The Chemistry of Higher Fungi. III*.
Contribution to the Chemistry of the Genus Russula

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Received February 4, 1955

The aqueous ethanolic extract of Russula emetica contains at least six fluorescent compounds.

A description is given of the isolation and some properties of russularhodin, the red dye from Russula emetica.

From the botanical point of view the genus Russula is the most difficult of the family Agaricaceae. Some characteristic representatives of this genus have been the subject of earlier chemical investigations. Thus Bertrand and Bourquelot discovered the presence of tyrosinase in Russula nigricans, and it was subsequently shown that in certain species of this genus oxidases can be found in comparatively large quantities, and can be isolated in pure form. From the work of Bourquelot it is evident that certain species of the genus Russula contain 10—20 times as much mannitol as other higher fungi. Russula integra, for example, contains 19—20% of mannitol (of the weight of dry material). Qualitative descriptions of the coloring matters of this genus have been given by Weiss, Schröter and Bachmann, who extracted the red dye from Russula integra, R. emetica, R. alutacea, and R. aurata. With cold water, and after having precipitated proteins and other substances with alcohol, obtained an amorphous red mixture of dyes. This showed a blue to blue-green fluorescence, if isolated from Russula integra. On addition of very small quantities of mineral acids the fluorescence disappeared. Careful neutralization restored the fluorescence, which again disappeared at the slightest excess of alkali. This dye is also present in Russula nitida, R. chameleontina Fr., R. nauseosa Pers., and R. rubra (DC) Fr. In several of these species a yellow dye was also reported. According to Kober, in Russula emetica basic substances are also present, such as choline and, probably, muscarine. Later, the chemistry of pigments was investigated, also qualitatively, by Gautier; the findings of earlier authors were confirmed, but no new data given.


* A very valuable review on the chemistry of higher fungi was given in 1906 by Zellner.
*** These botanical data could be of value for further work in this field.
We have included *Russulae* in our investigations of the chemistry of higher fungi for many reasons, but especially because this interesting genus has not been investigated for half a century with newer chemical techniques. In a preparative manner we started our work with *R. emetica* Fr. The ethanolic extracts of this species showed a marked fluorescence; a cross-section of the fresh mushroom showed also an intensive fluorescence in the ultraviolet (355 μ). We selected some ten species of *Russulae* and found that all of them displayed a marked fluorescence (Fig. 1). Other genera (*Amanita*, *Boletus*, *Lactarius*, etc.) showed no fluorescence. This distribution of fluorescent compounds according to Fig. 1 is highly characteristic of all the hitherto investigated *Russulae*.

![Fig. 1. Cross-section through a Russula specimen in ultraviolet light: A, Red pigment layer (russularhodin) shows no fluorescence, but a strong absorption. B, zone immediately next to pigment layer (cca. 0.5 mm.), intensive blue fluorescence, C, D, pileus, lamellae and stipes all show an intensive yellow fluorescence.](image)

Further investigation of the ethanolic-aqueous solution of these fluorescent coloring matters have shown that *Russulae*, especially *R. emetica*, produce a series of very stable, fluorescent pigments and colorless compounds. By paper chromatography of the ethanolic extract, using butanol-water and phenol-water, as well as by electrophoresis on paper, at least six fluorescent compounds could be detected, with red, yellow, blue, violet, and purple fluorescence. We carried out paper chromatography and electrophoresis of ethanolic extracts of ten selected *Russulae* and found that some of the fluorescent compounds are common to all the selected specimens, while some are specific for a given species. It is, therefore, probable that paper chromatograms and electropherograms could be a valuable aid in the identification of *Russulae*.

From *Russula emetica* we isolated 14.5% of mannitol (based on the weight of dry mushroom). The red dye from the pilei of *Russula emetica* was also isolated from the ethanolic extract by adsorption on cellulose. When adsorbed on cellulose, and at pH 7, the dye showed an orange fluorescence. It was eluted from the cellulose powder or filter-paper with aqueous 5% acetic acid. Aqueous solutions of the eluted dye showed no fluorescence at pH 7, nor at any other pH. It is of a bright pink color when adsorbed on paper, showing an orange fluorescence in the ultraviolet; it is identical with the characteristic red color of the pilei of fresh *Russula emetica*. In future, we will refer to this dye as *russularhodin*. On basifying the aqueous solution of
russularhodin and subsequently neutralizing, the red color reappeared. After a prolonged boiling of an aqueous solution of russularhodin with hydrochloric acid, the red color disappeared. Neither was a reaction with an aqueous ferric chloride solution observed, nor with a ninhydrin solution. Russularhodin is soluble in water and glacial acetic acid, but is sparingly soluble in ethanol and other organic solvents. Hydrolysis with hydrochloric acid yielded no products with a positive ninhydrin reaction, neither did the hydrolysate show the presence of carbohydrates.

Using Craig’s procedure of countercurrent distribution, we attempted to separate, on a preparative scale, the ethanolic extract into its components. The results are given in Fig. 2. Russularhodin is found in tubes No 42—58 (Fig. 2, section c), together with the main quantity of mannitol. From a solvent mixture butanol-acetic acid-water we failed to separate russularhodin from mannitol.

Acknowledgment. We are indebted to Mr. V. Štetić, State Department of Forestry, Zagreb, for his kind interest in this work, and for his advice and suggestions for the collecting of biological material. We are further indebted to Prof. M. Slanić, who made available to us some of the original papers cited in the references, and to L. Butula and D. Sutić for assistance in earlier stages of this work.

EXPERIMENTAL

Russula emetica was collected in autumn 1953 in oak woods of the neighborhood of Zagreb. Fresh mushrooms (36.2 kg.) were peeled, and the red peelings, not thicker than 2 mm. (5.6 kg) were minced and kept with two parts of 96°/% ethanol for a fortnight, at 5—10°. The ethanol was then decanted off, replaced with fresh solvent and the same procedure repeated. The first and second extracts were combined, the fungi thoroughly pressed in a tincture press, and the filtrate added to the extracts. The ethanol was evaporated under reduced pressure at a temperature below 50°, and the fat removed by ether extraction. After further evaporation of the ethanolic-aqueous solution, mannitol (140 g.) could be isolated. The dark red syrupy residue was evaporated to dryness and used for further experiments (Extract A).

The peeled fungi (30.5 kg.) were minced and extracted in the same manner, and 452 g. of mannitol were isolated. By proceeding in the same manner as for extract A, 383 g. of ethanolic extract (Extract B) were obtained. From 36.2 kg. of fresh mushroom 1.63% of mannitol could be isolated.

Craig Countercurrent Distribution

The general methods outlined by Craig19 were used in the attempt to separate the fluorescent compounds from the extract A. The distribution was carried out in a 200-tube modification of Craig’s all-glass apparatus20, at 21°, using 9.22 g. of the ethanolic extract A of Russula emetica, in the solvent system n-butanol — acetic acid — water (16 : 5 : 20 by volume), and in 187 transfers. All solvents were distilled before use, and equilibrated in separatory funnels. In preparing the machine for fractionation 25 ml. of the lower phase were added to each of the tubes. The extract A was dissolved in the lower phase, and added to tubes 4—8. About six transfers were made per hour. The tube fractions were concentrated to dryness by vacuum distillation. The contents of the tubes were weighed. The peak concentrations were found in tubes 18, 37, and 42 after 187 transfers (Fig. 2). Electropherograms of the fractions from those tubes in which the purest substance was expected showed two or more components. Russularhodin and mannitol have practically the same partition coefficients in the solvent system used (Fig. 2, section c).
Paper Chromatography

Paper chromatography was carried out on Whatman paper No. 1, at 19°, using phenol-water as mobile phase, during 24 hours. A series of fluorescent spots appeared, with $R_F$ values between 0.52 and 0.70. The spot due to *Russularhodin* appeared as an adsorption comet, a phenomenon often encountered with dyes.

Paper Electrophoresis

Paper electrophoresis was carried out on Munktell Paper 20/150; the strip was 30×1.5 cm.; the buffer was $H_2BO_3$-NaOH-Na-acetate, pH 8.60, ionic strength 0.0482. Voltage on the electrodes 140 V, current density in the strip was 0.22 mA/cm. at the beginning, and 0.45 mA/cm. at the end of the electrophoresis. The duration of the electrophoresis was 20 hours (see Fig. 3). Strip I on Fig. 3 represents the results obtained with the ethanolic aqueous extract (2:1) of fresh peelings after five days' extraction. Strip II was obtained using the second extract after a month of extraction at 10°. Russularhodin is contained in section 2 of the strips.

Fig. 3. — Electropherogram of aqueous ethanolic extract of *Russula emetica* peelings. Strip I, first aqueous ethanolic extract (1:2) (after five days' extraction). Strip II, second extract (after a month of extraction). Fluorescence in the ultraviolet: (1) orange-red; (2) *Russularhodin*; (3) yellow; (4) crimson; (5) blue; (6) green; (7) blue; (8) violet.

Isolation of Russularhodin

A solution of Extract A (8 g.) in distilled water (150 ml.) was passed through a column of cellulose powder (17×2 cm., 16 g., Whatman, Standard Grade, B. Quality). The column was prepared by slurring the cellulose powder with distilled water in to a glass column. The adsorbent was thoroughly agitated to ensure removal of suspended air bubbles, and to produce a fine dispersion. The russularhodin was adsorbed at the top of the column. The column was washed with distilled water.
water (1200—1500 ml.) until the completely colorless washings showed no more fluorescence in the ultraviolet. The russularhodin was eluted with 50% acetic acid (800—1000 ml.), and the column remained faintly colored. The acetic acid solution was evaporated under reduced pressure at a temperature below 30°, and russularhodin remained as a dark red, semicrystalline solid. Yield 200 mg. of russularhodin from the peelings of 36 kg. of fresh fungi. Russularhodin gives no reaction with a 1% methanolic FeCl₃ solution, nor with a ninhydrin solution. When treated with hydrogen in statu nascendi (Zn + HCl) at room temperature, russularhodin was reduced and discolored in a short time.

**Acid Hydrolysis of Russularhodin**

Russularhodin (5 mg.) was refluxed with 1 N hydrochloric acid (5 ml.). After two hours the red color disappeared. The refluxing was continued for ten more hours, and the reaction mixture was chromatographed on paper after the second, fifth, tenth and twelfth hour of refluxing. In every instance this was carried out on Whatman No. 1 paper, at 190, and with phenol-water as mobile phase. The paper strips were developed with a ninhydrin solution, and also with an aniline phthalate solution. The results were negative in both cases, and therefore the hydrolysis of russularhodin, under the described conditions, yielded neither amino acids nor carbohydrates.

**Determination of Muscarine-Like Activity of Extract B.**

The determination of muscarine-like activity was carried out according to Kögl on Rana esculenta. Six determinations were made, and Extract B showed an activity of 8—10 Muscarine Units for 1 g. of extract.

**REFERENCES**

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IZVOD

Kemija viših gljiva. III. Prilog kemiji Russula

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Pokazano je, da vođeno-etanolni ekstrakt Russula emetica sadrži najmanje šest fluorescentnih spojeva.

Opisana je izolacija i neka svojstva rusularodina, karakteristične crvene boje Russula emetica.

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Primljeno 4. februara 1955.