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Biochemistry of the Wood-rotting Fungi

9. VOLATILE METABOLIC PRODUCTS OF *STEREUM SUBPILEATUM* BERK. & CURT.*

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(*Received* 12 *November* 1956)

Our researches on the volatile metabolic products of some of the wood-rotting fungi led us to examine an organism which was isolated from the staves of a beer barrel constructed of Persian oak, *Quercus castaneaefolia* C. A. Mey. A batch of about eighty casks made of Persian oak, after being in use for 2–5 years, developed a curious scented odour which contaminated the beer so badly that the casks had to be destroyed.

Examination of staves from the defective casks showed small internal pockets of incipient white rot. Isolations from these areas yielded a vigorous golden-orange culture of a basidiomycete (now listed as Forest Products Research Laboratory no. 271A) which was found to be identical with a culture of *Stereum subpileatum* originally isolated in the U.S.A. by the late L. O. Overholts of Pennsylvania State College. Later, sporophores of the fungus developed on portions of staves kept in a damp chamber, and the structure of these agrees well with the description of *S. subpileatum* Berk. & Curt. This fungus has been reported by Long (1915) as a common cause of a 'honeycomb' white-pocket rot in thirteen species of oak in the southern part of the U.S.A. He noted that the fungus can continue to grow in felled timber. It is highly probable that infection in the oak used for the manufacture of beer barrels was present at least in an incipient stage in the logs when they were felled.

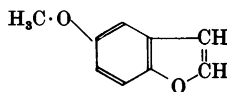
The production of a sweet musty odour by *S. subpileatum* has been described as a characteristic feature of this fungus. The odour from a culture is both penetrating and persistent and resembles a scent used in cheap soaps. It has been suggested that it may be compared with 'a mixture of wintergreen and apricots plus a trace of thymol'. There is little doubt that the metabolic products of the fungus growing in the wood were responsible for the contamination of the beer, even though the casks were lined inside with a vegetable pitch.

In order to investigate the metabolic products of *S. subpileatum* responsible for the characteristic odour, the fungus was cultured in the laboratory and the volatile products were separated from the mycelium and culture fluid by steam-distillation as in previous cases. As preliminary tests had shown that a carbonyl compound and an acid were present in the ether extract of the steam-distillate, the

* Part 8: Birkinshaw & Chaplen (1955).

distillate was extracted with ether and the ether solution was shaken with bisulphite and then with bicarbonate. The bisulphite solution, on acidification, afforded the free carbonyl compound, which was converted into the 2:4-dinitrophenylhydrazone. The carbonyl compound proved to be cinnamic aldehyde. Its identity was confirmed by preparation of the oxime. Acidification of the bicarbonate fraction afforded cinnamic acid, confirmed by mixed m.p. and by conversion into the dibromide.

The extracted ether solution contained a neutral crystalline product of m.p. 34°, having the characteristic odour of the cultures. It possessed the molecular formula $C_9H_8O_2$ and contained one methoxy group. It gave a negative reaction for carboxylic acid or ester and no colour with ferric chloride. The ultraviolet-absorption spectrum was close to that of coumarone; it was therefore probable that the substance was a methoxycoumarone. Of the known methoxycoumarones none has been reported as solid at room temperature except 5-methoxycoumarone (I). This substance was obtained in minute amount by Stoermer (1900) as an oil of b.p. 230–240°. He remarked on its peculiar sweet and clinging odour. It was later synthesized by Tanaka (1951), who records it as a solid of m.p. 32–33°.



(I)

The compound was synthesized by Tanaka's method and was found not to depress the m.p. of our natural product, on mixing. Confirmation of identity was sought by the action of bromine on the synthetic and natural products. It was expected that addition of bromine would occur at the double bond (2:3-addition) as with coumarone itself. Although the reaction was carried out at low temperature (−5°) in carbon disulphide with 2 atom-equivalents of bromine present, hydrobromic acid was evolved and two crystalline bromine compounds were obtained from both the synthetic and the natural product. These crystalline derivatives were (*a*) a colourless product, $C_9H_7O_2Br$, m.p. 76°, and (*b*) a yellow product, $C_9H_7O_2Br_3$, m.p. 80°. Evidently substitution had occurred in the benzene ring in both compounds and addition only in the yellow compound. The two products are therefore *x*-bromo-5-methoxycoumarone and 2:3:*x*-tribromo-5-methoxycoumaran respectively. The mono- and tri-bromo compounds from the natural 5-methoxycoumarone were identical with the mono- and tri-bromo compounds respectively obtained from the synthetic material (no depression in m.p. on mixing).

Cinnamic acid is known to occur in nature in various plant oils and resins. It is produced in the form of the methyl ester by one of the higher fungi, *Lentinus lepideus* (Birkinshaw & Findlay, 1940).

Cinnamaldehyde is a constituent of many essential oils (e.g. oil of cinnamon, cassia oil, patchouli oil) but has not hitherto been recorded as a fungal metabolic product. It may be the precursor of the cinnamic acid present in the cultures of *Stereum subpileatum*. The aldehyde could be converted into the acid by the action of a fungal enzyme or even by oxidation in the air.

5-Methoxycoumarone has not previously been reported as occurring in nature. Its production by a fungus recalls the hypothesis previously advanced that gentisic aldehyde is concerned in the fungal metabolic processes (Birkinshaw, 1953). It was suggested that it might be the common precursor of gentisyl alcohol and patulin, products of *Penicillium patulum*, as well as of gentisic acid, produced by *P. griseofulvum*. 5-Methoxy-2-hydroxybenzaldehyde derived by single *O*-methylation from gentisic aldehyde could, conceivably, by condensation with acetaldehyde and oxidation, be converted into 6-methoxycoumarin. The latter, by processes analogous to the chemical or thermal conversion of coumarin into coumarone, could then yield 5-methoxycoumarone. The oxygen methylation need not necessarily be the first step but could occur anywhere in the reaction chain. Cinnamic aldehyde (and hence cinnamic acid) could be derived by a similar condensation from benzaldehyde; this aldehyde, like gentisic aldehyde, has not yet been detected as a fungal product, but benzoic acid is known to be formed by *Penicillium roseo-purpureum* (Posternak, 1940).

EXPERIMENTAL

All m.p.'s are uncorrected. Elementary analyses are by Weiler and Strauss except where otherwise stated. Methoxy determinations were made by one of us (P.C.).

Organism

The culture used was F.P.R.L. no. 271 A, of *Stereum subpileatum* Berk. & Curt., isolated from a Persian-oak beer-barrel stave.

Culture conditions

Two media were employed: (1) aqueous malt extract (Vimaltol; A. Wander Ltd., London, W. 1), 4% (pH 5·6). This was sterilized by autoclaving at 110° for 30 min. (2) A synthetic medium as used for *Daedalea juniperina* (Birkinshaw & Chaplen, 1955), containing only glucose, Marmite and inorganic salts. It was sterilized by steaming on 3 successive days.

The medium was distributed (350 ml./flask) in 1 l. conical flasks plugged with cotton wool. The flasks were sterilized and inoculated by placing on the surface of the liquid a fragment of mycelium derived from a malt-agar slope. The flasks were incubated in the dark at 24°.

Culture characteristics

On the malt medium the mycelium, after 6 weeks, was orange-yellow in colour with deep-brown reverse. It was of a leathery nature but somewhat scanty, and did not cover the whole surface of the liquid in the flask. The culture filtrate was deep brown in colour and turbid. On the synthetic medium growth was very sparse. The colour of the mycelium was creamy white, with very little orange tint. The culture solution was light greyish brown and turbid. In both cases the cultures soon developed the characteristic odour which was immediately perceptible on entering the incubation room. As can be seen from Table 1, the products were qualitatively the same on the two media.

Harvesting and separation of volatile products

The method employed for one batch of fifty flasks (malt medium) will be described; a similar method was used for the other two batches.

The total contents of the flasks were subjected to steam-distillation. The disillate was extracted four times with one-quarter of its volume of ether. The ether extracts were dried (Na_2SO_4) and the ether was removed by distillation. The residual brown syrup (3·1 g.) rapidly crystallized on cooling. It was dissolved in ether and extracted six times with an equal volume of freshly prepared saturated $NaHSO_3$ solution. The ether was washed with water and the washings were added to the bisulphite solution. The ether was shaken six times with an equal volume of freshly prepared saturated $NaHCO_3$ solution and washed with water. The extracted ether solution was then dried over Na_2SO_4.

Treatment of bisulphite extract; isolation of cinnamaldehyde

The bisulphite extract was strongly acidified with conc. HCl and extracted four times with one-quarter of its volume of ether. The ether extract was dried and the ether was removed. The residual brownish yellow oil (0·5 g.) deposited a few small crystals on chilling. A portion (0·1314 g.) of this residue, when dissolved in a little ethanol and treated with an excess of 2:4-dinitrophenylhydrazine hydrochloride in 2N-HCl, gave 0·2311 g. of an orange-red 2:4-dinitrophenylhydrazone of m.p. 247–249°. Crystallization from acetic acid, then ethyl acetate, afforded deep-red needles, m.p. 251–252° decomp. [Found: C, 57·6; H, 3·8; N, 17·5; OMe, nil. Calc. for $C_{15}H_{12}O_4N_4$ (i.e. the dinitrophenylhydrazone of C_9H_8O): C, 57·7; H, 3·9; N, 18·0%]. An authentic specimen of cinnamaldehyde 2:4-dinitrophenylhydrazone of m.p. 252° produced no depression in m.p. when mixed with the above derivative of the natural product.

Another portion (0·12 g.) of the oil recovered from the bisulphite extraction was dissolved in ethanol (3 ml.) and treated with a solution of hydroxylamine hydrochloride (0·15 g.) and anhydrous sodium acetate (0·3 g.) in water (5 ml.). After several hours, the brown ppt. (90 mg., m.p. 85–90°) was collected and recrystallized successively from light petroleum, aqueous ethanol and light petroleum. Long thick colourless needles (50 mg.) of m.p. 136° were obtained. These did not depress the m.p. of authentic cinnamaldehyde oxime, m.p. 137°. The presence of cinnamaldehyde in the volatile products was thus confirmed.

Treatment of the bicarbonate extract; isolation of cinnamic acid

The aqueous bicarbonate extract was acidified with HCl. Colourless leaflets (0·38 g.) separated, m.p. 132–133°. The filtrate was extracted with ether and further acid (80 mg., m.p. 128–130°) was thus recovered. Recrystallization from water afforded colourless leaflets (0·4 g., m.p. 132°) which did not depress the m.p. (133°) of an authentic specimen of cinnamic acid on admixture. [Found (Schoeller): C, 72·7; H, 5·4. Equivalent by titration, 146. Calc. for $C_9H_8O_2$: C, 72·9; H, 5·4%; equivalent, 148]. Further confirmation of the identity of this substance was obtained by converting it into the dibromide. The acid (25 mg.) was dissolved in CS_2 and treated with drops of bromine until the latter was no longer decolorized. After removal of CS_2 the residue (35 mg.) consisting of stout rods was recrystallized from $CHCl_3$, then from light petroleum. The product was obtained as thick needles of m.p. 202°. It did not depress the m.p. of authentic cinnamic acid dibromide on admixture. The presence of cinnamic acid as a second metabolite is thus confirmed.

Treatment of the extracted ether solution; isolation of 5-methoxycoumarone

Removal of the solvent from the extracted ether solution left a golden-brown syrup (2·1 g.) which rapidly crystallized on cooling. On dissolution in methanol and dilution with

Table 1. *Cultural details and yields of volatile products of* Stereum subpileatum, *strain F.P.R.L. no.* 271 A

No. of flasks	Medium	Period of incubation at 24° (weeks)	Final pH	Residual glucose (%)	Wt. of ether extract of steam-distillate (g.)	Wt. (g.) and m.p. of crude 5-methoxy-coumarone	Wt. of crude cinnamic acid (g.)	Wt. of crude cinnamic aldehyde (g.)	Wt. of non-crystalline residue (g.)
18	Malt	11	4·2	—	1·2	0·56 (28–30°)	0·10	0·12	0·30
21	Synthetic	10	4·6	0·30	1·0	0·23 (29–31°)	0·17	0·33	0·30
50	Malt	10	4·4	—	3·1	1·4 (26–28°)	0·46	0·50	0·60

water, a light-brown oil separated which rapidly hardened on seeding. It was collected, washed with aqueous methanol and dried (1·2 g., m.p. 26–28°). Dilution of the mother liquor gave an oily solid from which further crystals were obtained by pressing on a porous plate. No further solid could be obtained by extraction of the porous plate or from the mother liquors. Recrystallization of the solid material from aqueous methanol afforded colourless shining leaflets of 5-methoxycoumarone of constant m.p. (34°); light-absorption max.: 221, 247, 293, 300·5 mμ in ethanol (log ϵ, 3·76, 3·99, 3·59, 3·55 respectively [Found: C, 72·9, 73·0; H, 5·6, 5·5; O, 21·4; OMe, 21·0. $C_9H_8O_2$ requires C, 72·95; H, 5·4; O, 21·6; OMe, 20·9%]. 5-Methoxycoumarone is only slightly soluble in cold but more soluble in hot water. It is only slightly soluble in aqueous NaOH. With cold conc. H_2SO_4 it gives a yellowish brown colour, with rapid charring. It decolorizes bromine in CS_2 and $KMnO_4$ in acetone. It has the characteristic odour produced by the cultures. The m.p. of authentic 5-methoxycoumarone (34°) synthesized for comparison was not depressed on admixture with this material. The ultraviolet-absorption spectra of the natural and synthetic products are also in close agreement.

Preparation of bromo derivatives of 5-methoxycoumarone

The natural 5-methoxycoumarone (0·3 g.) was dissolved in CS_2 (2 ml.) and cooled in an ice–salt bath so that the temperature did not rise above −5°. With vigorous stirring bromine (0·33 g., equivalent to 2 atoms of Br/mol.) in CS_2 (2 ml.) was added slowly. Fumes of HBr were evolved. The solvent was removed at room temperature and the residue was dried in a vacuum. Needle-shaped crystals formed in the greenish yellow syrupy residue. The material was taken up in light petroleum (10 ml.) which was decanted from a gummy residue. On evaporation of the petroleum, lemon-yellow needles (50 mg., m.p. 63–65°) of the 2:3:x-*tribromo-5-methoxycoumaran* were obtained. By further crystallizations and sublimation in a vacuum the m.p. was raised to 80° (Found: C, 28·3; H, 1·95; OMe, 8·4. $C_9H_7O_2Br_3$ requires C, 27·9; H, 1·8; OMe, 8·0%). This tribromo compound showed no depression in m.p. when mixed with the yellow product similarly prepared from authentic 5-methoxy-coumarone.

The residues from the crystallizations were combined (23 mg., m.p. 70–72°). Recrystallization from light petroleum and sublimation in a vacuum afforded 16 mg. of almost colourless needles of x-*bromo-5-methoxycoumarone*, m.p. 76° (Found: C, 47·7; H, 3·2 (Schoeller); Br, 35·8; OMe, 13·75. $C_9H_7O_2Br$ requires C, 47·6; H, 3·1; Br, 35·2; OMe, 13·7%). This product produced no depression in m.p. when mixed with the colourless monobromo derivative of authentic 5-methoxycoumarone. The third metabolic product isolated is therefore 5-methoxycoumarone.

Antibacterial activity of 5-methoxycoumarone

5-Methoxycoumarone (25 mg.) was dissolved by warming in sterile water (25 ml.) and quickly cooled. The solution remained clear long enough for dilutions in sterile heart broth–glucose (2%) from 1:2000 to 1:10 000 to be made. Two sets of broth tubes were inoculated with 24 hr. cultures of *Staphylococcus aureus* N.C.T.C. 6571 and *Escherichia coli* N.C.T.C. 86 respectively and incubated at 37° for 24 hr.

The inhibition of *Staph. aureus* was complete at 1:5000, partial at 1:8000 and nil at 1:10 000. With *Esch. coli* inhibition was complete at 1:5000, nil at 1:8000. Thus 5-methoxycoumarone has only weak antibacterial activity.

Syntheses

5-*Methoxycoumarilic acid* (*cf.* *Tanaka*, 1951). 5-Methoxy-salicylaldehyde (3 g.) prepared from quinol monomethyl ether by the method of Rubenstein (1925), diethylbromo-malonate (5 g.), anhydrous K_2CO_3 (3 g.) and ethyl methyl ketone (10 ml.) were refluxed together on a steam-bath for 5 hr. The main part of the solvent was distilled off, the residue was mixed with water, acidified with 2N-H_2SO_4 and the solution was extracted six times with one-quarter of its volume of ether. The residue from the ether (7·1 g.), a dark-brown syrup, was treated with ethanolic KOH (2 g./20 ml.) and refluxed for 1 hr. The product was dissolved in water and acidified with 2N-H_2SO_4. Amorphous light-brown material separated [0·8 g., m.p. 163–167° (decomp.)] and was collected. Ether extraction of the filtrate gave a syrup (2·3 g.). The hydrolysis with alkali was repeated on this material since it was calculated that the amount of alkali used (after Tanaka) in the first hydrolysis of the intermediate was insufficient. Further amorphous material (1 g.) was obtained, but not examined.

The filtrate from the first hydrolysis, which had been extracted with ether, when boiled and cooled deposited colourless needles. By a combination of boiling, cooling and concentrating under reduced pressure crystalline material (1·28 g.) of m.p. 214–215° was obtained. This was the required 5-methoxycoumarilic acid for which Tanaka gives the m.p. 212–213°. The filtrate from the second hydrolysis, on similar treatment, afforded a further quantity (0·65 g.) of m.p. 212–214°.

5-*Methoxycoumarone*. 5-Methoxycoumarilic acid (0·5 g.), Cu powder (0·11 g.) and quinoline (10 ml.) were refluxed for 30 min. After cooling, the mixture was diluted with ether, filtered from the Cu and extracted five times with 0·5 vol. of 2N-HCl and twice with water. Removal of the ether left a golden-brown syrup (0·36 g.). By treatment of this syrup dissolved in ethanol with water a slightly sticky solid (0·3 g., m.p. 32–33°) was obtained, which was purified by distillation at 70–75°/16 mm. The product, finally crystallized from ethanol–water (1:1), was in the form of colourless irregular plates of constant m.p. (34°), yield 0·27 g.; light-absorption max., 220, 246, 293, 300·5 mμ in ethanol; log ϵ, 3·77, 3·98, 3·57, 3·51 respectively.

Bromination of 5-methoxycoumarone. The method used, based on that of Stoermer & Kahlert (1902) for the preparation of coumarone dibromide, has already been described in reference to the natural product. Two bromo-derivatives were again obtained. These were (1) 2:3:x-Tribromo-5-methoxycoumaran, yellow needles m.p. 79–80° [Found: C, 28·8; H, 2·0; Br, 61·5; OMe, 7·9 (Schoeller); C, 27·5; H, 2·3; Br, 61·1. Mol.wt. (cryoscopic in camphor), 376. $C_9H_7O_2Br_3$ requires C, 27·9; H, 1·8; Br, 62·0; OMe, 8·0%; mol.wt. 387]. (2) x-Bromo-5-methoxycoumarone, colourless irregular plates, m.p. 76° [Found: C, 47·3, 48·1; H, 3·4, 3·3; Br, 33·1, 34·4; OMe, 13·6 (Schoeller); C, 47·8; H, 3·2; Br, 36·4. $C_9H_7O_2Br$ requires C, 47·6; H, 3·1; Br, 35·2; OMe, 13·7%].

SUMMARY

1. *Stereum subpileatum* Berk. & Curt., a basidiomycete found growing in Persian-oak beer barrels and causing contamination of the beer, was cultured on a malt medium and on a glucose–Marmite–salts medium. The steam-volatile metabolic products, which were qualitatively the same on both media, were examined.

2. Three metabolic products were isolated and identified: cinnamaldehyde, cinnamic acid and 5-methoxycoumarone. 5-Methoxycoumarone when treated with bromine in carbon disulphide afforded two crystalline products: (*a*) colourless x-bromo-5-methoxycoumarone, m.p. 76° and (*b*) yellow 2:3:x-tribromo-5-methoxycoumaran, m.p. 80°.

3. 5-Methoxycoumarone is the product mainly responsible for the penetrating characteristic odour of the fungus.

4. 5-Methoxycoumarone exhibited only weak antibacterial activity against *Staphylococcus aureus* and *Escherichia coli*.

We are indebted to Professor H. Rapoport and the University of Berkeley, California, for the C, H and O determinations on 5-methoxycoumarone. The Uvispek spectrophotometer used in this work was purchased by means of a grant from the Central Research Fund of London University.

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