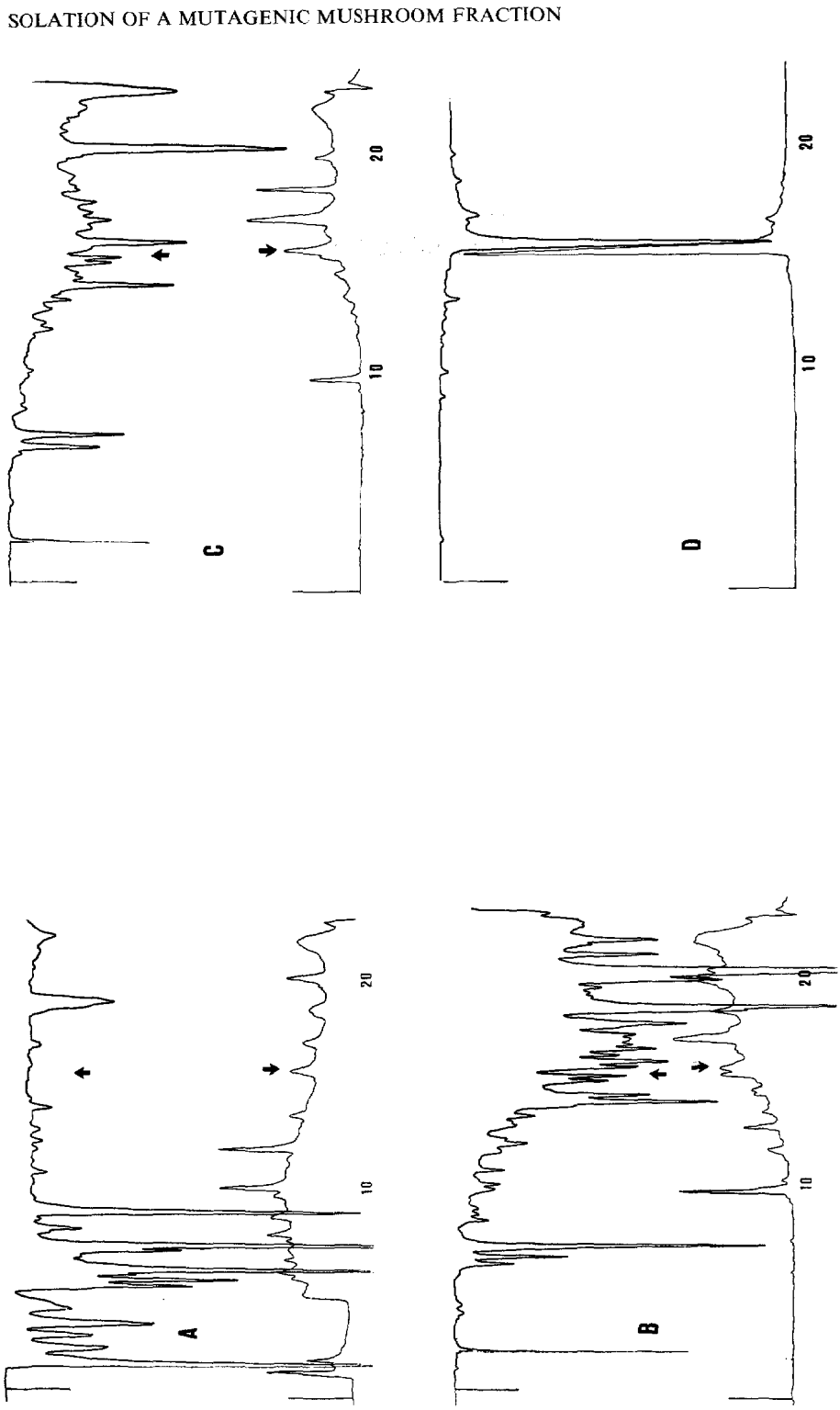

Fig. 1. Chromatograms of different extracts of *Lactarius necator*. The upper curve corresponds to 254 nm and the lower to 405 nm. For other conditions see text. The arrows denote the active fraction. a, 200 μl of aqueous extract (0.5 and 0.05 a.u.f.s.); b, 20 μl of 500 times concentrated ether extract (0.5 and 0.05 a.u.f.s.); c, 20 μl of 500 times concentrated ether extract of acidified $NaHCO_3$ extract (0.5 and 0.05 a.u.f.s.); d, 2 μg of the active compound (0.2 and 0.1 a.u.f.s.).

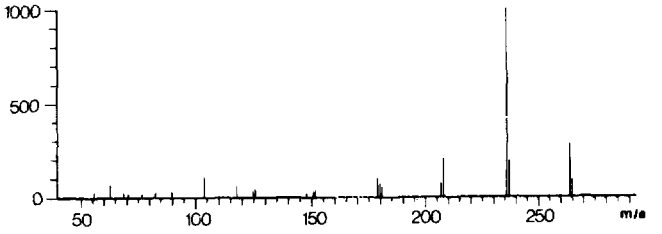

Fig. 2. EI mass spectra of the mutagen from *Lactarius necator*.

The concentration of the compound in mushrooms is about 2 mg/kg and it is stable for at least a day at room temperature at pH values between 1.0 and 11.0. The yield from the separation is low because only the crystallized material and not the precipitated material can be used for identification.

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