I wish to thank Dr M. Dixon, F.R.S., for suggesting the problem and for many valuable suggestions throughout the course of the work. I am grateful to Dr E. Webb for his help and advice. I am indebted to Mr B. Boon for carrying out the ultracentrifuge determinations and to Mr B. R. Slater for his help and co-operation with the electrophoresis experiments and for carrying out the diffusion measurement. I also wish to thank Mr D. Gardiner for much assistance during the preparation of diaphorase. This paper is published with the permission of the South African Council for Scientific and Industrial Research, to whom I am grateful for the award of a Research Bursary.

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Studies in the Biochemistry of Micro-Organisms

102. QUADRILINEATIN (1:2-DIFORMYL-5-HYDROXY-3-METHOXY-4-METHYLBENZENE), A METABOLIC PRODUCT OF *ASPERGILLUS QUADRILINEATUS* THOM & RAPER*

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Aspergillus quadrilineatus Thom & Raper, a species in the *A. nidulans* group, has been shown by Howard & Raistrick (1955) to produce in the perithecia a crystalline polyhydroxyanthraquinone pigment which has been named asperthecin. By degradative methods Neelakantan, Pocker & Raistrick (1957) have demonstrated that asperthecin is either 3:4:5:6:7-pentahydroxy-2-hydroxymethylanthraquinone or 3:4:5:7:8-pentahydroxy-2-hydroxymethylanthraquinone.

Howard & Raistrick (1955) noted the almost immediate production of a copious, heavy, dark-orange precipitate on the addition of Brady's reagent (2:4-dinitrophenylhydrazine in dilute hydrochloric acid) to the culture solution from strain S.M. 297 of the same organism. They recrystallized

* Part 101: Neelakantan, Pocker & Raistrick (1957).

the precipitate from acetone and obtained orange–red needles of m.p. 235° (decomp.) but did not examine them further. The investigation of the product responsible for this reaction forms the subject of the present communication. A description of the earlier stages of this work was incorporated in a Ph.D. thesis of London University (Lahoz-Oliver, 1955).

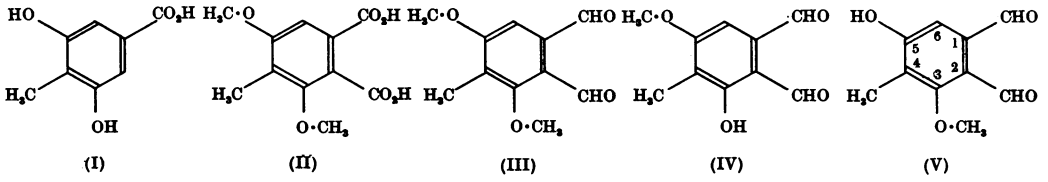
The separation of the product from the culture fluid was most readily accomplished by adsorption on active charcoal. The product could be extracted by means of ether from the charcoal after drying. The solid residue from the ether extract was recrystallized from benzene, which afforded a pure colourless crystalline product which has been named quadrilineatin.

Analysis of quadrilineatin, m.p. 172°, for elementary composition, methoxyl and *C*-methyl groups suggested the empirical formula, $C_{10}H_{10}O_4$, containing one methoxyl and one *C*-methyl group. The substance is neutral in reaction and possesses one hydroxyl group which can be acetylated or methylated. It affords a mono- and a bis-2:4-dinitrophenylhydrazone and a dioxime, indicating the presence of two reactive carbonyl groups. Oxidation with Doeuvre's (1927) reagent (alkaline mercuric iodide in potassium iodide solution) affords a crystalline monobasic acid, $C_{10}H_{10}O_5$, which still gives carbonyl reactions, indicating the presence of at least one formyl group in quadrilineatin. Oxidation of quadrilineatin with alkaline hydrogen peroxide gives in small yield a monobasic acid, isomeric but not identical with that obtained by the Doeuvre reaction. This acid also gives carbonyl reactions. This points to the presence of a second formyl group in quadrilineatin. The functions of all the oxygen atoms are thus accounted for; they are present, one as phenolic hydroxyl, one as methoxyl and two as formyl groups. Since a *C*-methyl group was detected and since benzene derivatives are readily obtained from quadrilineatin we may write the formula as $C_6H(CH_3, OH, OCH_3, 2CHO)$.

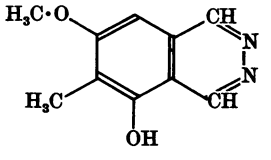
Considerable light was thrown on the arrangement of the substituents in the benzene nucleus by oxidation of the methyl ether of quadrilineatin with cold potassium permanganate. This produced a dibasic acid $C_{11}H_{12}O_6$ [represented as $C_6H(CH_3, 2\,O{\cdot}CH_3, 2\,CO_2H)$], which was an *o*-phthalic acid since it readily formed an anhydride. This phthalic acid on demethylation with hydrobromic acid lost also a carboxyl group and yielded 3:5-dihydroxy-*p*-toluic acid (I) identical with an authentic specimen. Owing to the symmetry of the molecule the carboxyl group eliminated must have occupied the 2-position, the phthalic acid precursor having the structure 3:5-dimethoxy-4-methylphthalic acid (II). For further confirmation the acid of structure (II) was synthesized and found to give no depression in m.p. when mixed with the phthalic acid obtained by permanganate oxidation of *O*-methylquadrilineatin. The anhydrides of these two acids were similarly compared and found to be identical. The two carboxyl groups of the phthalic acid must have arisen from the oxidation of the two formyl groups. Quadrilineatin methyl ether must therefore be an *o*-dialdehyde of structure (III).

On the evidence so far adduced, quadrilineatin must have structure (IV) or (V), depending on which of the two methoxyl groups present in the methyl ether (III) is found as free hydroxyl in quadrilineatin. An immediate clue to help decide this point is the fact that quadrilineatin gives no colour reaction with ferric chloride. It is inconceivable that a phenolic hydroxyl group in the *ortho* position to a formyl group should not give a colour. Further, neither of the two isomeric oxidation acids $C_{10}H_{10}O_5$ obtained, one by Doeuvre oxidation and the other by alkaline hydrogen peroxide oxidation, gives a ferric chloride reaction, yet one of these must have a carboxyl group in the 2-position (CHO at 1), adjacent to the 3-position, occupied by OH or $O{\cdot}CH_3$. If OH were the substituent at C-3 a strong ferric chloride colour would be expected (cf. salicylic acid). Hence the substituent at C-3 must be methoxyl, C-5 being reserved for the hydroxyl group. Quadrilineatin is therefore 1:2-diformyl-5-hydroxy-3-methoxy-4-methylbenzene (V).

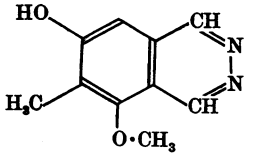
Confirmatory evidence was obtained by treating quadrilineatin with hydrazine or semicarbazide. These reagents afford a phthalazine $C_{10}H_{10}N_2O_2$ crystallizing ($+\,0{\cdot}2H_2O$ even after drying at 100°) in slightly yellow needles of m.p. 226–228° and giving virtually no colour reaction with ethanolic ferric chloride (which incidentally supports the formulation of quadrilineatin as a phthalaldehyde). The phthalazine obtained should have the structure 5-hydroxy-7-methoxy-6-methylphthalazine (VI) or 7-hydroxy-5-methoxy-6-methylphthalazine (VII) according as which of the two possible positions is occupied by the methoxyl group.



In the study of cyclopaldic acid by Birkinshaw, Raistrick, Ross & Stickings (1952) the phthalazine of established structure (VI) was obtained by the action of hydrazine on the acid, one molecule of carbon dioxide being lost in the process. This phthalazine had m.p. 260–262° (decomp.) and gave an orange–red colour with ethanolic ferric chloride. It is obviously different from the isomeric product obtained from quadrilineatin, which must therefore



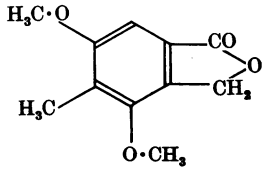
(VI)



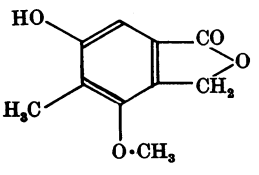
(VII)

have the structure (VII), confirming structure (V) for quadrilineatin. When these two phthalazines obtained from different sources were subjected to demethylation by hydrobromic acid, the products (isolated as the hydrobromide + $\frac{1}{2}C_2H_5 \cdot OH$ in each case) both melted at 238–240° (decomp.) and were identical since they showed no depression on mixing. This observation again confirms the correctness of the general structure ascribed to quadrilineatin.

When quadrilineatin is warmed with dilute sodium hydroxide it readily isomerizes to a phthalide, an internal Cannizzaro reaction to be expected from an *o*-dialdehyde. In order to determine which of the formyl groups is oxidized and which reduced in the rearranged form, the free hydroxyl group of the phthalide was methylated. The product of m.p. 158° could have either of two possible structures, 5:7-dimethoxy-6-methylphthalide or 4:6-dimethoxy-5-methylphthalide. The phthalide of the former structure had already been synthesized by Birkinshaw *et al.* (1952), when it was found to melt at 172–172·5°. The first possibility thus appeared to be excluded. The second possible structure, 4:6-dimethoxy-5-methylphthalide (VIII), was synthe-



(VIII)

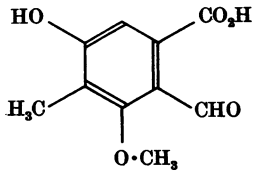


(IX)

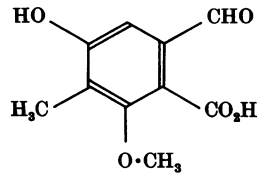
sized by chloromethylation of 3:5-dimethoxy-*p*-toluic acid, followed by hydrolysis and ring closure. It melted at 158° and gave no depression in melting point when mixed with the phthalide obtained as described from quadrilineatin. Therefore the phthalide formed from quadrilineatin under alkaline conditions must have the structure 6-hydroxy-4-methoxy-5-methyl phthalide (IX).

In the earlier work on quadrilineatin (Lahoz-Oliver, 1955) small amounts of a product of higher melting point were frequently obtained from the mother liquors on recrystallization of crude quadrilineatin with toluene or xylene as solvent. This was thought to be a second metabolic product. It has now been shown that this higher-melting product is identical with the phthalide obtained as described above. It must be regarded as an artifact and not as a second metabolite, since when benzene was used as solvent for crystallization and precautions were taken to avoid prolonged heating the phthalide could not be detected. Possibly traces of alkali in the glass of the vessels used, together with the temperature effect, conduced to its formation.

The two isomeric acids obtained by oxidation of one or other of the two formyl groups of quadrilineatin with Doeuvre's reagent and with alkaline hydrogen peroxide respectively must have the constitution (X) or (XI). In order to decide how these



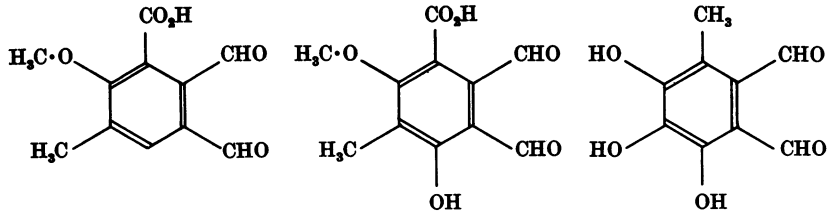
(X)



(XI)

structures should be allocated the acid produced by Doeuvre oxidation was subjected to reduction by zinc dust in methanol containing sulphuric acid. The –CHO group was reduced to $-CH_2 \cdot OH$ and a phthalide was obtained. This was methylated to a dimethoxyphthalide which was found to be identical (no m.p. depression on mixing) with the 5:7-dimethoxy-6-methylphthalide of m.p. 172–172·5° previously synthesized (Birkinshaw *et al.* 1952). This phthalide must have arisen from structure (XI), hence the acid produced by the Doeuvre reaction is 6-formyl-4-hydroxy-2-methoxy-3-methylbenzoic acid. The acid obtained by alkaline hydrogen peroxide oxidation of quadrilineatin must therefore have the alternative structure 2-formyl-5-hydroxy-3-methoxy-4-methyl benzoic acid (X).

Three other phthalaldehyde derivatives have been recorded as fungal metabolites: gladiolic acid (XII) from *Penicillium gladioli* (Brian, Curtis & Hemming, 1948; Grove, 1952; Raistrick & Ross, 1952), cyclopaldic acid (XIII) from *P. cyclopium* (Birkinshaw *et al.* 1952) and flavipin (XIV) from



(XII) (XIII) (XIV)

Aspergillus flavipes and *A. terreus* (Raistrick & Rudman, 1956). Of these cyclopaldic acid (XIII) is most closely related to quadrilineatin as regards type and arrangement of substituents; in fact norquadrilineatin would be identical with decarboxynorcyclopaldic acid.

Quadrilineatin was found to have only feeble antibacterial activity against *Staphylococcus aureus* and *Escherichia coli*. Since the three phthalaldehydes (XII), (XIII) and (XIV) were found to have considerable antifungal activity it became of interest to determine the behaviour of quadrilineatin in this respect. The antifungal activity, determined by Mr G. Smith, is recorded in the Appendix.

EXPERIMENTAL

All melting points are uncorrected. Elementary analyses and acetyl determinations are by Drs Weiler and Strauss, Oxford. Methoxyl determinations are by two of us (P.C. and R.L.-O.).

History of cultures

Five strains of *Aspergillus quadrilineatus* Thom & Raper were examined and compared. These were as follows: A 54a and A 54b were received in June 1942 from Dr K. B. Raper, of the Northern Regional Research Laboratory, Peoria, Illinois, and bore the designations NRRL 521 and NRRL 201 respectively. S.M. 200 and S.M. 297 were isolated from two different samples of Australian soil by Mrs S. Marcus in October 1950 and February 1951 respectively. G.A. 317 was isolated from Indian soil by Mr G. Agosti in August 1954.

The three soil strains were all isolated in this Department and were identified by Mr G. Smith. Since strain S.M. 297 gave the best yield of quadrilineatin (see Table 1) it was employed in the preparation of supplies of this metabolite needed for characterization and determination of molecular structure.

Cultural conditions

The culture medium employed throughout this work was the Raulin–Thom solution containing: glucose, 75 g.; tartaric acid, 4·0 g.; ammonium tartrate, 4·0 g.; $(NH_4)_2HPO_4$, 0·6 g.; $(NH_4)_2SO_4$, 0·25 g.; K_2CO_3, 0·6 g.; $MgCO_3$, 0·4 g.; $FeSO_4,7H_2O$, 0·07 g.; $ZnSO_4,7H_2O$, 0·07 g.; distilled water, 1·5 l. This was distributed in 1 l. conical flasks (350 ml./flask), which were plugged with cotton wool and sterilized by steaming on three successive days. The flasks were inoculated with a suspension in sterile distilled water of spores of the required strain, which had been cultivated on Czapek–Dox agar or wort–agar slopes. The inoculated flasks were incubated at 24° in the dark.

All the strains examined grew well but showed minor differences in appearance and reactions as recorded below.

Strain A 54a. The mycelium was folded, buff-brown in colour, with a deep purplish brown reverse. The pale-straw-coloured culture solution with Brady's reagent gave an immediate turbidity, followed by deposition of a fairly heavy orange–red ppt. It gave a slight brown colour with aqueous $FeCl_3$.

Strain A 54b. The upper surface of the mycelium was greyish brown, the under surface greyish white. The pale-yellow culture solution gave no turbidity or ppt. with Brady's reagent except on standing. There was no colour with $FeCl_3$.

Strain S.M. 200. The mycelium was dark yellowish brown on the upper, deep brown on the under surface. The golden-yellow culture solution gave only a slight orange ppt. with Brady's reagent and no reaction with $FeCl_3$.

Strain S.M. 297. The deeply-folded brown mycelium had a deep purplish brown reverse. The golden-yellow culture solution gave an immediate heavy red ppt. with Brady's reagent and a slight brown colour with $FeCl_3$.

Strain G.A. 317. The heavily folded mycelium was light buff-brown with a deep purplish brown reverse and showed numerous transpiration drops. The golden-yellow culture solution gave an immediate heavy red ppt. with Brady's reagent and no immediate colour with $FeCl_3$; a slight brown colour developed on standing.

The strains employed for the production of asperthecin were S.M. 200 and S.M. 297, as recorded by Howard & Raistrick (1955), who give further details about these two strains.

Harvesting of cultures and isolation of product

The method of harvesting one of the later batches of strain S.M. 297, as evolved when procedure had been standardized, is described below. It represents the typical method of working up and isolation of the product. For strain G.A. 317 a somewhat different procedure had to be adopted. This is also described.

Typical method as applied to strain S.M. 297. After incubation for a period of about 30 days the contents of the batch of flasks was mixed and filtered. The filtrate was stirred with activated charcoal (3 g./l.) for 2 hr.; the charcoal was recovered by filtration and dried *in vacuo* over conc. H_2SO_4. The filtrate no longer gave a ppt. with Brady's reagent. The dried charcoal was then exhaustively extracted with ether in Soxhlet extractors. The combined solid residues (6·65 g.) recovered from the ether were recrystallized once from benzene to yield needles (6·4 g.) of m.p. 169–170°. The tarry residues from the ether extracts and the mother liquor from the crystallization showed no tendency to crystallize and were not further examined. No evidence of a second,

higher-melting product was obtained. By further crystallization from benzene or water and sublimation in a high vacuum the m.p. was raised to a constant value of 172° (with slight decomp.).

Method applied to strain G.A. 317. The culture solution of strain G.A. 317 had given a heavy ppt. with Brady's reagent in the preliminary tests and appeared therefore to be a promising source of quadrilineatin. However, when a batch of ninety-eight flasks of this strain was harvested and the culture solution was treated with charcoal as above, it was found that the ether extract from the charcoal was a tar (12 g.), which showed no sign of crystallization even on prolonged cooling. It was adsorbed from benzene (200 ml.) on acid-washed alumina (200 g.), and the column was eluted successively with benzene (1 l.), benzene–ether (1:1, 1 l.) and ether–methanol (1:1; 500 ml.). The first two eluates were combined and the solution was concentrated to small volume, when crystalline material (3·6 g., m.p. 155–160°) was obtained. Removal of the solvent from the final eluate gave only a deep-brown tar (8 g.), from which no crystalline material could be obtained even by repeated chromatography. Recrystallization of the crystalline solid from benzene gave needles (3·15 g.; m.p. 168–170°) of quadrilineatin.

Properties of quadrilineatin

The pure product forms colourless needles of m.p. 172° (decomp.), and empirical formula $C_{10}H_{10}O_4$, light-absorption max. 250 and 297–300 mμ in ethanol (log ε 4·27, 3·71 respectively). It readily dissolves in hot water but is only sparingly soluble in the cold. It is very soluble in acetone and ether, and fairly so in cold ethanol. It is only slightly soluble in boiling $CHCl_3$ and in boiling light petroleum (b.p. 40–60°). It may be recrystallized from benzene. In aqueous or ethanolic solution no colour reaction is observed with aqueous or ethanolic $FeCl_3$. It is not soluble in a freshly-prepared cold saturated solution of $NaHCO_3$, but is soluble on warming to give a yellow solution. It is readily soluble in aqueous Na_2CO_3 to give a yellow solution and aqueous NaOH to give a yellowish brown solution, decolorized on acidification. It does not reduce Fehling's solution but does reduce ammoniacal $AgNO_3$ soln. It gives a ppt. of Hg when treated with Doeuvre's reagent (aqueous HgI_2 in KI and NaOH). It darkens somewhat on exposure to air. With the Gibbs (1927) reagent in borate buffer (pH 9·2) it gives a greenish blue solution almost immediately. This becomes more blue up to 1 hr., when the colour becomes permanent. It gives no reaction in the following tests for the $H_3C{\cdot}CO$-group: (*a*) iodoform reaction, (*b*) with sodium nitroprusside or (*c*) with *m*-dinitrobenzene in alkaline solution [Found (on a sublimed specimen, m.p. 172°): C, 62·2, 62·2; H, 4·9, 5·35; OMe, 15·8, 15·8; *C*-Me, 5·1; $C_{10}H_{10}O_4$ requires C, 61·8; H, 5·2; (one) OMe, 16·0; (one) *C*-Me, 7·7%].

Antibacterial activity. Quadrilineatin (50 mg.) was dissolved in hot sterile distilled water (15 ml.), cooled quickly and made up to 25 ml. to give a 1:500 dilution, from which further dilutions (two sets) starting at 1:1000 were prepared with sterile heart broth–2% glucose medium. These 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. With *Staph. aureus* there was no growth at 1:1000, partial growth at 1:2000, and full growth at 1:4000. With *Esch. coli* complete growth occurred at 1:2000. Quadrilineatin has thus only weak antibacterial activity against the strains tested.

Derivatives of quadrilineatin

Methyl ether. Quadrilineatin (0·08 g.) was dissolved in dry acetone (8 ml.); dry K_2CO_3 (1·6 g.) and dimethyl sulphate (0·8 ml.) were added to the solution. The mixture was refluxed for 2 hr. The excess of K_2CO_3 was removed by filtration and the acetone was distilled off. The residual solid was treated with water. The dark-brown crystalline material was collected and purified by sublimation in high vacuum. Colourless needles of O-*methylquadrilineatin*, m.p. 146–148°, were thus obtained. The product could be recrystallized

Table 1. *Cultural details and yield of product from various strains of* Aspergillus quadrilineatus *grown on Raulin–Thom medium at* 24°

Strain	No. of flasks	Incubation (days)	Residual glucose (%)	Final pH	Yield (g.) and m.p. of crude solid extracted by ether from charcoal	Yield (g.) and m.p. of once-recryst. material	Wt. (g.) of residual non-cryst. material
G.A. 317	98	21	0·60	4·2	3·57* 155–160°	3·15 168–170°	8
A 54a	22	32	0·36	4·6	2·57 168–170°		
S.M. 200	21	32	0·71	4·8	0·41 169–171°	6·2 169–171°	0·64
S.M. 297	22	32	0·42	4·8	3·72 169–171°		
A 54b	25	32	0·42	4·4	Nil	—	—
S.M. 297	100	28	0·46	4·4	6·65 168–171°	6·41 169–170°	0·22
S.M. 297	100	28	0·33	4·6	8·40 168–170°	8·01 169–170°	0·36

* Isolated by chromatography from 12 g. of tar.

from light petroleum, b.p. 80–100° [Found: C, 63·1; H, 5·7; OMe, 30·4. $C_{11}H_{12}O_4$ requires C, 63·4; H, 5·8;(two) OMe, 29·8%].

Acetate. Quadrilineatin (0·4 g.) was heated for 45 min. at 140° (bath temp.) with anhydrous sodium acetate (0·8 g.) and acetic anhydride (2 ml.). Water (20 ml.) was added to the cooled mixture. The oil separating solidified on shaking the mixture. The solid was collected and dried (0·46 g., m.p. 120–132°). Recrystallization from light petroleum (b.p. 80–100°) with addition of decolorizing charcoal afforded irregular plates of *acetylquadrilineatin*, m.p. 140–143° [Found: C, 60·8; H, 5·3; $H_3C{\cdot}CO{\cdot}$, 21·0. $C_{12}H_{12}O_5$ requires C, 61·0; H, 5·1; $H_3C{\cdot}CO{\cdot}$, 18·2%].

Quadrilineatin bis-2:4-dinitrophenylhydrazone. 2:4-Dinitrophenylhydrazine (0·5 g.) was dissolved in warm conc. H_2SO_4 (1 ml.) and the solution was diluted with ethanol (10 ml.). This solution was mixed with a solution of quadrilineatin (0·2 g.) in ethanol (10 ml.). A cherry-red product separated at once, which changed to orange–red in 5 min. The crude crystalline bishydrazone was collected, washed with a little ethanol and dried; it had m.p. 246–247°. After three recrystallizations from butanol the *bis-2:4-dinitrophenylhydrazone* was obtained as orange–red needles of m.p. 262–263° (decomp.) [Found: C, 48·0, 47·6; H, 3·3, 3·1; N, 17·1, 17·8; OMe, 5·7. $C_{22}H_{18}O_{10}N_8$ requires C, 47·7; H, 3·3; N, 20·2; OMe, 5·6%]. A drop of dilute NaOH added to an ethanolic solution of the bishydrazone gave a red colour, indicating that the parent substance was not an α-dicarbonyl compound.

Mono-2:4-dinitrophenylhydrazone of quadrilineatin. This derivative was readily obtained from the culture solution after harvesting. The culture solution (1 l.) from strain S.M. 297 was treated with Brady's reagent [2:4-dinitrophenylhydrazine (0·4%) in 2N-HCl; 500 ml.]. A reddish orange amorphous ppt. was immediately produced. The mixture was kept overnight at 0°. The ppt. was collected and crystallized from nitrobenzene, giving crimson hair-like needles (0·71 g.) of the *mono-2:4-dinitrophenylhydrazone* of quadrilineatin of m.p. 234–235° (decomp.). Further recrystallizations from butanol did not raise the m.p. [Found: C, 51·5; H, 4·5; N, 14·5. $C_{16}H_{14}O_7N_4$ requires C, 51·3; H, 3·8; N, 15·0%]. With strain G.A. 317, the culture solution (100 ml.) was treated with Brady's reagent (200 ml.) and was left for 1 hr. with occasional shaking. The orange–red ppt. formed was collected and dried (m.p. 185–190°). The filtrate was left for 4 days and the brownish red ppt. was collected (0·1 g., m.p. 183–186°). The combined ppts. were crystallized from nitrobenzene, which raised the m.p. to 233–235° (decomp.). By fractional crystallization from butanol and dioxan the crystals were separated into two distinct products: (*a*) the mono-2:4-dinitrophenylhydrazone (0·15 g.), m.p. 235–236° (decomp.), jagged red needles; (*b*) the bis-2:4-dinitrophenylhydrazone (0·03 g.), m.p. 264° (decomp.), orange–red needles. These two products did not depress the m.p. of the mono- and the bis-2:4-dinitrophenylhydrazones respectively previously obtained from strain S.M. 297 culture fluid and from pure quadrilineatin [Found for (*a*): C, 51·2; H, 4·5; N, 14·7; OMe, 8·3. $C_{16}H_{14}O_7N_4$ requires C, 51·3; H, 3·8; N, 15·0; OMe, 8·3%. Found for (*b*): C, 47·7; H, 3·4; N, 20·0; OMe, 6·0. $C_{22}H_{18}O_{10}N_8$ requires C, 47·7; H, 3·3; N, 20·2; OMe, 5·6%].

Quadrilineatin dioxime. A solution of quadrilineatin (0·1 g.) in pyridine (0·15 ml.) mixed with a solution of hydroxylamine hydrochloride (0·1 g.) in ethanol (2 ml.) was refluxed for 3 hr. Water (25 ml.) was added and the solution was evaporated under reduced pressure to half volume. The product, in the form of brownish needles (0·1 g., m.p. 188–192°) was recrystallized from water with addition of charcoal. The *dioxime* was obtained as cream-coloured needles of m.p. 205–206° [Found: C, 53·8, 53·8; H, 5·5, 5·5; N, 12·1, 12·5. $C_{10}H_{12}O_4N_2$ requires C, 53·6; H, 5·4; N, 12·5%].

Oxidation of quadrilineatin

Doeuvre reaction: production of 6-formyl-4-hydroxy-2-methoxy-3-methylbenzoic acid. Quadrilineatin (0·51 g.) was suspended in water (5 ml.) and a solution of HgI_2 (3 g.) and KI (10 g.) in water (20 ml.) was added, followed by N-NaOH (40 ml.). The bright-yellow solution soon began to deposit Hg. It was shaken frequently. After 6 hr. all the crystalline solid had disappeared and the Hg precipitation appeared complete. The solution was filtered through kieselguhr to remove Hg, and the filtrate and washings were titrated to phenolphthalein with N-HCl. The titration value (32·0 ml.) showed that 8·0 ml. of N-acid had been produced in the reaction. (For the conversion

$$R{\cdot}CHO + HgI_2 + H_2O \rightarrow R{\cdot}CO_2H + 2HI + Hg,$$

which requires the production of 3 equiv. of acid, the calc. value is 7·87 ml.) The solution was acidified to Congo red with conc. HCl. On chilling, straw-coloured needles slowly separated and were collected (0·24 g., m.p. 174–176°). Recrystallization from water gave almost colourless needles (0·16 g.) of *6-formyl-4-hydroxy-2-methoxy-3-methylbenzoic acid*, m.p. 176°. The acid was soluble in aqueous $NaHCO_3$ and Na_2CO_3 to give colourless solutions and in cold 2N-NaOH to give a slightly yellow solution. Brady's reagent added to an ethanolic solution of the acid produced an immediate turbidity, followed by deposition of an orange–yellow ppt. With the Gibbs reagent the reaction was similar to that of the parent compound [Found on a specimen dried for 1½ hr. in high vacuum at 70°: C, 55·1; H, 5·1; OMe, 13·85%; equivalent by titration, 215. $C_{10}H_{10}O_5,\frac{1}{2}H_2O$ requires C, 54·75; H, 5·1; OMe, 14·2%; equivalent (monobasic), 219].

2:4-Dinitrophenylhydrazone. The mother liquors from the crystallization of the above acid were treated with an excess of Brady's reagent. After 6 hr. the ppt. was collected and dried [0·11 g., m.p. 263–266° (decomp.)]. Recrystallization from butanol and acetic acid gave microscopic orange–yellow needles (45 mg.) of *6-formyl-4-hydroxy-2-methoxy-3-methylbenzoic acid 2:4-dinitrophenylhydrazone*, m.p. 267–268° (decomp.) [Found (on a specimen dried at 120° in high vacuum); C, 49·2; H, 3·8; N, 13·7; OMe, 8·0. $C_{16}H_{14}O_8N_4$ requires C, 49·2; H, 3·6; N, 14·35; OMe, 7·95%].

Oxidation of quadrilineatin with alkaline hydrogen peroxide: production of 2-formyl-5-hydroxy-3-methoxy-4-methylbenzoic acid. Quadrilineatin (1 g.) was dissolved in 2N-NaOH, and to the deep-yellow solution was added H_2O_2 (3%; 7 ml.) in one portion. The colour of the solution changed quickly to wine-red and the temp. rose to about 40°. When the reaction mixture had cooled to room temp. it was acidified to Congo red with 2N-H_2SO_4, when a little oil separated and was removed. The solution was then treated with a slight excess of $NaHCO_3$ and extracted with ether (4 × ¼ vol.). Removal of the ether left a greenish brown amorphous residue (0·42 g.), which when recrystallized from water afforded needles (0·33 g., m.p. 169–170°) of unchanged quadrilineatin. The aqueous portion was now reacidified and the oil produced was extracted with 4 × ¼ vol.

of ether. The deep-brown tarry residue (0·45 g.) from the ether was subjected to sublimation in a high vacuum at 160–170°. The sublimate (55 mg.) on recrystallization from water formed colourless needles (25 mg.) of *2-formyl-5-hydroxy-3-methoxy-4-methylbenzoic acid*, m.p. 218°. This product gave a deep greenish blue colour with the Gibbs reagent after 5 min.; the colour deepened further up to 1 hr. [Found (on a specimen sublimed in high vacuum): C, 56·5; H, 4·8; OMe, 14·5%; equivalent by titration, 212. $C_{10}H_{10}O_5$ requires C, 57·1; H, 4·8; OMe, 14·8%; equivalent (monobasic), 210].

2:4-*Dinitrophenylhydrazone*. The mother liquors from the crystallization of the above acid were treated with an excess of Brady's reagent and allowed to stand for 6 hr. The ppt. (m.p. 235–237°) when recrystallized twice from benzene gave orange–red needles of *2-formyl-5-hydroxy-3-methoxy-4-methyl benzoic acid* 2:4-*dinitrophenylhydrazone*, m.p. 237° (decomp.) [Found: C, 49·5; H, 3·5; N, 14·1. $C_{16}H_{14}O_8N_4$ requires C, 49·2; H, 3·6; N, 14·35%].

Oxidation of quadrilineatin methyl ether with potassium permanganate: isolation of 3:5-*dimethoxy-4-methylphthalic acid*. The crude methyl ether of quadrilineatin, prepared as described above, in the form of a golden-yellow syrup (0·81 g.), was dissolved in purified acetone (40 ml.), and finely powdered $KMnO_4$ (3·1 g.) was added gradually with shaking until the colour remained for 3 hr. The solution was decolorized with a few drops of ethanol and the MnO_2 removed by filtration. A negligible amount of dissolved material was present in the acetone solution. The MnO_2 was extracted with hot water (3 × 30 ml.) and the aqueous extract was acidified to Congo red with HCl and extracted with ether (4 × ½ vol.). The sticky light-brown residue from the ether was crystallized from ethyl acetate–light petroleum and gave colourless needles (0·20 g.) of m.p. 202–204°, with sublimation and gas evolution, resetting on cooling and remelting at 160–162°. The initial m.p. was raised by recrystallization from water to 207–208°, remelting at 164–165°. The m.p. was not depressed on admixture with authentic 3:5-dimethoxy-4-methylphthalic acid (m.p. 206–207°) synthesized for comparison [Found: C, 54·6; H, 5·2; *C*-Me, 6·7; OMe, 25·6%; equivalent by titration, 120. $C_{11}H_{12}O_6$ requires C, 55·0; H, 5·0. *C*-Me, 6·3, two OMe, 25·8%; equivalent (as dibasic acid), 120].

Conversion into the anhydride. The 3:5-dimethoxy-4-methylphthalic acid (55 mg.) was heated at 220° for 20 min. The product rapidly solidified on cooling. On sublimation in high vacuum at 120–130°, colourless needles (35 mg.) of 3:5-dimethoxy-4-methylphthalic anhydride of m.p. 165–166° were obtained. The product did not depress the m.p. of the authentic anhydride on admixture [Found: C, 58·9; H, 4·5; OMe, 28·0. $C_{11}H_{10}O_5$ requires C, 59·4; H, 4·5; two OMe, 27·9%].

Demethylation and monodecarboxylation of 3:5-*dimethoxy-4-methylphthalic acid: isolation of* 3:5-*dihydroxy*-p-*toluic acid*. The dimethoxy acid (0·10 g.) was refluxed for 20 min. with HBr (46–48%; 5 ml.). All the solid dissolved to form a light-brown solution. Almost colourless needles separated on cooling (50 mg.; m.p. 262–264°). The product was recrystallized from ethyl acetate–light petroleum, yielding microcrystalline needles (30 mg.) of 3:5-dihydroxy-4-methylbenzoic acid, m.p. 264–265° with sublimation and darkening. The m.p. was unchanged after sublimation. The product gave no colour with aqueous or ethanolic $FeCl_3$ and did not depress the m.p. of authentic 3:5-dihydroxy-*p*-toluic acid on admixture [Found (on sublimed specimen): C, 56·15; H, 5·0%; equivalent by titration, 166. $C_8H_8O_4$ requires C, 56·4; H, 4·8%; equivalent (as monobasic acid), 168].

Methyl 3:5-*dihydroxy*-p-*toluate*. The above acid (30 mg.) was dissolved in ether and treated with a slight excess of diazomethane. The solvent was removed and the residue was recrystallized twice from benzene to give needles (15 mg.) of methyl 3:5-dihydroxy-*p*-toluate; m.p. 189°, which did not depress the m.p. of an authentic specimen on admixture [Found: C, 59·4; H, 5·8; OMe, 17·2. $C_9H_{10}O_4$ requires C, 59·3; H, 5·5; OMe, 17·0%].

Production of a phthalazine from quadrilineatin

Action of hydrazine hydrate. Quadrilineatin (0·20 g.) and hydrazine hydrate [50% (v/v) prepared from 98–100% hydrazine; 0·10 ml.] were mixed to give a golden-brown solution. Water (3 ml.) was added and the mixture was refluxed for 15 min. The deep-brown solution was concentrated to about half volume on the water bath, when golden-yellow needles separated and were collected (0·15 g., m.p. 225–228°). Recrystallization from aqueous ethanol (50%, v/v) gave slightly yellow needles of *7-hydroxy-5-methoxy-6-methylphthalazine*, m.p. 226–228° (decomp.). These were immediately soluble in cold 2N-HCl and gave no ppt. with Brady's reagent and only a very slight yellowish brown colour with $FeCl_3$ [Found (on a specimen dried 1½ hr. in high vacuum at 100°, no loss in weight): C, 62·1; H, 5·2; N, 14·2; OMe, 16·55. $C_{10}H_{10}O_2N_2,\frac{1}{2}H_2O$ requires C, 61·95; H, 5·4; N, 14·45; OMe, 16·0%].

Action of semicarbazide. Quadrilineatin (0·32 g.) in pyridine (1·6 ml.) was mixed with a saturated solution of semicarbazide hydrochloride in water. Almost at once a crystalline ppt. of short needles, m.p. 195–197° was obtained. Recrystallization from water raised the m.p. to 228°. The product gave no depression when mixed with the phthalazine obtained by means of hydrazine hydrate [Found: C, 62·25; H, 5·6; N, 13·8. $C_{10}H_{10}O_2N_2,\frac{1}{5}H_2O$ requires C, 61·95; H, 5·4; N, 14·45%].

Demethylation of the phthalazine: formation of 5:7-*dihydroxy-6-methylphthalazine hydrobromide*. This phthalazine (55 mg.) was refluxed with HBr (50%; 3 ml.) for 3 hr. From the golden-brown solution light-brown needles (45 mg.; m.p. 232–234°) separated. Two recrystallizations from ethanol–ether (1:1) gave colourless needles (25 mg.) of 5:7-*dihydroxy-6-methylphthalazine hydrobromide*, m.p. 238–240° (decomp.). The product persistently retains ethanol of crystallization [Found (on a specimen dried in low vacuum at room temp.): OEt, 14·4. $C_9H_9O_2N_2Br,C_2H_5\cdot OH$ requires OEt, 14·9%. Found (on a specimen dried to constant wt. in high vacuum at 100°): C, 43·1; H, 4·3; N, 9·8; Br, 28·0; OEt, 8·5. $C_9H_9O_2N_2Br,\frac{1}{2}C_2H_5\cdot OH$ requires C, 42·9; H, 4·3; N, 10·0; Br, 28·5; OEt, 8·0%]. The m.p. of a specimen prepared by demethylation of 5-hydroxy-7-methoxy-6-methylphthalazine prepared from cyclopaldic acid (see below) was not depressed on admixture with this compound.

Action of dilute alkali on quadrilineatin: production of 6-*hydroxy-4-methoxy-5-methylphthalide*. Quadrilineatin (0·2 g.) was refluxed for 30 min. with 2N-NaOH (20 ml.). The solution, originally light yellow, became brown in a few minutes. After cooling it was acidified with HCl. The crystalline deposit (0·19 g., m.p. 216–218°) was collected and recrystallized twice from water. 6-*Hydroxy-4-methoxy-*

5-methylphthalide was thus obtained as needles (0·15 g.) of m.p. 234–235° (with sublimation). The product gave an immediate stable brown colour with the Gibbs reagent and a pink colour with cold conc. H_2SO_4; on warming with conc. H_2SO_4 it gave a slight purplish tinge, changing to a stable light brown [Found (on a sublimed specimen): C, 61·7; H, 5·2; OMe, 16·0. Calc. for $C_{10}H_{10}O_4$: C, 61·8; H, 5·2; OMe, 16·0%].

Methylation of the phthalide: identification of product as 4:6-dimethoxy-5-methylphthalide. The phthalide (0·1 g.) was dissolved in dry acetone and refluxed for 6 hr. with anhydrous K_2CO_3 (2·5 g.) while methyl iodide (5 ml.) was added in portions. The acetone was removed and the residue suspended in water. Colourless needles (85 mg., m.p. 156–158°) were formed on acidification. These were collected and recrystallized twice from ethanol, giving 4:6-dimethoxy-5-methylphthalide (40 mg.) of m.p. 158°. The m.p. of authentic 4:6-dimethoxy-5-methylphthalide synthesized for comparison, was not depressed on admixture [Found: C, 63·3; H, 5·9; OMe, 30·0. Calc. for $C_{11}H_{12}O_4$: C, 63·4; H, 5·8; (two) OMe, 29·8%].

Identification of high-melting product previously obtained in crystallization of quadrilineatin. It was previously reported by one of us (Lahoz-Oliver, 1955) that small amounts of a second product (product *B*) melting at 225–228° were obtained when toluene was used for recrystallization of crude quadrilineatin. This was thought to be a second metabolic product derived from *A. quadrilineatus*. When the method of crystallization from toluene previously employed was repeated on some of the crude material (1·1 g.), colourless needles (0·6 g.) of quadrilineatin of m.p. 168–170° were obtained as the main crop. The mother liquors on further concentration afforded colourless needles (0·24 g.) of m.p. 228–230°, raised to 235–236° on further crystallization from water with the use of activated carbon. The m.p. was raised to the constant value of 236° by sublimation in high vacuum. This product did not depress the m.p. of the 6-hydroxy-4-methoxy-5-methylphthalide obtained by the action of 2N-NaOH on quadrilineatin [Found (on a sublimed specimen): C, 61·9; H, 5·1; OMe, 15·8: Calc. for $C_{10}H_{10}O_4$: C, 61·8; H, 5·2; OMe, 16·0%]. None of this phthalide was obtained when benzene was used as solvent for crystallization and precautions were taken to avoid overheating. The product must therefore be regarded as an artifact and not as a second metabolite.

Proof of the structure of the Doeuvre-oxidation acid as 6-formyl-4-hydroxy-2-methoxy-3-methyl-benzoic acid

Reduction of the Doeuvre acid: production of a phthalide. The Doeuvre-oxidation acid (0·21 g.) in methanol (5 ml.) was treated with Zn dust (0·2 g.) followed by H_2SO_4 (50%, v/v; 1·5 ml.). The mixture was warmed slightly and shaken intermittently for 15 min., when the solution no longer gave a ppt. with Brady's reagent. The solution was filtered, diluted with water and chilled. It deposited colourless lustrous needles (0·14 g., m.p. 193–195°). Further material (40 mg., m.p. 191–193°) separated as a second crop. The combined material on recrystallization from water formed colourless needles (0·15 g.) of *5-hydroxy-7-methoxy-6-methylphthalide*, m.p. 198° [Found (on material sublimed in high vacuum at 140–150°): C, 61·6; H, 5·1; OMe, 16·2%. Calc. for $C_{10}H_{10}O_4$: C, 61·8; H, 5·2; OMe, 16·0%].

Methylation of the phthalide: formation of 5:7-dimethoxy-6-methylphthalide. The phthalide (50 mg.) was dissolved in dry acetone (15 ml.) and dry K_2CO_3 (2·5 g.) was added. The mixture was refluxed for 6 hr. with addition of methyl iodide (5 ml.) in portions. After removal of the solvent the residue was treated with water and acidified with HCl; crystalline needles separated (45 mg., m.p. 137–138°). The m.p. was raised by recrystallization from water to 139–140°. The methoxyl content at this stage was 32·7%: $C_{11}H_{12}O_4$ (two OMe) requires 29·8%; $C_{13}H_{16}O_5$ (three OMe, assuming ring opening and esterification of the carboxyl group) requires 38·8%. It was probable that some esterification had occurred. The product (25 mg.) was therefore refluxed for 4 hr. with ethanolic KOH (10% solution; 5 ml.). The solution was evaporated with addition of water and acidified. Colourless needles (20 mg., m.p. 169–171°) separated and were recrystallized from water. Sublimation in high vacuum at 100–110° gave 5:7-dimethoxy-6-methylphthalide of m.p. 171·5°. The m.p. of authentic material obtained by a previous synthesis (Birkinshaw *et al.* 1952) was not depressed on admixture with this product [Found: C, 62·9; H, 5·7; OMe, 29·45. $C_{11}H_{12}O_4$ requires C, 63·4; H, 5·8; (two) OMe, 29·8%].

Syntheses

4:6-Dimethoxy-5-methylphthalide. Charlesworth & Robinson (1934) prepared this from 3:5-dimethoxy-*p*-toluic acid in three stages by the method of Fritsch (1897). The following method (chloromethylation) accomplishes the reaction in one step. 3:5-Dimethoxy-*p*-toluic acid (0·2 g.), acetic acid (2 ml.) and chloromethyl ether (0·5 ml.) were heated under reflux at 80° for 8 hr. The initial light-brown solution gradually became pink and crystalline material separated. The mixture was treated with N-HCl (50 ml.) and refluxed for ½ hr. Most of the solid material had then dissolved. The solution was filtered from a little tar and extracted with ether (3 × ½ vol.). The combined ether extracts were shaken with 2N-Na_2CO_3 (2 × 5 ml.). Acidification of the Na_2CO_3 extract afforded unchanged starting material (0·11 g.). From the ether a crystalline residue (0·08 g.) was obtained, which on recrystallization from ethanol afforded needles (45 mg.) of 4:6-dimethoxy-5-methylphthalide, m.p. 158°, the same as that recorded by Charlesworth & Robinson (1934) [Found: OMe, 29·7. Calc. for $C_{11}H_{12}O_4$: (two) OMe, 29·8%].

3:5-Dimethoxy-4-methylphthalic acid. This acid was prepared from the phthalide by $KMnO_4$ oxidation according to the method of Charlesworth & Robinson (1934), who record the m.p. as 195–196° (decomp.). Recrystallization from water gave prismatic rods, m.p. 206–207° with gas evolution and sublimation, resetting and remelting at 165–166° [Found: OMe, 25·75. Calc. for $C_{11}H_{12}O_6$: (two) OMe, 25·8%].

3:5-Dimethoxy-4-methylphthalic anhydride. The acid (25 mg.) was held at 230° for 10 min. and then sublimed in high vacuum at 100–110°. The product was recrystallized from light petroleum, giving colourless needles of the anhydride, m.p. 166°, the same as that recorded by Charlesworth & Robinson (1934) [Found: OMe, 27·65. Calc. for $C_{11}H_{10}O_5$: (two) OMe, 28·0%].

Demethylation of 5-hydroxy-7-methoxy-6-methylphthalazine: formation of the monohydrobromide of 5:7-dihydroxy-6-methylphthalazine. 5-Hydroxy-7-methoxy-6-methylphthalazine (55 mg.), prepared by the action of hydrazine hydrate on cyclopaldic acid as described by Birkinshaw *et al.* (1952),

was refluxed for 2½ hr. with HBr (46–48%; 3 ml.). All the solid had then dissolved. On cooling, light greyish brown needles separated which still contained methoxyl (3·1%). The refluxing with HBr was continued for a further 1½ hr. Needle crystals (35 mg.; m.p. 232–234°) were obtained, of which the methoxyl content was nil. Recrystallization from ethanol–ether (1:1) with charcoal gave colourless needles (22 mg.) of the hydrobromide of 5:7-dihydroxy-6-methylphthalazine, m.p. 238–240° (decomp.), retaining ½ mol. of ethanol even when dried at 100° in high vacuum [Found: C, 43·1; H, 4·2; N, 9·7; Br, 28·2; OEt, 7·9). $C_9H_9O_2N_2Br$, $\frac{1}{2}C_2H_5\cdot OH$ requires C, 42·9; H, 4·3; N, 10·0; Br, 28·5; OEt, 8·0%].

SUMMARY

1. *Aspergillus quadrilineatus* Thom & Raper, when grown on Raulin–Thom solution, produces in the culture medium a hitherto undescribed fungal metabolite, which has been named quadrilineatin.

2. Quadrilineatin, $C_{10}H_{10}O_4$, forms colourless needles of m.p. 172° (decomp.). From the study of its functional derivatives and oxidation products, a number of which are described, quadrilineatin has been allocated the structure 1:2-diformyl-5-hydroxy-3-methoxy-4-methylbenzene.

3. Quadrilineatin is thus a member of the group of substituted *o*-phthalaldehydes, of which three, namely gladiolic acid, cyclopaldic acid and flavipin, were previously known as fungal metabolites.

4. Quadrilineatin shows only weak antibacterial activity against *Staphylococcus aureus* and *Escherichia coli*.

The light-absorption values were determined by means of a Hilger Uvispek spectrophotometer purchased by means of a grant from the Central Research Fund of London University.

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APPENDIX

Anti-Fungal Tests on Quadrilineatin

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Quadrilineatin was tested by the method described by Brian & Hemming (1945) and used previously in this Laboratory for determining the fungistatic activity of cyclopaldic acid and related substances. The procedure is to determine the lowest concentration that will inhibit the germination of the conidia of *Botrytis allii* Munn. A series of twofold dilutions in 'germination medium' plus spore suspension were made, starting at 1 in 25 000. There was complete inhibition at the highest concentration only, 1:25 000 (≡40 μg./ml.), partial inhibition up to 1:100 000 and virtually 100% germination at higher dilutions. The substance is therefore considerably less active than cyclopaldic acid, which showed complete inhibition at a dilution of 2·5 μg./ml., and gladiolic acid and flavipin, which inhibited completely at 10 μg./ml.

A second series of tests were carried out to determine the activity of quadrilineatin against the 'damping-off' fungus *Pythium debaryanum* Hesse. A primary series of twofold dilutions in sterile water were made. Tubes containing exactly 14 ml. of Czapek–Dox agar were prepared and sterilized. To each of these, with the agar melted and then cooled to 45°, was added 1 ml. of aqueous solution. The primary dilutions were made so that on dilution 1:15 a series of dilutions in agar starting at 1:12 500 were obtained. The concentration required for the first of the primary dilutions was greater than the solubility of the substance in water. Complete