Reinvestigation of Disulfiram-Like Biological Activity of *Coprinus atramentarius* (Bull. *ex* Fr.) Fr. Extracts

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Abstract □ A ninhydrin-positive aqueous fraction isolated from *Coprinus atramentarius* (Bull. *ex* Fr.) Fr. showed significant disulfiram-like activity in mice. The isolation of this fraction from the mushroom crude extract and a convenient pharmacological test for following the physiological activity in each fraction are described.

Keyphrases □ *Coprinus atramentarius*—mushroom crude extract, aqueous fraction isolated, biological activity evaluated □ Mushrooms—*Coprinus atramentarius* crude extract, methanolic fraction isolated, biological activity evaluated □ Alcohol-potentiating activity—evaluated in methanolic fraction of extract of *Coprinus atramentarius* mushroom

Among several species of *Coprinus*, *Coprinus atramentarius* (Bull. *ex* Fr.) Fr. has given rise to the greatest interest because of its ability to sensitize humans to ethanol (1–4). However, controversial conclusions about this potentiation have been reported (5–8). Isolation of disulfiram from *C. atramentarius* (9) was not confirmed (10, 11). Although no disulfiram was detected, recent investigations unequivocally confirmed that polar extracts of *C. atramentarius* significantly potentiate the action of ethanol in mice (12, 13). Recently, an active constituent isolated from *C. atramentarius* increased blood acetaldehyde levels in mice (14).

To investigate the chemical structure of the active compound(s), a ninhydrin-positive fraction was isolated from a crude extract of *C. atramentarius*[1]. Its disulfiram-like activity in mice was determined using a convenient pharmacological test.

EXPERIMENTAL

Collection of Mushrooms—*Coprinus atramentarius* (Bull. *ex* Fr.) Fr. was collected[2] on lawns around Brussels during the autumn of 1972. The mushrooms were stored in ethanol at −18° for 1.5–3 months before extraction.

Apparatus—GLC was performed on an instrument[3] equipped with a dual flame-ionization detector and using 2-m × 4-mm (i.d.) glass columns packed with 80–100-mesh Chromosorb W coated with 10% SE 30.

The following conditions were used. For fatty acid methyl esters, the column temperature was 150–200° programmed at 5°/min and the carrier gas was 30 ml of nitrogen/min. For silyl derivatives of amino acids, the column temperature was 50–200° programmed at 7.5°/min and the carrier gas was 20 ml of nitrogen/min. In both cases, the injector and detector temperature was 250°.

Mass spectra were recorded after direct inlet in the source of the mass spectrometer[4].

PMR spectra[5] were obtained in deuterochloroform, with tetramethylsilane as the internal standard.

Ion-exchange chromatography was carried out on a two-column automatic amino acid analyzer[6], as described by the manufacturer. A cation-exchange resin[7] (sulfonated polystyrene) was used.

Evaporations were performed in a rotary evaporator at temperatures lower than 40°.

Reagents—For preparative TLC (thickness of 2 mm), 70 g of silica gel[8] or 60 g of cellulose[9] on 20 × 40-cm glass plates was used. Prepared chromatoplates were prewashed by developing with methanol–water (1:1 v/v) and then were activated by heating for 2 hr at 120° (silica gel) or 60° (cellulose).

Analytical TLC was carried out on the same adsorbents (thickness of 0.25 mm; 5 g on 20 × 20-cm glass plates).

Cellulose powder[10] was used for percolation of the ethanol–water extract (Scheme I).

All solvents and reagents were analytical grade[11].

Methods—*Extraction Procedures*—The general treatment of the crude extract in Procedure I is described in Scheme I.

In addition, two separate analyses were performed on crushed fresh mushrooms. In Procedure II, mushrooms (500 g) were treated by steam distillation under nitrogen to obtain a distillate volume of 200 ml. In Procedure III, mushrooms (500 g) were extracted by shaking with 1 liter of benzene. The benzene extract was dried on anhydrous sodium sulfate and evaporated to 10 ml.

Chromatographic Studies—The 10-ml final benzene extract of Procedure III was screened for disulfiram by partition paper chromatography as described by Simandl and Franc (9) and by TLC performed on silica gel, with benzene as solvent and 0.5% $PdCl_2$ aqueous solution as the reagent spray. It was not possible to identify disulfiram under these conditions, but an unknown compound was detected at the previously reported (9) R_f value corresponding to disulfiram. Therefore, the total benzene extract was subjected to preparative TLC on silica gel with benzene as solvent. The pertinent band at R_f 0.6 was eluted with chloroform to afford, after evaporation, 200 mg of a compound, mp 68–72°.

The mass spectrum of the isolated compound showed a molecular ion at *m/e* 882.

Glycerol was detected directly by dehydration to acrolein (15). After methanolysis, the methyl esters of the constitutive fatty acids were identified by GLC as methyl stearate and methyl linoleate in a ratio of 1:2. These two esters were purified by preparative TLC, using the same conditions as described for the initial compound. Further evidence of the chemical structure of these purified fatty acids methyl esters was obtained by direct comparison of their PMR and mass spectra with those of authentic samples.

These results suggested that the compound under investigation was a triglyceride containing one molecule of stearic acid for two of linoleic acid. Because of the biological inactivity of this triglyceride, the relative position of the fatty acids was not studied.

Five hundred milligrams of the ninhydrin-positive fraction (Fraction C_3, Scheme I) was isolated from 10 g of Fraction C_2 by means of preparative TLC, first on silica gel (100 plates) and then on cellulose

[1] *Note added in proof:* Since this paper was accepted, the disulfiram-like constituent of *C. atramentarius* was identified as N^5-(1-hydroxycyclopropyl)-glutamine by two groups: G. M. Hatfield and I. P. Schaumberg, *Lloydia*, **38**, 489(1975), and P. Lindberg, R. Bergman, and B. Wickberg, *J. Chem. Soc. Chem. Commun.*, **1975**, 946. These works confirmed our preliminary results, which located the active compound in a fraction containing amino acids.

[2] Identified at the Botanical Institute of the Free University of Brussels. Specimens, preserved at −18°, are available for inspection in this laboratory.

[3] Packard-Becker model 421.

[4] AEI model MS 902.

[5] Jeol 100 MHz.

[6] Technicon sequential multisample amino acid analyzer (TSM).

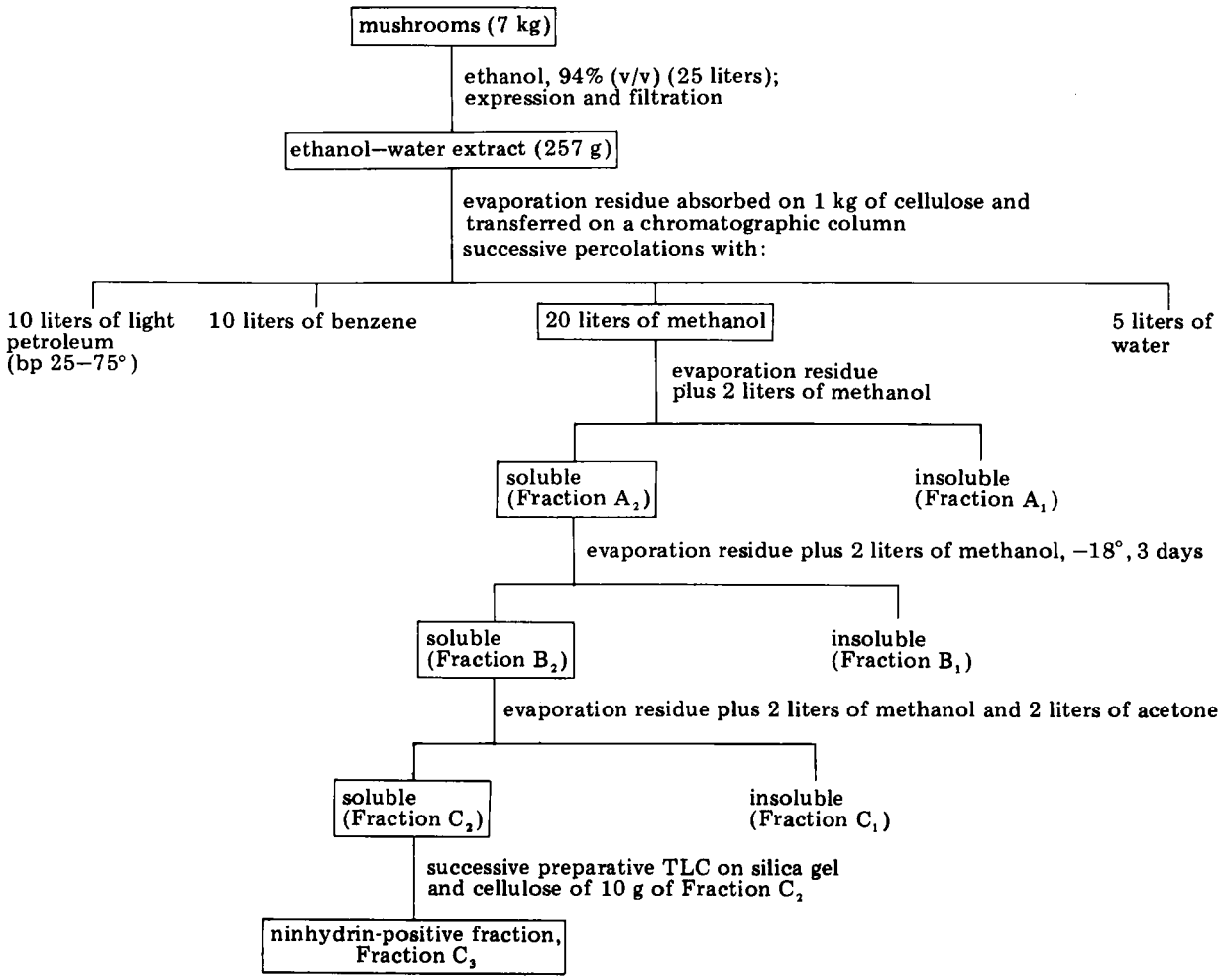
[7] Chromobeads C_3 Technicon resin.

[8] Silica gel H, Merck type 60.

[9] Cellulose Macherey-Nagel MN 300.

[10] Cellulose Macherey-Nagel MN 2100 ff.

[11] Merck.



Scheme I—Extraction Procedure I for the crude extract from C. atramentarius

(20 plates). The silica gel plates were developed with ethyl acetate–2-propanol–methanol–water (5:2:1.5:1.5 v/v). The cellulose plates were developed with the same solvent system in 1:1:1:1 (v/v) proportions. The physiologically active band (R_f 0.25 on silica gel and R_f 0.45 on cellulose) was eluted from the adsorbent by methanol–water (1:1 v/v).

Identification of the main amino acids present in Fraction C_3 was carried out by TLC on cellulose under the conditions described for a commercial device[12]. Visualization of amino acids after TLC was performed by separate spraying of ninhydrin, isatin, Ehrlich, anisidin, iodoplatinate, and nitroprussiate reagents (16). For betaines, Bregoff-Delwiche's modification of Dragendorff's reagent was used (17).

Further identification of the amino acids of Fraction C_3 was obtained by ion-exchange chromatography and by GLC of silyl derivatives.

Experimental Conditions—*Methanolysis of Fatty Acids*—A 100-mg sample, dissolved in 10 ml of dry 0.5 *M* methanolic hydrochloric acid, was placed into a glass-stoppered vial at 75–80° overnight. The methyl esters were extracted twice with 10 ml of *n*-hexane.

Silylation of Amino Acids—Twenty milligrams of sample was dispersed in 1 ml of *N,O*-bis(trimethylsilyl)acetamide and 3 ml of carbon tetrachloride in a stoppered vial and heated at 50° until a clear solution was obtained.

Pharmacological Test—The investigated fraction was dissolved or dispersed in 0.4% tragacanth or 1% polysorbate 80 aqueous dispersions. Following the method of Coldwell *et al.* (13), the dose levels used for any fraction were calculated from: (*a*) the active amount of the precedent fraction and (*b*) the weight of the fraction under investigation, which was assumed to contain at least 80% of the activity shown by the precedent fraction.

Female albino mice (Swiss species), 23 ± 1.5 g, were allowed free access to water but no food 15 hr before the test. They were divided into two groups of seven animals each. Mice of Group I were given 32 ml of solution/kg by stomach tube administration. Group II (controls) received an equal volume of the corresponding vehicle without the mushroom fraction.

Four hours after this treatment, both groups received orally a solution of 9.5% (w/w) absolute ethanol in water (2 g of ethanol/kg). Oral administration of diluted ethanol–water solutions causes a lack of balance in mice. After an induction period of 2–3 min, a sufficient loss of the grasping reflex on a narrow rod is seen (18). Under these experimental conditions, this loss of reflex was observed at 110 ± 20 min (n = 40) for control mice. This period was significantly increased in mice receiving disulfiram before ethanol administration (Table I).

In Procedure I (Scheme I), any investigated fraction giving a significant increase of this period was considered as active (Table I). Further purification of a fraction was achieved if: (*a*) p was lower than 0.01 (Student t test between the results of Groups I and II), and (*b*) this statistical test gave a similar conclusion when applied to the results of a second experiment with the same fraction.

RESULTS AND DISCUSSION

Extraction Procedure III—Contrary to the work of Simandl and Franc (9), the crude benzene extract from *C. atramentarius* failed to show the presence of disulfiram. A compound characterized as a triglyceride, with one molecule of stearic acid for two of linoleic acid, was isolated from the benzene extract. This compound was physiologically inactive when tested in mice at a dose of 200 mg/kg po (Table

[12] Merckotest, CCM Merck 3345 for plasmatic amino acids.

Table I—Pharmacological Activity of Fractions Related to Extraction Procedures I, II, and III, Ergothionein, and Disulfiram

Tested Material	Weight of Fraction, g	Dose Used, mg/kg	Pharmacological Activity, Time (Minutes) of Loss of Reflex	
			Group I	Group II (Control)
Procedure I:				
Ethanol–water extract	257	2400	210	91
Light petroleum extract	38	445	126	113
Benzene extract	1	12	107	113
Methanol extract	44	515	238	130
Fraction A_1	2.5	37	112	93
Fraction A_2	41	515	253	97
Fraction B_1	3	40	131	127
Fraction B_2	38	515	233	95
Fraction C_1	14.5	200	98	114
Fraction C_2	23	310	276	104
Fraction C_3	0.5	150	335	129
Water extract	160	1870	111	121
Procedure II	200 ml of distillate from 500 g of mushrooms	32 ml/kg	99	123
Procedure III:				
Triglyceride	0.2	200	110	105
Ergothionein		200	123	128
Disulfiram		40	292	105

I). This result is supported by the inactivity of the light petroleum extract obtained by Procedure I (Scheme I and Table I).

Extraction Procedure II—To detect physiologically active volatile compound(s) that might have been overlooked using Procedures I and III, the aqueous distillate from extraction Procedure II was administered to mice (32 ml/kg po). This distillate was inactive (Table I).

Extraction Procedure I—The initial ethanol–water extract, prepared from the mushroom material, was subjected to a systematic fractionation (Scheme I), with all steps guided by pharmacological assays. Fraction C_2 was found to exhibit the biological activity present in the starting mushroom material. Further separation of Fraction C_2 (10 g), using preparative TLC, afforded the physiologically active ninhydrin-positive Fraction C_3 (500 mg).

Fraction C_3 exhibited physiological activity at doses (Table I) that were nontoxic when administered with or without ethanol (*i.e.*, no deaths were observed up to 2 months following these experiments). TLC and GLC of Fraction C_3 showed the presence of amino acids and of two unidentified compounds which could be connected to betaines [positive reaction with Bregoff-Delwiche's modification of Dragendorff's reagent (17) after TLC].

Ion-exchange chromatography allowed the identification of the following 20 amino acids (listed in order of decreasing concentrations): glutamine, alanine, threonine, serine, valine, proline, glutamic acid, tyrosine, glycine, aspartic acid, isoleucine, taurine, asparagine, leucine, tryptophan, citrulline, lysine, histidine, arginine, and 3-methylhistidine. Amino acids related to some central nervous system activity were actively sought. Ibotenic acid, muscazon, and muscimol, which were identified in many species of *Amanita* (19), could not be detected in Fraction C_3 by TLC or ion-exchange chromatography (comparison with authentic samples).

In this connection, ergothionein, found together with hercynin in *C. atramentarius* (20), *C. comatus* (21, 22), and *C. micaceus* (23) was tested on mice. Although this amino acid appeared only in trace amounts on thin-layer chromatograms of Fraction C_3, doses of 200 mg/kg of authentic compound were tested on mice and were inactive (Table I).

These results suggest that the structure of the disulfiram-like compound(s) of *C. atramentarius* could be connected to some particular oligopeptides, amino acids, and amines such as tryptamine derivatives. Further work is underway to isolate and characterize the compound(s) responsible for the disulfiram-like activity exhibited by Fraction C_3.

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