absence of arylcarbinol groups and may be taken to indicate a large number of C-C bonds between the phenylpropane units of the artificial lignin. In view of the readiness with which eugenol forms dehydrodieugenol (II) it might be suspected that a large number of diphenyl links would occur in eugenol lignin, but the low yield of dehydrodivanillin (III) shows that this is not the case. Despite similarities of the eugenol lignin to gymnosperm lignin it does differ from natural lignin in the very important aspects mentioned above and certainly cannot be accepted as a true lignin. This should emphasize the danger of drawing conclusions about natural lignification from experiments in artificial systems.

SUMMARY

1. Artificial lignin has been produced on oat-coleoptile segments and potato parenchyma from purified eugenol in buffered aqueous solution containing hydrogen peroxide.

2. The colour reaction of the eugenol lignin with phloroglucinol, its behaviour in the 72 % (w/w) sulphuric acid lignin determination and its ultimate composition were all similar to these characteristics of gymnosperm lignin. Its ultraviolet- and infrared-absorption spectra were similar to those of gymnosperm lignin.

3. Unlike natural lignin, the eugenol lignin has no *p*-hydroxybenzyl alcohol or other arylcarbinol groups that can be ethylated.

4. On oxidation with nitrobenzene the eugenol lignin gave only a low yield of vanillin (5 %). The yield of dehydrodivanillin was about the same as from lignin.

5. It is concluded that eugenol lignin is a lignin-like substance, but that it differs from natural lignin in important respects. Conclusions about natural lignin and lignification drawn from experiments with eugenol lignin must therefore be made with the utmost caution.

The author wishes to thank Dr A. B. Wardrop and Mr G. W. Davies for preparation of lignified coleoptiles and the suggestion that larger quantities of eugenol lignin could be produced on potato parenchyma. He is grateful to Mrs M. Szilagyi for technical assistance. The microanalyses were carried out by the Australian Microanalytical Service.

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Biosynthesis of Griseofulvin: the Methylated Benzophenone Intermediates

By A. RHODES, B. BOOTHROYD, MOIRA P. MCGONAGLE AND G. A. SOMERFIELD
Glaxo Laboratories Ltd., Sefton Park, Stoke Poges, Bucks

(Received 16 February 1961)

Consideration of structural evidence led Birch & Donovan (1953) to suggest that the skeletons of many natural phenolic compounds were derived, at least in part, by the head-to-tail linkage of acetic acid units. Two types of ring closure were dis-

tinguished: an aldol type, leading notably to orcinol derivatives, and a *C*-acylation type, leading principally to phloroglucinol derivatives. It was suggested that griseofulvin arose from the head-to-tail linkage of seven acetic acid units and sub-

sequent ring closure of both types, one leading to the phloroglucinol ring and the other to the (potential) orcinol ring. Birch, Massy-Westropp, Rickards & Smith (1958) subsequently observed that [1-¹⁴C]acetic acid was incorporated into griseofulvin to the extent of about 1%, with the expected distribution of isotopes.

Barton & Cohen (1957) have proposed a chlorinated benzophenone 5-chloro-6:4'-dihydroxy-2:4:2'-trimethoxy-6'-methylbenzophenone as an intermediate in the biogenesis of griseofulvin. They postulated that one-electron-transfer oxidizing agents would oxidize the benzophenone to the diradical, which would then be coupled to give the intermediate dehydrogriseofulvin and finally griseofulvin by enzymic reduction. Day, Nabney & Scott (1960) later demonstrated this mechanism by chemical synthesis of griseofulvin.

Hockenull & Faulds (1955) showed that the methoxyl groups of griseofulvin arose by methyl transfer, presumably through the usual biochemical C₁-donor system. Uniform distribution of isotope labelling between the three methoxyl groups suggested that methylation occurred late in the biosynthesis of griseofulvin from a relatively stabilized methyl pool.

Examination by McMaster, Scott & Trippett (1960) of the residual material after extraction of griseofulvin from fermentation broths of mutant strains of *Penicillium patulum* led to the isolation and characterization of a number of new metabolites: dehydrogriseofulvin, griseophenone A, griseoxanthone C, griseophenone Y and dechlorogriseofulvic acid, of which the first two were already known to be intermediates in the chemical synthesis of griseofulvin.

Meanwhile, we had identified the chlorination stage in the biosynthesis of griseofulvin. Extension of this work showed that griseofulvin formation involved a number of benzophenones and that side reactions leading to xanthenes could intervene. Biochemical aspects and the structures of these griseofulvin intermediates and by-products are now described. The compounds have been given trivial names to agree with the nomenclature of McMaster *et al.* (1960).

EXPERIMENTAL

Ultraviolet spectra were measured with a Unicam SP. 500 or a Hilger Uvispek spectrophotometer, infrared spectra with either a Perkin-Elmer model 21 or Infracord spectrophotometer. Woelm acid alumina (supplied by L. Light and Co. Ltd.), deactivated to the Brockmann grade quoted in the text, was used. Melting points were determined in tubes and are corrected.

Organisms. Two mutant strains (C4/30, GA766) of *P. patulum* Bainier-Thom C.M.I. 39,809 were used.

Production of inoculum for shake-flask fermentations. The

required strain was grown on slopes of Czapek-Dox agar in cylindrical glass bottles (3½ in. × 1 in.) until sporulation had occurred (7 days at 24°). A small quantity of culture was then inoculated by loop into a 250 ml. conical flask containing 60 ml. of the medium: 2% of brown sugar; 1% of AnalaR CaCO₃; 3.8% of corn-steep-liquor solids; pH was adjusted to 5.5 with NaOH and the flask sterilized at 15 lb./in.² for 20 min. After inoculation, the flask was shaken for 48 hr. at 25° on a rotary shaker and 2 ml. of the resulting vegetative mycelium was used to inoculate each flask of fermentation medium.

Standard method of fermentation. This was done in 250 ml. conical flasks containing 40 ml. of the medium: 11% of lactose; 0.3% of KCl; 0.4% of KH₂PO₄; 0.8% of AnalaR CaCO₃; corn-steep liquor to give 0.075% of N and distillers' solubles to give 0.075% of N. The flasks were sterilized by autoclaving for 20 min. at 15 lb./in.². For any given fermentation treatment, pH was determined on the contents of one flask, and this was subsequently adjusted to pH 4.8 with a noted volume of sterile 1% H₂SO₄. This volume of acid was then added to all other flasks of that treatment, thus giving uniform pH for the beginning of all fermentations. There were 3 or 4 replicate flasks of each treatment. Each flask was inoculated as already described and then shaken at 25° on a rotary shaker until the fermentation was completed.

Chromatographic assay. At intervals during the fermentation the contents of the flasks were sampled. Two replicate flasks were sampled at days 5, 7, 9 and 16 or 17, and the remainder at days 9, 12 and 16 or 17. Broth (1 or 2 ml.) was removed into test tubes and extracted by vigorous shaking for 5 min. with an equal volume of chloroform. The tubes were centrifuged and the chloroform layers removed into clean test tubes. The extracts were loaded (0.1 ml. at days 5, 7 and 9; 0.05 ml. at day 12; 0.025 ml. at day 16 or 17) on Whatman no. 1 papers (30 cm. × 50 cm.), which were developed by downward flow. The solvent system, benzene-cyclohexane-methanol-water (5:5:6:4, by vol.) was shaken thoroughly and allowed to separate for at least 2 hr. in a constant-temperature room (70° F). Acetic acid (1 vol.) was then added to 200 vol. of upper layer. The lower layer was used in the bottom of the tanks and the acidified upper layer in the troughs. Immediately before development, a jet of upper-layer solvent was directed just below the origin line on the paper so as to give a broad band of saturation in the path of development. Rates of flow were followed by using a solution (one drop) of α-naphtholbenzein as marker at each side of the papers. Development was usually complete in 6 hr. at 70° F. The papers were then dried and examined under ultraviolet light, first at 3660 Å and then at 2537 Å.

The benzophenone intermediates appeared as ultraviolet-quenching maroon-coloured spots under radiation of either wavelength. It was observed that the colour saturation of these spots under 3660 Å radiation could readily be graded on a scale of 0 to 5 and that these grades of intensity were accompanied by similarly increased areas. This was used as a rough quantitative method of expressing the relative amounts of benzophenone present.

The xanthenes and an unidentified substance (*P*) were graded under 2537 Å radiation on an arbitrary scale of 0 to 5. The xanthenes were assessed by the intensity of their yellow fluorescence and substance *P* by the degree of saturation of its purple ultraviolet-quenching colour.

Spectrophotometric assay. At the end of the fermentation periods (day 16 or 17) the broths were extracted as described below. The contents of each flask, along with 2 drops of octan-1-ol to prevent excessive frothing, were homogenized for approximately 1 min.: 10 ml. of homogenized broth and 80 ml. of acetone were shaken for 30 min. in 250 ml. stoppered flasks containing 10 glass beads (diameter 4–7 mm.); 0.5–1.0 ml. of the supernatant extract was transferred to a tube (6 in. $\times$ 1 in.) and evaporated to dryness in a stream of warm air; the residue was dissolved in 1 ml. of *NN*-dimethylformamide and 49 ml. of butyl acetate added; the solution was filtered through Whatman no. 1 paper; the extinction of the filtrate was determined at 288, 292, 296, 298 and 300 m μ . Similar determinations were made on a standard solution of pure griseofulvin in butyl acetate (containing an amount of dimethylformamide such as to make it comparable with the unknown sample). The griseofulvin content of the extracts was determined from the ultraviolet-absorption values by application of the six-point correction procedure of Daly (1961) as an improvement on the method for elimination of irrelevant absorption described by Ashton & Tootill (1956).

Isolation of griseophenone C. A chloride-deficient but otherwise standard fermentation medium with thiourea (30 μ g./ml.) added after 96 hr. was used in a 50 gal. vessel inoculated with strain C4/30, and the broth (110 l.; pH 7.6) was filtered on a rotary filter. The wet mycelium was extracted with acetone (2 $\times$ 30 l.) and the acetone extract evaporated *in vacuo* to 5 l. (A). The broth filtrate was adjusted to pH 3.0 with H_3PO_4 and extracted with chloroform (2 $\times$ 30 l.). The extract was evaporated *in vacuo* to 2 l. (B).

The acetone solution (A) was evaporated further until all the acetone had been removed, and the residual aqueous suspension (about 2 l.) was extracted with chloroform (3 $\times$ 1 l.); this chloroform solution and the filtered broth extract (B) were combined and evaporated under reduced pressure to about 1 l. A colourless solid (griseofulvin) was separated, and the chloroform solution was extracted with *N*-NaOH (3 $\times$ 500 ml.). The alkaline solution was acidified and extracted with ethyl acetate (3 $\times$ 500 ml.). The dried ethyl acetate solution was partly decolorized with activated charcoal and evaporated to about 20 ml. After 24 hr. at room temperature, brown prisms (3.0 g.), m.p. 168–174° (increased to 182–184° by repeated crystallization from ethyl acetate), were separated from the syrupy liquid, which yielded a brown solid (20 g.) on evaporation to dryness. This solid was dissolved in benzene (150 ml.) and filtered through a column of alumina (Grade IV, 150 ml.). Elution with benzene (500 ml.) and benzene-ethyl acetate (2:1, v/v; 500 ml.) yielded a pale-brown solid (9 g.), which was crystallized from ethyl acetate to yield pale-brown prisms (5.5 g.), m.p. 174–178°. Further elution with ethyl acetate yielded a dark-brown solid that could not be purified.

After several recrystallizations from ethyl acetate and finally from benzene, 2:6:4'-trihydroxy-4:2'-dimethoxy-6'-methylbenzophenone (griseophenone C) was obtained as pale-yellow prisms, m.p. 183–184° [Found: C, 63.1; H, 5.3; OMe, 20.2; mol.wt. (Rast), 276. $C_{18}H_{16}O_6$ requires C, 63.1; H, 5.3; OMe, 20.4%; mol.wt., 304], λ_{max} in ethanol 295 and 228 (infl.) m μ (ϵ 26 900 and 22 800 respectively), λ_{min} 247 m μ (ϵ 4360). The infrared spectrum (bromoform) had peaks at 3623, 3448 and 1639 cm.⁻¹.

The benzophenone (0.9 g.) was heated under reflux for

2 hr. with acetic anhydride (10 ml.) and pyridine (8 ml.); after dilution with water, a colourless solid was separated. After several recrystallizations from methanol, griseophenone C triacetate (0.52 g.) was obtained as colourless prisms, m.p. 155–156° (Found: C, 61.1; H, 5.2; acetyl group 30.8. $C_{23}H_{22}O_9$ requires C, 61.4; H, 5.1; acetyl group, 30.1%). The infrared spectrum (bromoform) had strong peaks at 1756–1750, 1650 and 1215 cm.⁻¹.

Methylation of griseophenone C. (i) A solution of griseophenone C (0.8 g.) in dry acetone (60 ml.) was heated under reflux for 24 hr. with anhydrous K_2CO_3 (2.5 g.) and dimethyl sulphate (2 ml.; a further 1 ml. was added after 12 hr.). The reaction mixture was diluted with water and extracted three times with ether. The ethereal solution was extracted with 2*N*-NaOH (3 $\times$ 20 ml.), washed with water, dried and evaporated to yield a yellow oil (0.78 g.). This oil was dissolved in benzene-light petroleum (b.p. 40–60°) (1:1, v/v; 12 ml.) and put on a column of alumina (Grade IV; 20 ml.). Elution with the same solvent (100 ml.) yielded a pale-yellow oil (0.74 g.), that crystallized from methanol to give colourless plates (0.40 g.), m.p. 123–125°. Further recrystallization from methanol yielded 6-hydroxy-2:4:2':4'-tetramethoxy-6'-methylbenzophenone, m.p. 124–125° (Found: C, 65.3; H, 6.1; OMe, 37.7. $C_{18}H_{20}O_6$ requires C, 65.1; H, 6.0; OMe, 37.4%), λ_{max} in ethanol 297 and 225 (infl.) m μ (ϵ 11 900 and 12 000 respectively), λ_{min} 250 m μ (ϵ 1550). The benzophenone gave a purple colour on treatment with ethanolic $FeCl_3$. The infrared spectrum (bromoform) had strong peaks at 1600 and 1580 cm.⁻¹, with a shoulder at 1613 cm.⁻¹. The alkaline extract was acidified and extracted with ether. Evaporation of the dried ethereal solution yielded a brown oil (0.08 g.) that could not be purified.

(ii) A solution of diazomethane (about 1 g.) in ether (30 ml.) was added at 0° to a solution of griseophenone C (1.5 g.) in tetrahydrofuran (15 ml.). After 40 hr. at 0°, the excess of diazomethane was decomposed and ether (70 ml.) was added to the ethereal solution before it was extracted with 2*N*-NaOH. Evaporation of the ethereal solution yielded a yellow oil (1.7 g.), which was chromatographed on alumina as described above. The resultant solid was crystallized from methanol to yield griseophenone C dimethyl ether as colourless prisms, m.p. and mixed m.p. 123–125°. The infrared spectra of the two samples were identical.

Oxidation of griseophenone C. A solution of CrO_3 (3.0 g.) in acetic acid (15 ml.) and water (6 ml.) was slowly added to a solution of griseophenone C (1.0 g.) in acetic acid (15 ml.), heat was evolved, and the temperature of the mixture rose to 60°. After 20 min. at 50°, water (200 ml.) was added to the mixture and the aqueous solution continuously extracted with benzene for 12 hr. Evaporation of the dried benzene solution yielded a brown solid (230 mg.), which was dissolved in benzene-light petroleum (b.p. 40–60°) (2:1, v/v; 5 ml.) and put on a column of alumina (Grade IV; 7 ml.). Elution with the same solvent (25 ml.) yielded a yellow solid (126 mg.), which was crystallized from methanol to yield 3-methoxytoluquinone (57 mg.), m.p. and mixed m.p. with an authentic sample prepared from griseofulvin (Grove, Ismay, MacMillan, Mulholland & Rogers, 1952a) 151.5–153.5° (Found: C, 62.8; H, 5.4; OMe, 20.3. Calc. for $C_9H_8O_3$: C, 63.2; H, 5.3; OMe, 20.1%). The infrared spectra of the two samples were identical.

Preparation of griseoxanthone C. (i) A solution of griseophenone C (0.5 g.) in a mixture of ethanol (10 ml.) and water (60 ml.) in a steel bomb was kept at 220° $\pm$ 10° for 6 hr.

A pale-yellow solid was separated from the cold mixture and crystallized from ethanol (25 ml.) as yellow needles (0.3 g.; yield 67%), m.p. 255–257°. The crystals were sublimed at $220^{\circ} \pm 5^{\circ}$ and 0.1 mm. Hg, and the sublimate was crystallized from ethanol to yield griseoxanthone C as yellow needles, m.p. 258–259° (Found: C, 66.1; H, 4.5; OMe, 11.4. Calc. for $C_{15}H_{12}O_5$: C, 66.2; H, 4.4; OMe, 11.4%), $\lambda_{\max}$ in ethanol 338 (infl.), 309, 270 (infl.) and 242 m μ (ϵ 9300, 23 200, 9300 and 37 800 respectively), $\lambda_{\min}$ 280 and 219 m μ (ϵ 12 000 and 17 400 respectively). The infrared spectrum had major peaks: 3330, 1666, 1626 and 1605 cm^{-1} (Nujol mull); 3521, 1653, 1618 and 1605 cm^{-1} (dimethyl sulphoxide); 3226, 1655 and 1605 cm^{-1} (tetrahydrofuran). McMaster *et al.* (1960) give m.p. 253–255° and $\lambda_{\max}$ 340, 309, 269, 242 m μ (ϵ 7000, 23 000, 9000 and 37 000 respectively).

(ii) A solution of griseophenone C (1.0 g.) in *n*-KOH (50 ml.) was heated under reflux and N_2 for 2 hr. The solution was cooled, acidified and extracted with ethyl acetate. Evaporation of the dried ethyl acetate solution yielded a yellow solid (0.85 g.), which was recrystallized from methanol to yield griseoxanthone C (0.76 g.; 85%), m.p. 257.5–259°.

Methylation of griseoxanthone C. A solution of griseoxanthone C (0.59 g.) in tetrahydrofuran was mixed at 0° with a solution of diazomethane (about 250 mg.) in ether (40 ml.). After 2 days at room temperature, the excess of diazomethane was decomposed and the dried ethereal solution evaporated to yield a yellow solid. The solid was recrystallized from methanol to yield lichexanthone (1-hydroxy-3:6-dimethoxy-8-methylxanthone) (0.57 g.; 92%), m.p. and mixed m.p. with a synthetic specimen (see below) 188–189.5° (Found: C, 67.3; H, 5.1; OMe, 21.8. Calc. for $C_{16}H_{14}O_5$: C, 67.1; H, 4.9; OMe, 21.7%). The two specimens had identical infrared spectra (Nujol mull and bromoform).

Synthesis of lichexanthone. Ethyl everminate (6.4 g.), m.p. 71.5–73°, prepared by the methylation of ethyl orsellinate, was hydrolysed with H_2SO_4 for 7 hr. at room temperature (cf. Fischer & Hoesch, 1912) to yield everminic acid (1.1 g.; 20%).

1:3-Dihydroxy-6-methoxy-8-methylxanthone was prepared by the method of Grover, Shah & Shah (1956) from the same quantities of starting materials. A yellow solid (0.19 g.) was obtained and had m.p. 252–258°, increased to 266–269° (decomp.) by several crystallizations from ethyl acetate–light petroleum. Grover *et al.* give m.p. 260–262°. This xanthone had m.p. 227–232° when mixed with griseoxanthone C.

1:3-Dihydroxy-6-methoxy-8-methylxanthone in acetone was methylated with diazomethane in ether (Grover *et al.* 1956) to yield lichexanthone, m.p. 188–189.5° after several crystallizations from ethyl acetate.

Isolation of griseoxanthone C from fermentation broth. The broth (800 ml.) from a chloride-deficient but otherwise standard fermentation medium was extracted with chloroform (3 $\times$ 300 ml.) and the chloroform solution evaporated to a small volume (about 40 ml.). After three extractions with 2*N*-NaOH, the chloroform solution was shown to contain only griseofulvin by paper chromatography and was discarded. The alkaline solution was acidified and extracted with chloroform. Evaporation of the dried chloroform extract yielded a brown solid (0.82 g.) that was dissolved in benzene (6 ml.) and put on a column of alumina (Grade V; 15 ml.). Elution with benzene (110 ml.) and benzene–ether (9:1, v/v; 70 ml.) yielded a brown gum that was shown to

be a mixture of griseoxanthone C and griseophenone C by paper chromatography. After several recrystallizations from methanol, griseoxanthone C (5 mg.) was obtained as yellow needles, m.p. 257.5–259°, undepressed on admixture with an authentic specimen. The two specimens had identical infrared spectra (Nujol mull) and the same R_F when run on the same paper chromatograms.

Extraction of griseophenones A and B. A standard shake-flask fermentation medium inoculated with strain C4/30 was harvested on the seventh day, when the chromatograms showed good yields of griseophenones A and B with minimum amount of griseophenone C. The mycelium was separated from the broth (5 l.), to which kieselguhr had been added to aid the separation, and the mycelium–kieselguhr was then extracted with acetone (2 $\times$ 2 l.). The acetone was removed by evaporation under reduced pressure and the residual aqueous suspension extracted with chloroform (3 $\times$ 400 ml.). The broth filtrate (4 l.) was also extracted with chloroform (3 $\times$ 1.2 l.), and the two chloroform solutions were combined and concentrated to a small volume (about 400 ml.). The chloroform solution was then extracted with 2*N*-NaOH (3 $\times$ 100 ml.); 2*N*-HCl (200 ml.) was slowly added to the cooled alkaline solution, which was then saturated with CO_2 . The resultant solution (pH 7.5) was extracted with chloroform (3 $\times$ 200 ml.), and the dried chloroform extract evaporated to yield a brown gum (4.9 g.). The gum was leached several times with boiling benzene (total 120 ml.), and the cooled solution filtered through alumina (Grade IV; 30 ml.). Elution with benzene (300 ml.) and benzene–ethyl acetate (4:1, v/v; 200 ml.) yielded a brown solid (3.9 g.).

Countercurrent separation of griseophenones A and B. The distribution was carried out in all-glass 50-tube apparatus (40 ml. each phase) with a benzene–methanol–water–*N*-HCl (200:200:100:1, by vol.) solvent system. The solid (3.9 g.), previously purified on alumina, was dissolved in upper phase (160 ml.) and put into tubes 1–4 of the apparatus, which had been filled with lower phase. The last tube of the apparatus was coupled to a fraction collector actuated when the machine was in the ‘decant’ position. After 210 transfers, the ultraviolet absorption of the upper-phase fractions at 295 m μ was measured and plotted to give the distribution curve shown in Fig. 1. Fractions 10–54 were combined and evaporated to yield a brown solid (1.58 g.), shown by paper chromatography to be griseophenone A. The solid was crystallized from benzene to yield 5-chloro-6:4'-dihydroxy-2:4:2'-trimethoxy-6'-methylbenzophenone (0.80 g.), m.p. 213–214° undepressed on admixture with an authentic specimen (Found: C, 57.7; H, 4.7; OMe, 26.4; Cl, 10.0. Calc. for $C_{17}H_{12}O_6Cl$: C, 57.9; H, 4.8; OMe, 26.4; Cl, 10.1%). McMaster *et al.* (1960) give m.p. 212–214° for griseophenone A. The two specimens had identical infrared spectra (tetrahydrofuran) and the same R_F when run on the same paper chromatogram.

Evaporation of fractions 55–120 yielded a yellow solid (1.22 g.) that was shown by paper chromatography to be griseophenone B. The solid was crystallized from benzene to yield 5-chloro-2:6:4'-trihydroxy-4:2'-dimethoxy-6'-methylbenzophenone (0.85 g.) as yellow prisms, m.p. 204.5–205.5° (Found: C, 56.9; H, 4.4; OMe, 18.3; Cl, 10.3. $C_{16}H_{12}O_6Cl$ requires C, 56.7; H, 4.4; OMe, 18.3; Cl, 10.5%), $\lambda_{\max}$ in ethanol 293 and 235 (infl.) m μ (ϵ 21 800 and 19 300 respectively), $\lambda_{\min}$ 255 m μ (ϵ 4900). The infrared spectrum had peaks at 3448, 1626 (shoulder), 1610 and 1575 cm^{-1} .

Fig. 1. The separation of griseophenones A, B and C by countercurrent distribution. A, B and C refer to the corresponding griseophenones.

(Nujol mull) and 3584, 3425, 1618 (shoulder) and 1608 cm^{-1} (tetrahydrofuran).

Fractions 121–129 were evaporated to yield a brown solid (64 mg.) that was found to be a mixture of griseophenones B and C on paper chromatography. Fractions 130–160 and the contents of tubes 26–50 were evaporated to yield a dark-brown solid (442 mg.), from which griseophenone C (146 mg.), m.p. 179–182°, was obtained on crystallization from benzene.

Extraction of griseophenones A, B and C and griseoxanthone C from large-scale fermentation residues. The residues were a dark-brown thick gum obtained by evaporating to dryness the mother liquors from a large-scale crystallization of crude griseofulvin.

The residues (about 250 g.) were dissolved in chloroform (1.3 l.), filtered to remove some resinous material and extracted with 0.5N-NaOH (total 2.5 l.). The chloroform solution was concentrated and, in the cold, impure griseofulvin separated. This process was repeated until the volume of the solution was about 100 ml. and crude griseofulvin (about 75 g.) was obtained. The chloroform solution was extracted with 2N-HCl (3 × 40 ml.), and the acid solution made alkaline with NaOH and then extracted with chloroform (3 × 50 ml.). Evaporation of the chloroform solution yielded a brown solid (5.1 g.) that was shown to contain a large amount of substance P by paper chromatography. The alkaline solution was adjusted to pH 7.5 with H_3PO_4 and extracted with chloroform (3 × 800 ml.). Evaporation of this chloroform solution yielded a dark-brown gum (31.5 g.) shown to contain griseophenones A, B, C and griseoxanthone C by paper chromatography.

The gum was dissolved in benzene (150 ml.) and filtered through alumina (Grade IV; 300 ml.). Elution with benzene (800 ml.) and benzene-ethyl acetate (4:1, v/v; 600 ml.) yielded a brown solid (19.0 g.) that was washed three times

with boiling benzene (total 60 ml.). The pale-brown solid that separated from the cold benzene extract and the solid left after the benzene washings were combined and separated by countercurrent distribution. The dark-coloured benzene solution was discarded.

Countercurrent distribution of extracts from large-scale fermentation residues. The distribution was carried out in five separating funnels by the 'diamond' pattern of Bush & Densen (1948) with a benzene-methanol-water-2N-HCl system (500:600:500:1, by vol.). The volumes of the upper phase (180 ml.) and the lower phase (270 ml.) for each equilibration were chosen so that the equation $\sqrt{(K_A r \times K_O r)} = 1$ [where K_A = partition coefficient of griseophenone A = 3.8 and K_O = partition coefficient of griseophenone C = 0.6 so that r = (volume of upper phase)/(volume of lower phase) = 2/3] was satisfied.

Some of the brown solid (8.3 g.) was dissolved in the upper phase (180 ml.) and was separated into ten fractions when the distribution was complete. The contents of each fraction were examined by paper chromatography and fractions of similar composition combined. Fractions 1–4 (1.70 g.) were crystallized from benzene to yield griseophenone C (1.17 g.), m.p. 173–176° increased to 181–183° by further crystallization from ethyl acetate-benzene. Fractions 7–10 (5.82 g.) were also crystallized from benzene to yield griseophenone A (2.06 g.), m.p. 210–212°. Paper chromatography of these benzene mother liquors showed the presence of griseoxanthone C and griseophenone A. The mother liquors were evaporated to leave a brown solid (3.7 g.). This solid and the remainder of the benzene-washed solid were subjected to another 'diamond' countercurrent distribution, with ten separating funnels. The solvent system was benzene-methanol-water-2N-HCl (600:600:300:1, by vol.), for which K_A is 1.78 and K_O is 0.34 so that r is 1.29, calculated from the above expression. The solid (9.5 g.) was dissolved in upper phase (256 ml.) and equilibrated with lower phase (200 ml.). Twenty fractions were obtained and treated as already described to yield: (a) Fractions 2–9, griseophenone C (819 mg.), m.p. 176–178° increased to 182–183° by further crystallization. (b) Fractions 10–19, griseophenone A (1.52 g.), m.p. 210–212°. The mother liquors were concentrated and a yellow solid (1.92 g.), m.p. 181–198°, was obtained. This solid was crystallized several times from methanol to yield griseoxanthone C (206 mg.), m.p. 256–258°.

Fractions 5 and 6 (719 mg.) from the first 'diamond' countercurrent distribution were shown to be a mixture of griseophenones A, B and C by paper chromatography and were fractionated by countercurrent distribution under the conditions described for the isolation of griseophenones A and B (see above). The crude, combined fractions were crystallized from benzene to yield griseophenones A (180 mg.), m.p. 212–213°, B (100 mg.), m.p. 188–190°, and C (170 mg.), m.p. 179–182°.

RESULTS

Observations on shake-flask fermentations. Chloride deficiency, induced by omission of potassium chloride from the standard fermentation medium, affected the metabolism of the two strains C 4/30 and GA 766. Biosynthesis of griseofulvin (R_f 0.85) was markedly inhibited in both, the final mean yields

being diminished by 78–81 %. The formation of a small amount of griseofulvin in the absence of added chloride was due to the presence of some chloride in the corn-steep liquor and distillers' solubles used as medium ingredients. The diminution in griseofulvin biosynthesis was accompanied by altered proportions of griseophenones, griseoxanthones and substance *P*.

Strain C4/30, in the standard fermentation medium, produced griseophenone C (R_f 0.25), griseophenone B (R_f 0.45) and griseophenone A (R_f 0.60), together with a substantial quantity of *P* (R_f 0.10). The effect of chloride deficiency could be seen by the fifth day and resulted in a marked accumulation of griseophenone C, but an absence of griseophenone B and a substantial diminution in griseophenone A. By the twelfth day there was also substantial diminution in substance *P*. These changes in the chloride-deficient fermentation broth were associated with the advent of griseoxanthone C (R_f 0.75) at the ninth day and its gradual accumulation up to the seventeenth day.

Strain GA766 behaved essentially in the same way except that larger amounts of griseophenone C, griseophenone B and substance *P* were formed in the standard fermentation broth. Enhanced synthesis of griseophenone C by this strain masked the effect of chloride deficiency on griseophenone C formation at the seventeenth day, but accumulation of this material between the fifth and twelfth days was obvious. This strain was also notable for the synthesis of a new substance, 'griseoxanthone B' (R_f 0.80), in the standard fermentation broth by the seventeenth day, but formation of this substance was prevented by chloride deficiency.

Subsequently it was shown that these effects of chloride deficiency could be simulated by addition of thiouracil or copper-chelating substances, such as thiourea, sodium diethyldithiocarbamate, picrolic acid, rubeanic acid and salicylaldehyde, or by reduced aeration.

It was more difficult to determine the effects of chloride deficiency with the parent strain, *Penicillium patulum* Bainier–Thom CMI 39,809, as it had a much lower potential for griseofulvin production. Nevertheless, a considerable quantity of griseophenone C was observed at the seventh day in chloride-deficient fermentation broths.

The accumulation of griseophenone C when griseofulvin biosynthesis was inhibited by deficiency of chloride suggested that this benzophenone might be the substrate for the chlorination reaction. If this were so, then addition of chloride at various times to a chloride-deficient fermentation broth would be expected to permit identification of further griseofulvin intermediates by observation of the sequence to removal of the metabolic block.

An experiment was performed with strain C4/30 when 0.3 % of potassium chloride was added at either the fifth, seventh or ninth day to a chloride-deficient standard fermentation broth. The fermentation medium without added potassium chloride showed by the fifth day the typical chloride-deficient metabolic picture already described. Addition of potassium chloride to the chloride-deficient fermentation broth at the fifth day resulted within 48 hr. in a diminution of griseophenone C accompanied by the presence of griseophenone B in large quantity and a small increase in griseophenone A. This diminution in griseophenone C was associated with absence of griseoxanthone C, as opposed to its appearance in the chloride-deficient fermentation broth at the seventh day and its gradual accumulation thereafter. Delay in the addition of potassium chloride to the chloride-deficient fermentation broth until the seventh or ninth day gave essentially similar results as addition at the fifth day, but resumption of the chlorination reaction suffered a lag period that increased progressively with the span of chloride deficiency.

The rate at which the chlorination of griseophenone C progressed was reflected in the mean yields of griseofulvin on the sixteenth day. The chloride-deficient medium afforded only 22 % of the mean amount of griseofulvin in the standard fermentation medium but addition of potassium chloride to the chloride-deficient fermentation broth at the fifth, seventh or ninth day increased the relative mean yields to 66, 29 and 26 % respectively.

Substitution of potassium iodide, bromide or fluoride at various concentrations in place of potassium chloride additions to similar chloride-deficient fermentation media did not yield any detectable quantity of iodo-, bromo- or fluoro-griseofulvin, although it seemed that iodide, and more particularly bromide, took part to a small extent in the halogenation reaction, since they led to the appearance of traces of ultraviolet-quenching substances having R_f values similar to that of griseophenone B. It may therefore be concluded that in this strain the halogenation of griseophenone C is specific for chlorine, but that iodine, and to a greater extent bromine, can participate to a minor degree.

Since bromide and iodide had replaced chloride in the chloride-deficient fermentation medium to a small extent only, it seemed possible that these halogens might in fact antagonize the chlorination reaction in the griseofulvin fermentation.

The addition of either 0.21 % of potassium bromide or 0.18 % of potassium iodide to the standard fermentation broth already provided with abundant chloride resulted in accumulation of griseophenone C and concomitant diminution of substance *P* and

griseophenones B and A, thus presenting a picture similar to that already observed in chloride-deficient conditions. Similarly, the metabolic block caused by bromide or iodide resulted in markedly decreased biosynthesis of griseofulvin, the yields being only 36 and 52 % respectively of that in the standard fermentation medium, accompanied by griseoxanthone C formation in the later stages of fermentation.

None of the experiments in which the chlorination reaction was inhibited provided any evidence for a major biosynthetic route from griseophenone C to dechlorogriseofulvin. This was subsequently confirmed by growth of strain C4/30 in a chloride-deficient fermentation medium prepared from substantially chloride-free corn-steep liquor (British Patent 773,453), although in this instance small amounts of dechlorogriseofulvin were observed by chromatography of broth extracts. This substance had slightly lower R_f and darker-blue ultraviolet fluorescence than griseofulvin and the crude solid gave a blue-violet colour when treated with concentrated nitric acid (MacMillan, 1953).

Further information was obtained by identifying the methylation stages in griseofulvin biosynthesis. With strain C4/30, addition of 0.1 % of choline chloride to the standard fermentation medium at 0, 48 or 96 hr. resulted in markedly faster biosynthesis of griseophenone C. The increased rate of formation of griseophenone C on addition of a methyl donor suggested that a folic acid antagonist, such as aminopterin, might have an inhibitory action on methylations in the griseofulvin fermentation. The standard fermentation medium, however, usually produced substantial quantities of griseophenone A, which might mask the inhibitory effect of aminopterin if this were exerted at some point in benzophenone synthesis.

Under conditions of optimum chloride concentration but with lactose reduced to 7 %, aminopterin (2, 4 or 8 $\mu\text{g./ml.}$ added at 0 hr.) diminished biosynthesis of griseofulvin, the mean yields falling to between 64 and 73 % of that from the standard fermentation medium, and led to accumulation of griseophenones B and C between the twelfth and eighteenth days. Aminopterin, however, also depressed the overall biosynthesis of griseophenones and substance P during the earlier stages of fermentation. Nevertheless, the ratio of griseophenone A to griseophenone B was substantially less throughout the aminopterin treatment.

The results obtained with the same additions of aminopterin to a chloride-deficient medium were similar to those obtained in a chloride-deficient medium without aminopterin: thus griseophenone C accumulated together with griseoxanthone C. These changes were accompanied by diminutions in biosynthesis of griseophenone A and substance P

and by absence of griseophenone B, as expected. Aminopterin, however, superimposed its inhibitory action on these effects of chloride deficiency and caused accumulation of griseophenones A and B, but now accompanied by diminution in griseophenone C and griseoxanthone C production during the earlier stages of fermentation. The inhibitory effects of aminopterin, however, were masked in the later stages of fermentation by the metabolic block imposed by chloride deficiency. In these circumstances, aminopterin had thus little effect on overall biosynthesis of griseofulvin, as this was already effectively limited to about 20 % of that in the standard fermentation broth.

DISCUSSION

Characterization of griseophenone C. It has been shown above that some phenolic metabolites of griseofulvin (I) accumulate when the normal fermentation is inhibited. In the absence of added

chloride, griseophenone C accumulated to the exclusion of the other metabolites. This substance contained two methoxyl groups. The infrared spectrum of its triacetate had peaks (1756–1750 and 1215 cm.^{-1}) consistent with the presence of phenolic acetate groupings and another peak (1650 cm.^{-1}) consistent with the carbonyl frequency of an aryl or diaryl ketone with several methoxyl substituents. A similar peak (1613 cm.^{-1}) in the dimethyl ether may be attributed to a carbonyl group hydrogen bonded with an *ortho* hydroxyl group, which is also consistent with its insolubility in alkali. Similar hydrogen bonding must occur in the parent substance to give the lowered frequency (1639 cm.^{-1}) of the carbonyl peak. The ultraviolet spectrum of the metabolite is similar to that of methyl phloracetophenone recorded by Morton & Sawires (1940) and, in part, to that of griseofulvin (Grove, MacMillan, Mulholland & Rogers, 1952b). It was probable that griseophenone C was a trihydroxydimethoxymethylbenzophenone.

On oxidative degradation, similar to that used on griseofulvin by Grove *et al.* (1952a), the benzophenone was readily oxidized, and under mild conditions chromic oxide oxidation yielded 3-methoxy-

toluquinone. The isolation of this toluquinone proves that one aromatic ring of the benzophenone is substituted with one hydroxyl, one methoxyl and one methyl group, and, by analogy with griseofulvin, the other aromatic ring is probably a phloroglucinol residue of which one hydroxyl group has been methylated.

Some benzophenones may be degraded to substituted benzoic acids when fused with potassium hydroxide, but fusion of this benzophenone yielded a small quantity of a non-acidic yellow crystalline solid. This yellow solid was also obtained when the benzophenone was heated with potassium hydroxide solution under various conditions or with water at 220° in a closed tube. An almost quantitative yield was obtained when the benzophenone was heated under reflux with *N*-potassium hydroxide for 2 hr. Methanol had been eliminated and the ultraviolet spectrum of the substance was similar to those reported by Yates & Stout (1958) for 1-hydroxyxanthenes. A monomethyl ether of the substance was identical with a specimen of lichexanthone (II; R = R' = Me) synthesized by the method of Grover *et al.* (1956).

Only two structures are possible for xanthenes whose monomethyl derivatives are lichexanthone. One of these, 1:3-dihydroxy-6-methoxy-8-methylxanthone (II; R = H; R' = Me), was an intermediate in the synthesis of lichexanthone and was different from the substance derived from the metabolite. Thus it follows that this substance, griseoxanthone C, must have the structure 1:6-dihydroxy-3-methoxy-8-methylxanthone (II; R = Me; R' = H). [McMaster *et al.* (1960) isolated griseoxanthone C from the residue obtained by evaporation of mother liquors from the crystalliza-

tion of crude griseofulvin. The work described above was carried out concurrently with theirs, but independently of it.] The position of the methoxyl group in this xanthone fixes the position of the methoxyl group in the phloroglucinol residue of the benzophenone. The methoxyl group already proved to be present in the other ring of the benzophenone was therefore eliminated in the formation of griseoxanthone C and must have been *ortho* to the carbonyl group. From the structure of griseoxanthone C, the methyl group must also be *ortho* to the carbonyl group, so that the structure of griseophenone C is 2:6:4'-trihydroxy-4:2'-dimethoxy-6'-methylbenzophenone (III; R = R' = H).

The elimination of methanol from a benzophenone to yield a xanthone has been reported by Barton & Scott (1958), who isolated a xanthone carboxylic acid from dihydroerodin after treatment with boiling *N*-sodium hydroxide, and by Hassall & McMorris (1959), who obtained a dichloroxanthone when dihydroeodoxin was kept at 125° for 5 min. with sulphuric acid (80 %). Nishikawa (1937) had isolated a substance that was probably a xanthone carboxylic acid from the potassium hydroxide fusion of sulochrin, a benzophenone from the mycelium of *Oospora sulphurea-ochracea*. In these examples, the elimination of methanol has been facilitated by an acid or base, but we know of no previous reports of the elimination of methanol in a sealed tube at high temperature. This method was first described by Dutta & Watson (1911) for the preparation of xanthenes by elimination of water from *oo'*-dihydroxybenzophenones, and Grover, Shah & Shah (1955) have reported that the conversion is almost quantitative.

Characterization of griseophenone B. Griseophenone B had a molecular formula $C_{16}H_{15}O_6Cl$, identical with that of griseophenone C when chlorine is substituted for one hydrogen. Since it has also been shown that griseophenone B is formed from griseophenone C during the fermentation and is subsequently converted into griseophenone A, it follows that griseophenone B is 5-chloro-2:6:4'-trihydroxy-4:2'-dimethoxy-6'-methylbenzophenone (III; R = Cl; R' = H).

Biosynthesis of griseofulvin. It may be concluded from the above that biosynthesis of griseofulvin proceeds from griseophenone C through griseophenones B and A to griseofulvin.

Chlorination has been shown to be a key reaction in this biosynthesis. The marked accumulation of griseophenone C accompanied by absence of griseophenone B and a marked fall in griseofulvin yield under chloride-deficient conditions strongly suggests that griseophenone C is the substrate for the chlorination reaction. This is supported by the observation that chloride ions supplied to a chloride-deficient fermentation medium that has accumu-

lated griseophenone C at the expense of griseofulvin formation can restart griseofulvin biosynthesis by progression first through griseophenone B and then through griseophenone A. Further support for this metabolic sequence is found in the fact that accumulation of griseophenone B in the presence of aminopterin is accompanied by increase in griseophenone C and a corresponding depression in the relative amount of griseophenone A formed.

The chlorination reaction in which griseophenone C is converted into griseophenone B appears to be similar to the chlorination stage in the fermentation of chlorotetracycline: both reactions are inhibited by bromide or iodide ions, thiouracil, reduced aeration or copper-chelating substances (Di Marco & Pennella, 1959), such as thiourea, sodium diethyldithiocarbamate, picrolonic acid, rubeianic acid and salicylaldehyde.

The two methoxyl groups of griseophenone C appear to be formed through the usual biochemical C_1 -donor system, because formation of this intermediate is accelerated by choline chloride but inhibited by aminopterin, and the latter might be expected to block methylation owing to antagonism of folic acid. Similarly, the same C_1 -donor system appears to be involved in the methylation reaction whereby griseophenone B is converted into griseophenone A, since this also is inhibited by aminopterin.

The aminopterin-induced metabolic block between griseophenone A and griseofulvin is unlikely to be due to interference with a methylation process, since this benzophenone already contains all the methoxyl groups of griseofulvin. It is possible that biosynthesis of griseofulvin from griseophenone A may be an oxidation-reduction reaction dependent on folic acid.

There are several side reactions that can intervene in the biosynthesis of griseofulvin. Thus whenever griseophenone C reaches a high concentration, regardless of the method of inhibition employed, formation of griseoxanthone C soon follows. Similarly, it was observed that strain GA766 formed 'griseoxanthone B', but its formation was prevented under chloride-deficient conditions.

Griseoxanthone C has been shown to be readily formed *in vitro* from griseophenone C by elimination of methanol. Similarly, 'griseoxanthone B' has been shown to be formed readily from griseophenone B *in vitro*, but we have, as yet, only recognized this substance by paper chromatography. It has an orange-yellow fluorescence under ultraviolet light and R_F 0.80, compared with the golden-yellow fluorescence and R_F 0.75 of griseoxanthone C. It seems likely that 'griseoxanthone B' may contain chlorine, since it is formed *in vitro* from griseophenone B and its biosynthesis is inhibited by the chloride-deficient conditions that caused accumulation of griseoxanthone C. It is therefore possible

that griseoxanthones C and B formed during our fermentations may be due to elimination of methanol from griseophenone C and griseophenone B respectively, in side reactions initiated when the subsequent reactions leading to griseofulvin are rate-limiting.

Another possibility is that the griseoxanthones may be formed *in vivo* by removal of water. Thus a benzophenone precursor of griseophenone C might lose water to give an unmethylated xanthone, which in turn could give griseoxanthone C by methylation or 'griseoxanthone B' by chlorination and methylation.

None of the experiments in which the chlorination reaction was inhibited provided any evidence for a major biosynthetic route from griseophenone C to dechlorogriseofulvin, although a small amount of this compound was observed when substantially chloride-free corn-steep liquor was used in the chloride-deficient fermentation medium. This side reaction might vary quantitatively with the strain of organism used and could account for the dechlorogriseofulvin isolated from surface cultures by MacMillan (1953). Subsequent hydrolysis of dechlorogriseofulvin could afford the dechlorogriseofulvic acid found by McMaster *et al.* (1960).

SUMMARY

1. Inhibition by appropriate methods of griseofulvin shake-flask fermentations and paper chromatography of the broth extracts showed that decreased yield of griseofulvin was accompanied by increased amounts of other metabolites.

2. Chlorination was shown to be a key reaction in griseofulvin biosynthesis. Inhibition of this reaction resulted in accumulation of griseophenone C, 2:6:4'-trihydroxy-4:2'-dimethoxy-6'-methylbenzophenone.

3. Chlorination of griseophenone C in the course of the fermentative production of griseofulvin afforded griseophenone B, which was shown to be 5-chloro-2:6:4'-trihydroxy-4:2'-dimethoxy-6'-methylbenzophenone.

4. Griseophenone B was shown to be methylated in the fermentation to give griseophenone A, which was found to be 5-chloro-6:4'-dihydroxy-2:4:2'-trimethoxy-6'-methylbenzophenone.

5. Griseophenone A was shown to be an intermediate in griseofulvin biosynthesis, and its conversion into griseofulvin was inhibited by aminopterin.

6. A side reaction in which griseoxanthone C (1:6-dihydroxy-3-methoxy-8-methylxanthone) was formed, intervened whenever griseophenone C reached a high concentration. Griseoxanthone C was isolated after a fermentation in which the chlorination of griseophenone C was inhibited. The

same compound was also prepared in good yield by various methods from griseophenone C, and its monomethyl ether was identical with lichexanthone.

7. The sequence established for the biosynthesis of griseofulvin is: griseophenone C $\rightarrow$ B $\rightarrow$ A $\rightarrow$ griseofulvin.

We thank Dr M. E. Peel (Allen and Hanburys Ltd) for a generous gift of ethyl orsellinate and Dr A. I. Scott (University of Glasgow) for a specimen of griseophenone A and for a copy of his paper with Dr W. J. McMaster and Dr S. Trippett before publication. We also thank Mrs S. Tarrant and Mrs M. Mannerings for invaluable technical assistance during all parts of the work concerned with fermentation and assay of the broth extracts.

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Studies of Fatty Acid Oxidation

7. THE EFFECTS OF FATTY ACIDS ON THE PHOSPHATE METABOLISM OF SLICE AND MITOCHONDRIAL PREPARATIONS OF RAT LIVER*

BY K. AHMED† AND P. G. SCHOLEFIELD‡

McGill-Montreal General Hospital Research Institute, 3619 University Street, Montreal, P.Q., Canada

(Received 3 November 1960)

The mechanism of fatty acid oxidation in mammalian tissues has been the subject of detailed investigation in recent years (see, for example, the review by Lynen, 1955), and it has been shown that they must undergo an initial activation step involving the participation of adenosine triphosphate to form acyl-coenzyme A derivatives. It was reasonable therefore to infer that inhibition of regeneration of adenosine triphosphate would lead to inhibition of fatty acid oxidation such as has been shown to occur in the presence of agents which uncouple oxidation from phosphorylation

* Part 6: Scholefield (1958).

† Colombo Plan Fellow.

‡ Research Associate of the National Cancer Institute of Canada.

(Cross, Taggart, Covo & Green, 1949; Lehninger, 1951). Quastel & Wheatley (1933) observed that fatty acids when present at concentrations above an optimum value gave rise to an inhibition of respiration of guinea-pig-liver slices. Lehninger (1945, 1951) made a similar observation on the oxidation of fatty acids by liver particles, and suggested that the fatty acids might act as uncoupling agents. It was later shown that decanoate decreases the phosphate/oxygen ratio for pyruvate oxidation in rat-brain and -kidney mitochondria (Scholefield, 1956) and that fatty acids stimulate the latent adenosine-triphosphatase activity of liver mitochondria (Pressman & Lardy, 1956). Further work (Creaser & Scholefield, 1960) showed that decanoate strongly inhibits phosphate incorporation into all