15. BIOCHEMISTRY OF THE WOOD-ROTTING FUNGI

1. METABOLIC PRODUCTS OF *LENTINUS LEPIDEUS* FR.

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THE fungus *Lentinus lepideus* Fr. is a frequent cause of decay in worked timber, particularly in imperfectly creosoted material. It is often associated with the rotting of railway sleepers, telegraph poles and paving blocks, which it is able to attack on account of its tolerance of a comparatively high concentration of creosote. It is also an important factor in the decay of timber in mines. In culture it frequently produces well formed crystals in the medium or on the walls of the culture vessel and it has a strong, sweet, aromatic odour which is said to resemble that of Peru balsam. The present investigation was undertaken in order to identify the crystalline substance formed and if possible to determine the cause of the characteristic odour.

As the products under investigation were presumably volatile, the experimental material—Scots pine sapwood decayed by *L. lepideus*—was subjected to steam distillation. Only the matter volatile in steam was examined. The steam distillate deposited the crystals characteristic of the organism and the aqueous solution on extraction with ether afforded an oil having the characteristic odour. The crystalline substance was shown to be methyl *p*-methoxycinnamate. The oily extract was distilled *in vacuo* and the distillate was found to be a mixture of esters containing, in addition to methyl *p*-methoxycinnamate, esters of cinnamic and anisic acids. The cinnamic acid was present as the methyl ester since the distilled oil when treated with bromine gave the dibromide of methyl cinnamate. It was not possible to determine the alcohol associated with anisic acid owing to the small amount available. Methyl cinnamate has a sweetish aromatic odour not unlike that of *L. lepideus* and is no doubt responsible in part for the characteristic smell of the fungus.

The products isolated from *L. lepideus*, namely the methyl esters of cinnamic and *p*-methoxycinnamic acids and an ester of anisic acid, are structurally closely related and afford further examples of the production of methylated products by fungi. Many examples of methoxyl and methyl ester groups have already been noted amongst the products of the lower fungi.

The questions then arose as to (1) whether the products isolated are present in sound wood, (2) whether the esters described are characteristic of the organism and obtainable on other media which support growth of *L. lepideus* or whether they represent degradation products of some substance or substances present in wood alone and therefore obtainable by growth of the organism only on that medium.

Experiments were performed with the object of deciding these questions. Steam distillation of sound Scots pine sapwood afforded a volatile oil which could not be induced to yield crystals. Further, after saponification of the oil no trace of the acids obtainable after growth of *L. lepideus* could be detected. Hence it may be concluded that the esters identified do not pre-exist in the wood.

A culture of *L. lepideus* grown on malt agar was subjected to steam distillation. Although growth was poor and the examination of the culture was unavoidably delayed it was found that from the material volatile in steam it was possible to isolate a small amount of methyl *p*-methoxycinnamate which was satisfactorily identified. Thus this ester and probably the other esters formed by *L. lepideus* are characteristic products of the fungus and are not formed by degradation of wood alone.

It appears that methyl *p*-methoxycinnamate has not hitherto been recorded as occurring in nature, although the ethyl ester of *p*-methoxycinnamic acid was found by van Romburgh [1900, 1; 1902] to be the chief constituent of the essential oil of *Kaempferia galanga* Linné. *p*-Methoxycinnamic acid also occurs (in some form of combination) in "leptandra", the dried rhizome and roots of *Veronica virginica* Linné (*Leptandra virginica* Nuttall), a plant indigenous to the greater part of North America [Power & Rogerson, 1910]. "Leptandra" is used medicinally. The acid was obtained by cold alkaline hydrolysis of the chloroform extract of a resinous portion non-volatile in steam. It is not likely to have been present as the methyl ester, which is volatile in steam. Cinnamic acid was also present among the hydrolysis products of leptandra.

Cinnamic acid is found in nature in a wide variety of plant products, particularly in ethereal oils and resins. It is usually encountered in the form of esters. The methyl ester has been reported in the ethereal oil from the rhizome of *Alpinia malaccensis* [van Romburgh, 1900, 2] and in wartara oil [Schimmel & Co., 1901]. It is present to the extent of 48% in the root oil of *Alpinia galanga* [Ultée, 1911] and forms the chief constituent of the ethereal oil from the leaves of *Ocimum canum* Sims [Rouré-Bertrand Fils, 1914; Schimmel & Co., 1914]. Cinnamic acid or its esters are constituents of certain balsams. It occurs (chiefly as benzyl ester) in Peru balsam which *L. lepideus* is said to resemble in odour.

Anisic acid occurs in all ethereal oils which contain anethole from which it is produced by atmospheric oxidation. The occurrence in nature of esters of this acid does not appear to have been recorded.

As already stated *L. lepideus* is remarkably tolerant to relatively high concentrations of creosote and indeed is outstanding amongst the wood-rotting fungi in this respect. Thus it can attack wood which has been impregnated with solutions containing up to 4% of coal tar creosote, i.e. which contains up to 15 kg. of creosote per cubic metre of wood. There may be some connexion between this property of the fungus and its power to elaborate aromatic substances of the type which have been isolated.

It is possible that what we may term the "benzenoid environment" due to the metabolic products described has rendered the fungus immune to the toxic action usually associated with such substances and hence resistant to certain constituents of creosote itself. It is noteworthy, however, that two of the acids formed, namely *p*-methoxycinnamic and anisic acids, are methoxyl derivatives of phenolic acids. It is recognized that methylation is a method employed by nature, particularly in the plant kingdom, for detoxicating substances which would prove harmful to the organism. It may be that the phenolic acids or their esters are the primary metabolic products of the fungus and that these

are then methylated and so rendered harmless. Granted the power of achieving this methylation of metabolic products, the fungus may apply the same method to the phenolic constituents in creosote which are presumably the most active fungicidal agents present and thus effect at any rate a partial detoxication. We have at present no evidence as to whether either of these hypotheses is correct.

EXPERIMENTAL

Organism

A culture of *Lentinus lepideus*, which was originally isolated by Dr Liese at the Forestry School, Eberswalde, Germany, was used. This particular isolation was chosen because it appeared to form crystals in culture more readily than any of the other strains available. It may be noted that this isolation is the one which it has been agreed to use for standard laboratory tests on the toxicity of wood preservatives.

Method of culture

The fungus was grown on 5% malt agar in special Kolle flasks. After the surface of the agar was covered with mycelium four blocks of Scots pine sapwood measuring $5 \times 2.5 \times 1.5$ cm., which had been sterilized in an autoclave at 15 lb. pressure, were introduced into each flask. These were supported on low glass rests so that they came into contact only with the mycelium and did not touch the agar. The flasks containing the sample blocks were then kept in an incubator at 22° for 3 months, and at the end of this period the blocks were removed from the flasks and submitted to analysis.

Since it was considered undesirable to oven-dry the blocks before the experiment, their original oven-dry weight was estimated by drying a large number of similar blocks of the same size which had been cut from the same pieces of wood. In this way the average oven-dry weight of a single sample block was found to be 7.93 g.

Treatment of infected wood

The Scots pine sapwood (118 blocks) which had been inoculated with *L. lepideus* was placed in a 5 l. flask and subjected to steam distillation for 6 working days. From the results of previous experiments it was estimated that the blocks had lost about 20% of their oven-dry weight as a result of the decay brought about by this fungus during 3 months at 22°, so that the dry weight of material used for analysis was estimated at approximately 748 g. Crystals separated in the distillate and were collected after chilling. The yield decreased each day, that obtained on the 6th day being only 0.06 g. The total yield of crude crystalline material (A) was 1.29 g., M.P. 85.5–86.5°.

The filtrates from the crystals were extracted with ether. The liquid extract remaining after removal of ether had the characteristic sweet ethereal smell of *L. lepideus*. This residue (wt. 1.62 g.) was distilled at 2–3 mm., yielding fraction I (B), B.P. 94–110°, wt. 0.83 g. clear yellowish oil, and fraction II, B.P. 110–122°, wt. 0.23 g. pale yellow cryst. solid M.P. 70–85°.

The residue in the distillation flask had darkened considerably and finally became a black tarry mass.

Fraction II on recrystallization from 1 ml. of methyl alcohol gave 0.18 g. of material melting at 87–89° and identical with the crystalline material (A) obtained directly from the steam distillate by filtration.

Examination of the crystalline material (A)

The crude material, M.P. 85.5–86.5°, was recrystallized from methyl alcohol and then had M.P. 88°. (Found: C, 68.87, 68.80; H, 6.19, 6.36; OMe, 30.8, 30.8%. N, zero. Mol. wt. (Rast camphor method), 204, 206. $C_{11}H_{12}O_3$ (with 2OMe) requires C, 68.74; H, 6.30; OMe, 32.29%. Mol. wt. 192.)

Saponification. Substance A was insoluble in cold aqueous NaOH. It was hydrolysed by heating 0.2 g. with 5 ml. of *N* methyl alcoholic KOH for 30 min. under reflux. The solid first went into solution, then crystals of the K salt separated. These were dissolved by addition of about 25 ml. of water and the mixture was acidified with HCl. The precipitated acid was collected and dried *in vacuo*—wt. 0.16 g., M.P. 168–170°. On recrystallization from benzene it gave needles of M.P. 172–173°. The melt was cloudy but it cleared at 187°. Sublimation *in vacuo* did not further raise the M.P. (Found: C, 67.45; H, 5.47; OMe, 17.2%. Equiv. by micro-titration, 180.6. $C_{10}H_{10}O_3$ (1OMe) requires C, 67.41; H, 5.66; OMe, 17.4%. Equiv. (for monobasic acid), 178.2.)

Substance A is evidently the monomethyl ester of an acid $C_{10}H_{10}O_3$ with one methoxyl group.

Action of $KMnO_4$ on substance A. The substance (0.19 g.) was dissolved in 50 ml. of pure acetone and finely ground $KMnO_4$ was added in small portions with shaking. As the solution became slightly warm it was cooled under the tap. After 0.6 g. of $KMnO_4$ had been added the oxidation became very slow. The solution was filtered and the MnO_2 was washed with acetone and dried. It was then extracted with water and the clear filtrate was acidified with HCl. The precipitated acid was collected, washed and dried. It weighed 0.095 g., M.P. 181–183°. The M.P. after recrystallization from water was raised to 182–183°. A further yield was obtained from the acetone by continuing the addition of $KMnO_4$. (Found: C, 63.55; H, 5.42; OMe, 20.2%. Equiv. by titration, 151.2. $C_8H_8O_3$ (1OMe) requires C, 63.17; H, 5.30; OMe, 20.4%. Equiv. (as monobasic acid), 152.1.)

This substance obtained by oxidation proved to be anisic acid since it showed no depression in M.P. when mixed with an authentic specimen of that substance. The saponification acid would therefore be *p*-methoxycinnamic acid, confirmed by the fact that it gave no depression in M.P. when mixed with authentic material synthesized for comparison by the method of Knoevenagel [1898]. The synthetic acid had the same M.P. 172–173° and likewise gave a turbid fluid on melting which became clear at 188°. The original substance A is thus methyl *p*-methoxycinnamate. It showed no depression in M.P. when mixed with the synthetic ester of the same M.P.

Examination of the distillate B

The mol. wt. (Rast) of the liquid distillate in two determinations was 159 and 150. The methoxyl content by Zeisel's method was 24.5%. As it seemed probable that it was also an ester it was subjected to alkaline hydrolysis.

Saponification by cold methyl alcoholic KOH. The substance (0.1046 g.) was treated with 2.00 ml. of approximately *N* KOH in MeOH and 1 ml. of MeOH was added. It was left stoppered at room temperature for 3 days and then titrated, a control (without substance) receiving similar treatment. The titration value of the acid produced was 0.520 ml. giving an apparent mol. equiv. of 201 for the original substance. The neutralized solution was diluted with water and acidified. An acid was precipitated which was separated after chilling the solution. The crystalline acid (0.03 g.) had M.P. 97–110°. On recrystallization

from light petroleum (B.P. 60–80°) it had M.P. 124–127·5° and equiv. by micro-titration of 145. The amount obtained was too small for further recrystallization and as from the molecular equiv. the hydrolysis appeared to have been incomplete another hydrolysis was undertaken on a fresh portion using aqueous NaOH.

The liquid (0·1934 g.) was treated with 5 ml. of aqueous *N* NaOH. It gave a bright golden yellow colour. After 1·5 hr. under reflux all the oil had gone into solution. Titration now gave an apparent equiv. for the original substance of 160. The acid (0·12 g.) was precipitated with HCl and collected. It had M.P. 110–126° but proved even less amenable to purification by crystallization from light petroleum than the preceding sample, whereas it was too soluble in other solvents. The acids were converted into *p*-nitrobenzyl esters, but these also proved difficult to fractionate by crystallization. Accordingly it was decided to revert to the method of saponification with methyl alcoholic KOH in the cold, which, although not proceeding to completion, gave a purer product.

The oil (0·2503 g.) was treated with 5 ml. of *N* methyl alcoholic KOH and a further 2 ml. of methyl alcohol were added. The mixture was left for 3 days at room temperature. The solution was deep reddish brown immediately after addition of the KOH solution, but lightened on keeping. Titration gave an apparent mol. equiv. of 199, showing that again hydrolysis was not complete. The acids were precipitated as an emulsion which crystallized on chilling. The crystals were collected and dried. They weighed 0·095 g. and had M.P. 100–103° after softening at 92°. After seven recrystallizations from light petroleum during which the M.P. was gradually raised to 129–130°, the weight was only 7 mg. When mixed with pure cinnamic acid of M.P. 133°, the M.P. was not depressed. (Found: C, 73·46; H, 5·52%. Calc. for cinnamic acid (C₉H₈O₂), C, 72·95; H, 5·44%.)

The mother liquor from the final crystallization of the above acid was evaporated and the crystals were dissolved in about 2 ml. of carbon disulphide and treated with minute drops of bromine until the colour of the bromine persisted. During this treatment crystals separated. These were collected, recrystallized from light petroleum (B.P. 50–60°) and then from chloroform. There was considerable loss from the latter solvent. A synthetic sample of dibromide was prepared in similar manner from cinnamic acid. The two specimens were heated side by side together with a mixture of the two. All melted together at 200–201° with some decomposition. Thus the product obtained by the action of bromine is undoubtedly cinnamic acid dibromide, confirming the presence of cinnamic acid in the hydrolysate.

Further examination of the hydrolysis products of liquid B

The whole of the remaining material from the mother liquors of crystallization of cinnamic acid and the ether extract of the aqueous filtrate obtained after the initial precipitation with HCl were combined and further hydrolysed with aqueous NaOH. The material (0·22 g.) was treated with 5 ml. of *N* NaOH and boiled for 1·5 hr. under reflux. A back titration showed that organic acid equivalent to 1·43 ml. of *N* alkali was present. The acids were precipitated with HCl and collected (weight 0·11 g.).

The acids were heated under reflux with about 15 ml. of light petroleum (B.P. 50–60°). Some remained undissolved. The mixture was filtered and the undissolved material, M.P. 160–175°, recrystallized from benzene. It now had M.P. 178–180°, raised by recrystallization from water to 180–182°. Mixed with

authentic anisic acid of m.p. 183°, the m.p. was 182–183°. Hence the product is anisic acid. (Found: C, 63.44; H, 5.31 %. Calc. for anisic acid ($C_8H_8O_3$), C, 63.17; H, 5.30 %.)

Preparation of the dibromide of methyl cinnamate from product B

Product B (0.19 g.) in about 2 ml. of carbon disulphide was treated with minute drops of bromine until the latter was no longer decolorized. There was an evolution of HBr which should not occur with mere addition of bromine, probably due to some unidentified product in the mixture of esters. The carbon disulphide was removed *in vacuo* leaving a mixture of crystals and oil. The crystals were washed with a little ethyl alcohol to remove the oil and drained. The colourless compact prisms (0.15 g.) so obtained had m.p. 114–115.5°, raised on recrystallization from ethyl alcohol to 116.5–118°. Yield 0.10 g. When mixed with synthetic material of the same m.p., prepared by addition of bromine to methyl cinnamate, there was no depression. (Found: Br, 50.04, 50.11 %. Calc. for $C_{10}H_{10}O_2Br_2$, Br, 49.64 %.)

This affords conclusive evidence that the cinnamic acid occurs as the methyl ester. The alcohol associated with the anisic acid has not been determined. It seems likely that the anisic acid ester will prove to be methyl anisate, however, since the other two acids identified are present as methyl esters. This also accords with the mol. wt. of the mixed liquid esters (product B), namely 159, 150.

Examination of volatile products of L. lepideus grown on malt agar

The fungus was cultivated on a layer of malt agar (5 %) in Kolle flasks. After 10 weeks' incubation at 22° the flasks were placed in the refrigerator to await examination, which took place 4 weeks later. The growth was poor and, owing to the delay in examination, only small yields of products were expected. The whole of the material, fungus and agar, was subjected to steam distillation. The first distillate (1800 ml.) did not deposit crystals on chilling so it was extracted with ether. After evaporation of the ether there remained a small amount of sticky crystals (0.03 g.). Further steam distillation gave only 0.01 g. of material. The combined ether extracts were subjected to vacuum sublimation from a bath at 40°. The crystals obtained had m.p. 73–77° raised by second and third sublimations to 84–86.5 and 86–88° respectively. The purified product showed no depression in m.p. when mixed with authentic methyl *p*-methoxycinnamate. Thus it is evident that methyl *p*-methoxycinnamate is produced by *L. lepideus* on media other than wood. Owing to the small amount of product obtained on steam distillation no attempt was made to identify other esters formed.

Volatile products from wood

743 g. of fresh chips of Scots pine sapwood were subjected to steam distillation. Ether extraction of the distillate gave an oil (1.2 g.) which showed no sign of crystals. This was distilled *in vacuo* at 1–2 mm. and gave two fractions: I, B.P. 55–75°, 0.72 g.; II, B.P. 75–86°, 0.23 g. Both fractions were clear yellow oils which did not crystallize.

As it was thought that traces of the products sought would be more readily obtained crystalline in the form of free acids, both oily fractions were subjected to alkaline hydrolysis by boiling with 20 ml. of *N* NaOH for 2.5 hr. The unsaponifiable matter was then extracted with ether and the aqueous portion acidified with HCl. No crystals were obtained on acidification and ether extraction of the acids gave an oil in which there was only a trace of doubtfully

crystalline material. This portion was subjected to vacuum sublimation but no solid, only an oil, was obtained. Thus it appears that the identified products obtained from cultures of *L. lepideus* do not pre-exist in the wood. They are therefore true metabolic products of the fungus.

SUMMARY

The wood-rotting fungus *Lentinus lepideus* Fr., when grown on Scots pine sapwood, gives rise to volatile metabolic products. Amongst these methyl *p*-methoxycinnamate, methyl cinnamate and an ester of anisic acid (probably the methyl ester) have been detected. These products are shown to be absent from uninfected wood. Methyl-*p*-methoxycinnamate has been identified amongst the products obtained by growth of *L. lepideus* on a malt-agar medium.

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