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Studies in the Biochemistry of Micro-organisms

97. FLAVIPIN, A CRYSTALLINE METABOLITE OF *ASPERGILLUS FLAVIPES* (BAINIER & SARTORY) THOM & CHURCH AND *ASPERGILLUS TERREUS* THOM*

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The Aspergilli are divided by Thom & Raper (1945), mainly on morphological characters, into fourteen groups, two of which are of interest in the present communication. The *Aspergillus flavipes* group consists of a single species, *A. flavipes* (Bainier & Sartory) Thom & Church. The closely related *A. terreus* group includes three species, of which the commonest and most important is *A. terreus* Thom and its three varieties, *boedijni* (Bloch.) n.var., *floccosus* Shih and *aureus* n.var. It is interesting to note that old cultures of *A. flavipes* often resemble *A. terreus*, the two species being readily distinguished, however, when young cultures are examined.

We have found that culture filtrates of one strain each of *A. flavipes* grown on Raulin–Thom solution and of *A. terreus* grown on Czapek–Dox solution give an intense blue–black ferric reaction and an immediate heavy red precipitate with Brady's reagent (2:4-dinitrophenylhydrazine in aqueous 2N hydrochloric acid). We have isolated in a state of purity the metabolic product responsible for these reactions and propose for it the trivial name flavipin, since it does not appear to have been described previously.

There appears to be no published record of the isolation of any metabolic product of *A. flavipes*, although there are a number of references in the literature to the formation by this species of culture filtrates having an antibacterial spectrum similar to that of penicillin and containing a substance which is destroyed by penicillinase. In marked contrast, different strains of *A. terreus* have been shown to produce a large variety of metabolites, among which are: itaconic acid (Calam, Oxford & Raistrick, 1939); itatartaric acid $HO_2C.C(OH)(CH_2.OH).CH_2.CO_2H$ (Stodola, Friedkin, Moyer & Coghill, 1945); expansine, $C_7H_6O_4$ (Kent & Heatley, 1945); terrein, $C_8H_{10}O_3$, and citrinin, $C_{13}H_{14}O_5$ (Raistrick & Smith, 1935); geodin, $C_{17}H_{12}O_7Cl_2$, and erdin, $C_{16}H_{10}O_7Cl_2$ (Raistrick & Smith, 1936); and terreic acid, $C_7H_6O_4$, from *A. terreus* var. *aureus* n.var. (Pansacker, Philpot, Jennings & Florey, 1947).

* Part 96: Galarraga, Neill & Raistrick (1955).

Production of flavipin

The influence of cultural conditions, and particularly of the composition of the culture medium used, on the course of mould metabolism is well illustrated by a comparison of the metabolism of *A. flavipes* with that of *A. terreus*. Thus we have shown that, although good yields of flavipin are produced by *A. flavipes* when grown on Raulin–Thom solution, no flavipin could be detected when this species was grown on Czapek–Dox solution. *A. terreus*, on the contrary, failed to produce any flavipin on Raulin–Thom solution, though it gave moderate yields on Czapek–Dox solution. It was further shown that, whereas the optimum incubation period at 24° for maximum production of flavipin is 12–14 days with *A. flavipes* grown on Raulin–Thom medium, the corresponding time is 7–9 days for *A. terreus* grown on Czapek–Dox medium. The results obtained showed clearly that flavipin cannot be regarded as an 'end' product of the metabolism of either species, since, after the concentration of flavipin in the culture fluid had reached a maximum, flavipin was decomposed at about the same rate as the glucose in the medium

was metabolized, and, in the case of *A. terreus* grown on Czapek–Dox solution, almost all the flavipin present at the peak of production had completely disappeared after 21 days' incubation.

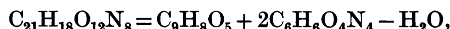
For the bulk preparation of flavipin, cultures of *A. flavipes* on Raulin–Thom solution were harvested after 12 days' incubation at 24°. The culture fluid was separated by filtration from the mould mycelium, lightly acidified with hydrochloric acid and exhaustively extracted with ether. The yellow–orange ether extract was dried and evaporated, giving crops of crude flavipin which were purified either by crystallization or, preferably, by sublimation at 140° in high vacuum. The yields of flavipin which were obtained were 5–6 g. from 100 flask cultures of *A. flavipes*, each containing 350 ml. of Raulin–Thom solution, and about 2 g. from the same number of flask cultures of *A. terreus* on Czapek–Dox solution.

Properties and structure of flavipin

Flavipin, $C_9H_8O_5$, sublimes as small pale-yellow rods, m.p. 233–234° (decomp.). It has no optical activity. It contains three active hydrogen atoms and one methyl group attached to carbon but no methoxyl group. It dissolves in aqueous sodium bicarbonate giving a yellow solution and in sodium hydroxide forming a solution which is initially cherry-red but quickly becomes brown. It has strong antifungal properties but is only weakly antibacterial.

The following reactions of flavipin have some diagnostic value: an intense but unstable blue–black ferric reaction in aqueous solution; an immediate dark-red precipitate with Brady's reagent; reduction of ammoniacal silver nitrate at room temperature and of Fehling's solution on boiling; a green coloration and a green precipitate with sodium–mercury amalgam in ethanolic solution, indicative of three vicinal phenolic groups (Bargellini, 1919).

The following crystalline functional derivatives of flavipin have been prepared and characterized: (*a*) pentaacetylflavipin hydrate, $C_{19}H_{20}O_{11}$, colourless rods, m.p. 180°, insoluble in cold aqueous sodium hydroxide and giving no ferric colour and no precipitate with Brady's reagent; (*b*) monomethyl ether, $C_{10}H_{10}O_5$, yellow needles, m.p. 196–197°; (*c*) diflavipin hexamethyl ether monohydrate, $C_{24}H_{30}O_{11}$, colourless needles, m.p. 138·5–139°, dissolves slowly in aqueous sodium hydroxide, gives no ferric reaction, but, on treatment with Brady's reagent, gives (*d*) trimethylflavipin bis-2:4-dinitrophenylhydrazone, $C_{24}H_{22}O_{11}N_8$, orange needles, m.p. 216–217°; (*e*) flavipin anhydro-2:4-dinitrophenylhydrazone, $C_{15}H_{10}O_7N_4$, deep-red rods, m.p. above 300°; (*f*) flavipin 2:4-dinitrophenylhydrazine derivative,



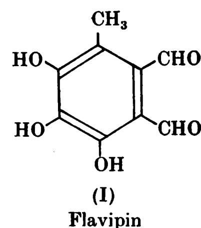
$$C_{21}H_{18}O_{12}N_8 = C_9H_8O_5 + 2C_6H_6O_4N_4 - H_2O,$$

red rods, m.p. 242–243° (decomp.); (*g*) hydrazine derivative of flavipin,

$$C_9H_8O_3N_2 = C_9H_8O_5 + N_2H_4 - 2H_2O,$$

by treatment of flavipin with hydrazine hydrate, yellow rods, m.p. above 300°, gives a blue–black ferric reaction but no precipitate with Brady's reagent.

The experimental evidence so far presented justifies the working hypothesis that the molecule of flavipin contains the following groups: one *C*-CH_3 group actually estimated; three hydroxyl groups, possibly vicinal; two carbonyl groups, probably aldehydic and adjacent to each other to explain the formation of the derivative $C_9H_8O_3N_2$ on treatment with hydrazine hydrate. The empirical formula for flavipin, $C_9H_8O_5$, may therefore be expanded to $C_6(CH_3)(OH)_3(CHO)_2$, and the probability emerges that flavipin is a fully substituted benzene derivative, i.e. a diformyltrihydroxymonomethylbenzene. Further, if the inferences made from the Bargellini test and from the hydrazine product of flavipin are correct, i.e. that the three hydroxyl groups and the two formyl groups are vicinal respectively to each other, only one benzenoid structural formula for flavipin is possible, i.e. structure (I). It will be shown later that this structure explains satisfactorily the formation of the functional derivatives described above.



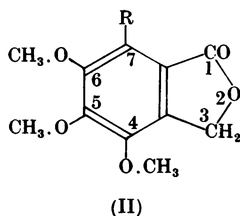
(I)
Flavipin

In order to establish structure (I) on a much sounder basis, a number of reaction and oxidation products of flavipin and its derivatives were investigated.

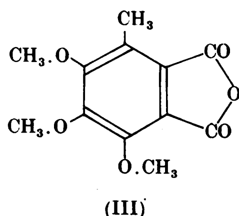
When flavipin was oxidized with alkaline hydrogen peroxide a very vigorous and deep-seated reaction took place, leading to the complete disruption of the molecule. The oxidation products identified were: (i) carbon dioxide equivalent to about 1·5 molecules per molecule of flavipin; (ii) formic acid equivalent to about 75 % of 2 molecules. This finding gives strong support to the view that flavipin contains two formyl groups; (iii) methylmalonic acid, $HO_2C.CH(CH_3).CO_2H$, confirming the presence of a *C*-CH_3 group in flavipin;

and (iv) hydroxymalonic acid (tartronic acid), $HO_2C.CH(OH).CO_2H$.

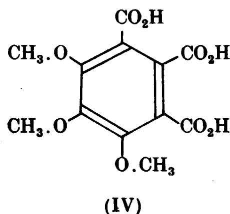
The crude, oily, methylated flavipin, obtained by exhaustive methylation with dimethyl sulphate and anhydrous potassium carbonate in acetone, was refluxed with 40 % aqueous sodium hydroxide. A good yield of 4:5:6-trimethoxy-7-methylphthalide (II, $R = CH_3$) was isolated from the reaction products. The identity of the phthalide, which has not been described previously, was established by comparison with a synthetic specimen prepared by catalytic reduction with palladium and hydrogen of 7-chloromethyl-4:5:6-trimethoxyphthalide (II, $R = CH_2Cl$) prepared by chloromethylation of the trimethyl ether of gallic acid with formaldehyde and concentrated hydrochloric acid (Edwards, Perkin & Stoyle, 1925; King & King, 1942).



(II)



(III)

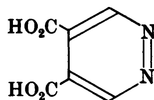


(IV)

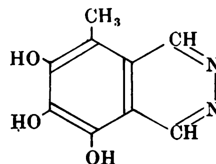
The crude, oily, methylated flavipin was oxidized with cold alkaline aqueous potassium permanganate. Two of the resulting oxidation products were identified as 4:5:6-trimethoxy-3-methylphthalic anhydride (III) and 4:5:6-trimethoxybenzene-1:2:3-tricarboxylic acid (IV), the identity of each of which was established by comparison with authentic specimens prepared by alkaline potassium permanganate oxidation respectively of 4:5:6-trimethoxy-7-methylphthalide in the cold and 7-chloromethyl-4:5:6-trimethoxyphthalide in boiling solution.

Finally, pure crystalline diflavipin hexamethyl ether hydrate was oxidized with cold alkaline potassium permanganate and gave 4:5:6-trimethoxy-3-methylphthalic anhydride (III).

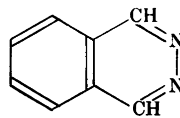
Hence, the relative positions of the methyl group and the three hydroxyl groups in the flavipin molecule are established, and it now remains to confirm that the two remaining positions in the benzene ring are occupied by –CHO groups, as is postulated in structure (I) and is indicated in the reaction products (II, $R = CH_3$) and (III). This was done by oxidizing with hot alkaline permanganate the phthalazine derivative of flavipin, $C_9H_8O_3N_2$ (VI), which was prepared by treating flavipin with hydrazine hydrate. The resulting oxidation product (V) was identified as pyridazine-4:5-dicarboxylic acid by comparison with an authentic specimen prepared as described by Gabriel (1903) by hot permanganate oxidation of phthalazine (4:5-benzopyridazine) (VII), which was synthesized by treating phthalaldehyde with hydrazine hydrate (Gabriel & Pinkus, 1893).



(V)



(VI)

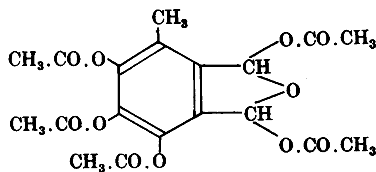


(VII)

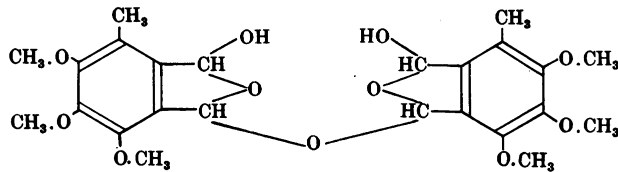
The conclusion that flavipin is a substituted *o*-phthalaldehyde is supported by the experimental finding mentioned previously that methylated flavipin is readily converted by boiling aqueous sodium hydroxide into 4:5:6-trimethoxy-7-methylphthalide (II, $R = CH_3$), since *o*-phthalaldehyde itself is well known to undergo rearrangement under alkaline conditions to give *o*-hydroxymethylbenzoic acid, from which its lactone, phthalide, is readily formed on acidification.

Flavipin may therefore be described systematically as 1:2-diformyl-4:5:6-trihydroxy-3-methylbenzene (I). This unusual formulation, containing as it does five reactive substituents, is reflected in the unusual nature of some of its functional derivatives. Thus the product of the acetylation of flavipin with acetic anhydride and either sodium acetate or concentrated sulphuric acid as catalyst is not a simple triacetyl derivative. It has the formula $C_{19}H_{20}O_{11}$, contains five acetyl groups and an extra molecule of water, and since it does not react with 2:4-dinitrophenylhydrazine, except on long standing and therefore probably because of hydrolysis, we suggest structure (VIII) for it.

Diflavipin hexamethyl ether monohydrate, $C_{24}H_{30}O_{11}$, is, as a molecular-weight estimation showed, a double molecule and its properties are illustrated in structure (IX). This structure is, however, not to be regarded as definitive since a number of isomers are possible.



(VIII)

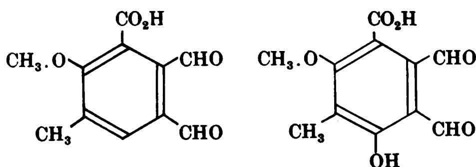


(IX)

The products formed by the interaction of 2:4-dinitrophenylhydrazine with flavipin and its derivatives are somewhat confusing. When the reaction is carried out in hot glacial acetic acid flavipin gives a product, $C_{21}H_{18}O_{12}N_8$, crystallizing in red rods; this, however, does not appear to be a bis-2:4-dinitrophenylhydrazone, which would require the formula $C_{21}H_{16}O_{11}N_8$. It may be the 2:4-dinitrophenylhydrazine salt of a mono-2:4-dinitrophenylhydrazone, since *o*-phthalaldehyde, when treated similarly, gives only a mono-2:4-dinitrophenylhydrazone. In contradistinction, diflavipin hexamethyl ether monohydrate in ethanol solution gave, with 2:4-dinitrophenylhydrazine in aqueous 2N hydrochloric acid, trimethylflavipin bis-2:4-dinitrophenylhydrazone, $C_{24}H_{22}O_{11}N_8$, as orange needles. Finally, when flavipin was heated in butanol solution with 2:4-dinitrophenylhydrazine the resulting product was flavipin anhydro-2:4-dinitrophenylhydrazone, $C_{15}H_{10}O_7N_4$, in deep-red rods.

Fungistatic properties of flavipin

Two other derivatives of *o*-phthalaldehyde have been described as mould metabolic products, i.e. gladiolic acid (X) from *Penicillium gladioli* Machacek (Brian, Curtis & Hemming, 1948; Grove, 1952; Raistrick & Ross, 1952) and cyclopaldic acid (XI) from *P. cyclopium* Westling (Birkinshaw, Raistrick, Ross & Stickings, 1952).

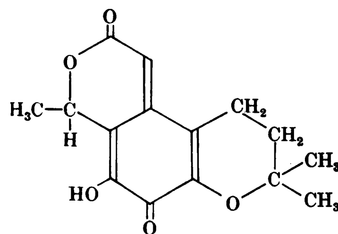


(X) (XI)

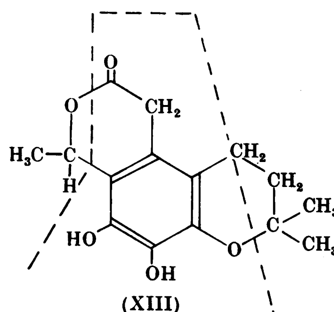
It is interesting therefore to find that all three substances have pronounced fungistatic properties. When tested here against the conidia of *Botrytis allii* Munn in a germination medium at pH 3·5 by the technique described by Brian & Hemming (1945), 95–100 % inhibition of germination was produced by flavipin at a concentration of 10·0 μg./ml., by gladiolic acid at 10·0 μg./ml. and by cyclopaldic acid at 2·5 μg./ml. Under the same conditions, and at a concentration of 1·25 μg./ml., flavipin produced 95–100 % inhibition of germination of the conidia of the same strain of *Aspergillus flavipes* as was used in its preparation.

Metabolic significance of flavipin

Derivatives of 1:2:3-trihydroxybenzene (pyrogallol), of which flavipin is obviously one, occur only rarely as fungal metabolic products. Bernhard & Albrecht (1947) isolated gallic acid (3:4:5-trihydroxybenzoic acid) as a metabolite of *Phycomyces blakesleeanus* Burgeff grown on a solution containing glucose, asparagine, yeast extract and aneurin in addition to mineral salts. Fuscin and dihydrofuscin were first described as antibacterial metabolites of the mould *Oidiodendron fuscum* Robak by Michael (1948), and Birkinshaw, Bracken, Michael & Raistrick (1951) described a number of their derivatives and breakdown products, one of which was shown to be 3:4:5-trihydroxyphenylacetic acid. Barton & Hendrickson (1956) have correlated this experimental work in terms of constitutional formulae for fuscin (XII) and dihydrofuscin (XIII) and have confirmed their deductions by synthesis.

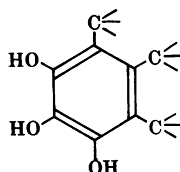


(XII)
Fuscin



(XIII)
Dihydrofuscin

The origin of the 3:4:5-trihydroxyphenylacetic acid breakdown product of dihydrofuscin is indicated by the broken line in structure (XIII). Further, it is clear that flavipin, fuscin and dihydrofuscin are closely related structurally, since they all potentially contain the basic structure (XIV).



(XIV)

EXPERIMENTAL

Cultures

(i) *Aspergillus flavipes* (Bainier & Sartory) Thom & Church, strain London School of Hygiene and Tropical Medicine (L.S.H.T.M.), catalogue no. S.M. 884, was isolated here by Mrs S. Marcus in 1952 from soil collected by her at Sidmouth.

(ii) *Aspergillus terreus* Thom, strain L.S.H.T.M., catalogue no. A. 465, was isolated here by Mrs Marcus in 1951 from an Australian soil sample no. 52.1, kindly made available to us by Professor E. G. Hallsworth, University of Nottingham.

Both these cultures were identified by Mr George Smith of this Department.

Cultural conditions and characteristics

(i) Raulin–Thom culture solution (glucose, 75·0 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, 1500 ml.) was used exclusively for the cultivation of *A. flavipes* and for the bulk preparation of flavipin.

(ii) Czapek–Dox culture solution (glucose, 50·0 g.; $NaNO_3$, 2·0 g.; KH_2PO_4, 1·0 g.; KCl, 0·5 g.; $MgSO_4,7H_2O$, 0·5 g.; $FeSO_4,7H_2O$, 0·01 g.; distilled water, 1000 ml.) was used for the cultivation of *A. terreus*.

Batches of 1 l. conical flasks, plugged with cotton wool and each containing 350 ml. of either solution (i) or (ii), were prepared and sterilized by steaming at 100° for 0·5 hr. on each of 3 consecutive days. The contents of the flasks were then inoculated with a spore suspension in sterile water of the appropriate mould grown for 14 days on malt-agar slopes, three flasks being sown from each slope. The inoculated flasks were incubated in the dark at 24°.

After 7 days' incubation the flask cultures of *A. flavipes* on Raulin–Thom solution contained a rather thin, granular, brown–purple mycelium with a purple-brown reverse and a deep-brown, clear culture fluid. After 14 days the mycelium had become folded and almost black and the dark-brown culture fluid contained much debris. The culture fluid in each case gave an intense purple–black or blue–black colour with aqueous $FeCl_3$, slowly fading to purple–brown, and an immediate, very heavy, dark-red, amorphous precipitate with Brady's reagent (0·32 % 2:4-dinitrophenylhydrazine in aqueous 2N-HCl).

The well-formed mycelium in the 7-day cultures of *A. terreus* on Czapek–Dox solution was dull pale-yellow with an orange–yellow reverse and showed numerous orange-coloured drops after 14 days' incubation. The culture fluid was orange–brown in colour and gave reactions with $FeCl_3$ and with Brady's reagent which were very similar to but less intense than those given by the corresponding culture fluids of *A. flavipes* grown on Raulin–Thom solution.

When *A. flavipes* was grown on Czapek–Dox solution, although the mould grew normally, no indication of the formation of flavipin could be obtained since the culture fluid after either 7 or 14 day's incubation gave negative ferric and Brady reactions. Contrariwise, when *A. terreus* was grown on Raulin–Thom solution, no reaction indicative of flavipin in the culture fluid could be obtained.

Course of metabolism

(i) *A. flavipes.* In order to follow the course of metabolism of *A. flavipes* and to determine the optimum time for harvesting, fifty flasks of Raulin–Thom medium were inoculated with this strain and incubated at 24°. Five sets of ten flasks, chosen at random, were harvested after incubating for 7, 9, 12, 14 and 16 days respectively. The contents of each set of ten flasks were combined and filtered, and the following estimations were carried out on the filtrates. (1) pH. (2) Residual glucose, estimated polarimetrically, the initial concentration being 5·0 %. (3) Crude flavipin. The culture filtrate was acidified with conc. HCl (3 ml./l.) and extracted three times with ether, $\frac{1}{3}$ vol. of ether being used each time. The yellow–orange ethereal extract was dried over anhydrous Na_2SO_4 and filtered and the solvent removed. The dried residue of crude flavipin was weighed. Its weight × 10 is recorded in Table 1, column 4. (4) An antifungal assay was carried out by using spores of *Botrytis allii* as

Table 1. *Course of metabolism of* Aspergillus flavipes *strain* S.M. 884 *grown on Raulin–Thom solution at* 24°

In columns 5–11, 0 = no germination, 1 = less than 1 % germination, 3 = 50–90 % germination, and 4 = 90–100 % germination.

Incubation period (days)	pH of culture filtrate	Residual glucose (%)	Crude flavipin (g./100 flasks)	Antifungal assay against *Botrytis allii* Dilution of culture filtrate						
				1/4	1/8	1/16	1/32	1/64	1/128	1/256
7	3·1–3·4	3·50	3·05	0	0	0	4	100 % germination		
9	3·1–3·4	3·45	5·36	0	0	0	1	100 % germination		
12	3·1–3·4	3·38	7·20	0	0	0	1	3	100 % germination	
14	2·9–3·1	3·10	7·39	0	0	0	1	4	100 % germination	
16	2·9–3·1	3·00	4·50	0	0	0	1	4	100 % germination	

the test organism and the germination medium of pH 3·5 as described by Brian & Hemming (1945). All the five culture filtrates gave the characteristic intense blue–black ferric colour and a heavy deep-red precipitate with Brady's reagent. At no time did the culture filtrates show appreciable antibacterial activity against either *Staphylococcus aureus* or *Escherichia coli*. Table 1 indicates clearly that the optimum incubation period is 12–14 days.

(ii) *A. terreus*. The course of metabolism of *A. terreus* was followed in a similar fashion to that of *A. flavipes*, with the exception that an antifungal assay was not carried out. The results obtained are given in Table 2. The pH of the culture filtrate was between 6·1 and 6·4 in all nine tests, in all of which the characteristic ferric colour and red precipitate with Brady's reagent were also observed.

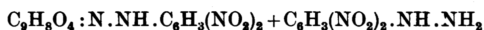
Table 2 indicates clearly that, in this case, the optimum incubation period is 7–9 days, after which time flavipin and glucose are metabolized at approximately the same rate.

Isolation of flavipin

The culture fluid from each batch of flask cultures (usually 50–100) of *A. flavipes* on Raulin–Thom solution, harvested after 12 days' incubation at 24°, or of *A. terreus* on Czapek–Dox solution after 9 days' incubation, was separated from the mycelium by straining through muslin and was then clarified by filtration through a thin layer of kieselguhr.

The *A. flavipes* culture filtrates, pH 3·1–3·4, were acidified with conc. HCl (1 ml./300 ml. of filtrate) and extracted with ether in a large countercurrent extraction apparatus with a bath temperature of 55°, four such extractions being necessary. The *A. terreus* filtrates, pH 6·1–6·4, gave a cleaner product when ether extracted without prior acidification, five extractions being necessary. The ether extracts from either mould were combined, dried over anhydrous Na_2SO_4 and evaporated to low bulk (50–100 ml.). The solid crude flavipin, m.p. 190–215°, which separated was collected, together with a second small crop obtained on further concentration. Complete removal of the ether from the final mother liquors gave an intractable gum. Details of a number of batches of *A. flavipes* are given in Table 3, and of *A. terreus* in Table 4.

It will be shown later that the most satisfactory method of purification of crude flavipin is by sublimation in a high vacuum at a bath temperature of 140°. Hence it became necessary to prove that this treatment does not lead to any decomposition of flavipin. This was done as follows. The culture filtrate (1500 ml.) from five flasks of *A. flavipes* grown on Raulin–Thom solution for 12 days was treated with Brady's reagent (310 ml.). After standing for 0·5 hr. the resulting red precipitate was separated by centrifuging and was washed on the centrifuge twice with water, twice with ethanol and once with ether. The resulting solid was repeatedly crystallized from dimethylformamide, giving finally dark-red needles (49 mg.), m.p. 242–243° (decomp.) not depressed on admixture with the 2:4-dinitrophenylhydrazine derivative of the same m.p. prepared from flavipin purified by vacuum sublimation (see p. 401). The two specimens had also the same colour, crystalline form and analysis and gave the same purplish-red Neuberg reaction. (Found, on sample prepared from culture filtrate: C, 44·2; H, 3·2; N, 19·3. $C_{21}H_{18}O_{12}N_8$, i.e.



$$C_9H_8O_4:N.NH.C_6H_3(NO_2)_2+C_6H_3(NO_2)_2.NH.NH_2$$

requires C, 43·9; H, 3·2; N, 19·5%.)

Table 2. *Course of metabolism of* Aspergillus terreus *strain* A. 465 *grown on Czapek–Dox solution at* 24°

Incubation period (days)	Residual glucose (%)	Crude flavipin (g./100 flasks)
7	3·00	1·58
8	2·80	1·30
9	2·49	1·84
10	2·38	1·27
11	1·74	1·20
13	1·19	0·63
14	0·95	0·37
17	0·58	0·38
21	0·20	0·13

Table 3. *Yields of flavipin from* Aspergillus flavipes *grown on Raulin–Thom solution for* 12 *days*

Batch no.	Residual glucose (%)	Yield of flavipin (g.)	No. of flasks in batch
1	3·48	5·37	98
2	3·46	3·65	84
3	3·71	4·57	100
4	3·55	5·33	97
5	3·50	5·87	100
6	3·28	4·58	100
7	3·10	5·60	97
8	3·25	6·30	97
9	3·46	5·60	100

Table 4. *Yields of flavipin from* Aspergillus terreus *grown on Czapek–Dox solution for* 9 *days*

Batch no.	Residual glucose (%)	Yield of flavipin (g.)	No. of flasks in batch
1	2·18	1·40	59
2	2·80	1·87	100
3	2·28	2·14	100

Purification of flavipin

Crude flavipin may be crystallized from ethyl acetate, benzene, $CHCl_3$ or acetic acid, or from mixtures of these. This method of purification is not very satisfactory, since many crystallizations are necessary to reach a constant melting point, probably because of the marked thermolability of flavipin (especially in hydroxylic solvents). However, a single sublimation in high vacuum at a bath temperature of 140° raised the m.p. from 180–215° to 227° and gave material suitable for preparative and degradative work. Three further sublimations gave a product with a constant m.p. of 233–234° and of analytical purity. Specimens purified by crystallization or by sublimation gave comparable analyses.

General properties of flavipin

Flavipin sublimes as small pale-yellow rods, m.p. 233–234° (decomp.) and crystallizes from solvents in yellow needles of the same m.p. (Found (*a*) on a recrystallized specimen from *A. terreus*: C, 55·2; H, 4·1; (*b*) on a sublimed specimen from *A. flavipes*: C, 54·9; H, 4·1; N, S, Cl, nil;

C-CH_3, 7·3; active H, 1·45%; mol.wt. (cryoscopic in dioxan), 194, 197. $C_9H_8O_5$ requires C, 55·1; H, 4·1; 1*C*-CH_3, 7·7; 3 active H, 1·54%; mol.wt. 196.) Flavipin shows no optical activity, is thermolabile and photosensitive. It is soluble in water, methanol, ethanol, ethyl acetate and acetone; slightly soluble in $CHCl_3$ and benzene, and insoluble in light petroleum (b.p. 40–50°). It dissolves slowly in cold aqueous $NaHCO_3$ to give a yellow solution from which it is precipitated on acidification. Its solution in aqueous NaOH is initially cherry-red in colour and rapidly changes through orange to brown. It gives an orange solution in cold conc. H_2SO_4. An ethanolic solution of flavipin gives with ethanolic $FeCl_3$ an unstable steel-blue colour, increasing momentarily in depth of colour on dilution with water and changing rapidly through reddish purple and orange to brown. An aqueous solution gives the following reactions: with aqueous $FeCl_3$, an intense but transient blue–black colour, rapidly becoming brown; with $CuSO_4$, a yellow precipitate insoluble in $CHCl_3$; with $SnCl_2$, a heavy, pale-yellow precipitate; with neutral lead acetate a red precipitate; and with Brady's reagent an immediate red precipitate. It immediately reduces ammoniacal $AgNO_3$, and Fehling's solution is turned to green in the cold and is reduced to Cu_2O at 100°. It is readily oxidized by Doeuvre's reagent with the formation of Hg, and by alkaline sodium hypoiodite with the formation of CHI_3. Flavipin gives a positive result in the Bargellini (1919) test, i.e. a green colour and a green precipitate with sodium amalgam in ethanol, indicative of three vicinal hydroxyl groups. It gives a negative result in the following reactions: (i) Schiff's reagent, the colour is not restored. (ii) The Gibbs (1927) test with 2:6-dichloroquinone chloroimide at pH 9·2. (iii) The nitrochromic acid test for primary and secondary alcoholic-OH groups (Fearon & Mitchell, 1932). (iv) No coupling with diazotized sulphanilic acid. When solid flavipin is exposed to the vapour of conc. NH_3 (*d*, 0·880) it quickly changes colour to dark brown. If then dissolved in aqueous conc. NH_3 it gives a deep olive–brown solution, slowly changing to brownish orange.

Flavipin exhibits strong antifungal properties in inhibiting the germination of *Botrytis allii* spores and, to an even greater extent, in inhibiting the germination of the spores of the strain of *A. flavipes* which produces it. It possesses only very weak antibacterial properties against either *Staphylococcus aureus* or *Escherichia coli*.

Functional derivatives of flavipin

Pentaacetylflavipin hydrate. A mixture of flavipin (0·2 g.), anhydrous sodium acetate (0·4 g.) and acetic anhydride (2 ml.) was kept at room temp. for 16 hr., since heating caused charring. The crude acetate (0·4 g.) recovered as a colourless, amorphous solid by the addition of ice, was crystallized from methanol (5 ml.) and cooled in acetone–solid CO_2, giving colourless crystals (65 mg.) which were recrystallized from methanol. The *acetate* is dimorphic, slow and undisturbed cooling giving rods, m.p. 180–180·5°, while normal crystallization gives a mixture of needles and rods, m.p. 179–180° after rearrangement to the rod form at 163–165°. (Found: C, 53·9; H, 4·85. $C_{19}H_{20}O_{11}$, i.e. $C_9H_3O_5,H_2O,5Ac$, requires C, 53·8; H, 4·75%.)

A mixture of flavipin (0·1 g.), acetic anhydride (1 ml.) and conc. H_2SO_4 (1 drop) was warmed for a few seconds, cooled and treated with ice. The recovered colourless, crystalline product (0·19 g.), on crystallization from ethanol, gave an acetate identical with that prepared by the previous method. [Found: C, 53·8; H, 4·7; *C*-CH_3, 18·2%; mol.wt. (cryoscopic in dioxan), 411. $C_{19}H_{20}O_{11}$ requires C, 53·8; H, 4·75; 6 *C*-CH_3, 21·25%; mol.wt. 424.]

The acetate is insoluble in cold aqueous 2N-NaOH, gives no colour with $FeCl_3$ and no precipitate with Brady's reagent, except on long standing, probably as a result of partial acid hydrolysis.

2:4-Dinitrophenylhydrazine derivative of flavipin. A solution of 2:4-dinitrophenylhydrazine (0·45 g.) in almost boiling acetic acid (8 ml.) was added to a suspension of flavipin (0·2 g.) in cold acetic acid (2 ml.). A red crystalline precipitate formed almost immediately. The mixture was cooled, shaken intermittently and filtered after 0·5 hr. The collected crystals were washed with acetic acid, then with ethanol and finally with ether. The damp crystals were crystallized from dimethylformamide, giving red rods (84 mg.), and addition of an equal volume of methanol to the acetic acid mother liquors gave a second crop (130 mg.). Recrystallization from dimethylformamide gave the 2:4-*dinitrophenylhydrazine derivative* as red rods, m.p. 242–243° (decomp.), which gave a purplish-red Neuberg reaction, i.e. colour of solution in ethanolic NaOH. (Found: C, 44·1; H, 3·2; N, 19·5. $C_{21}H_{18}O_{12}N_8$ requires C, 43·9; H, 3·2; N, 19·5%.)

o-*Phthalaldehyde mono-2:4-dinitrophenylhydrazone.* This derivative was prepared by treating phthalaldehyde (0·1 g.) in cold acetic acid (1 ml.) with 2:4-dinitrophenylhydrazine (0·22 g.) in hot acetic acid (4 ml.) exactly as described above. Crystallization of the crude product from dimethylformamide gave o-*phthalaldehyde mono-2:4-dinitrophenylhydrazone* as slender orange–yellow needles (0·1 g.), m.p. 198–199° (decomp.), which gave a purplish-red Neuberg reaction. (Found: C, 53·7; H, 3·25; N, 17·6. $C_{14}H_{10}O_5N_4$ requires C, 53·5; H, 3·2; N, 17·8%.)

Flavipin anhydro-2:4-dinitrophenylhydrazone. A solution of 2:4-dinitrophenylhydrazine (0·4 g.) in hot *n*-butanol (50 ml.) was added to a solution of flavipin (0·2 g.) in hot butanol (10 ml.), and the mixture was held at 80° for 5 min. The deep-red rods (0·26 g.) which separated were purified by chromatographing in benzene solution on a talc column and eluting with ether–benzene (50:50), followed by recrystallization from benzene. *Flavipin anhydro-2:4-dinitrophenylhydrazone* was thus obtained as deep-red rods, m.p. >300°, which give a pure-blue colour with Neuberg's reagent. (Found: C, 50·6; H, 3·0; N, 15·7. $C_{15}H_{10}O_7N_4$ requires C, 50·3; H, 2·8; N, 15·6%.)

Flavipin monomethyl ether. An excess of a solution of diazomethane in ether was added to a suspension of flavipin (0·2 g.) in ether. After standing at room temp. for 0·5 hr. the ether and residual diazomethane were removed. The resulting oil, dissolved in benzene (20 ml.), was chromatographed on acid-washed alumina and was eluted with $CHCl_3$. The residue obtained on removal of $CHCl_3$ was crystallized from $CHCl_3$ and sublimed in high vacuum at 130°, giving *flavipin monomethyl ether* (20 mg.) as stumpy yellow needles, m.p. 196–197°. (Found: C, 56·8; H, 4·8; OMe, 15·2. $C_{10}H_{10}O_5$ requires C, 57·1; H, 4·8; 1 OMe, 14·8%.) An ethanolic solution of the product gives with ethanolic $FeCl_3$ a reddish colour which quickly turns brown and finally green. It gives an orange precipitate with Brady's reagent. All attempts to obtain a crystalline derivative by longer methylation with diazomethane, even in the presence of methanol, failed.

Methylation of flavipin with dimethyl sulphate. (*a*) *Brief*

methylation. A mixture of flavipin (1 g.), dry acetone (75 ml.), anhydrous K_2CO_3 (4 g.) and dimethyl sulphate (3 ml.) was refluxed for 1 hr., when the initial orange colour had changed to pale yellow. The mixture was filtered and the acetone was removed from the filtrate. The residual oil was dissolved in ether (30 ml.), and the solution, on long standing, ultimately gave long, colourless needles (0·61 g.), m.p. 94–106°, which were purified by continuous extraction in a Soxhlet apparatus with ether, in which the substance is not very soluble. Repetition of this process finally gave the substance as colourless needles with a constant m.p. of 138·5–139°. (Found: C, 58·3, 57·9; H, 6·1, 6·3; OMe, 37·2, 37·6; mol.wt. cryoscopic in dioxan, 441. $C_{24}H_{30}O_{11}$, i.e. $[C_9H_5O_2(OMe)_3]_2$, H_2O requires C, 58·2; H, 6·1; 6 OMe, 37·7%; mol.wt. 494.) This substance, which, for convenience, may be called *diflavipin hexamethyl ether monohydrate*, gives no ferric colour but gives a crystalline orange precipitate with Brady's reagent. It dissolves slowly on shaking with aqueous 2N-NaOH.

A solution of the methyl ether (0·2 g.) in ethanol (10 ml.) was treated with Brady's reagent (15 ml.). The resulting orange precipitate (0·12 g.) was collected and recrystallized from ethanol, giving *trimethylflavipin bis-2:4-dinitrophenylhydrazone* as slender orange needles, m.p. 216–217° (decomp.), which gave an orange–red colour with Neuberg's reagent. (Found: C, 48·1; H, 3·7; N, 19·1; OMe, 14·9. $C_{24}H_{22}O_{11}N_8$ requires C, 48·15; H, 3·75; N, 18·8; 3 OMe, 15·6%.)

A mixture of flavipin (0·1 g.), m.p. 229–230° (decomp.), dry acetone (20 ml.) and anhydrous K_2CO_3 (1·0 g.) was refluxed for 0·5 hr. The portion undissolved in acetone was separated by filtration, dissolved in water (5 ml.) and acidified with conc. HCl. The resulting yellow precipitate A was collected, washed and dried. The acetone filtrate was concentrated and acidified with HCl, giving a yellow precipitate B, which was combined with precipitate A, total wt. 78 mg., m.p. 230–231° (decomp.). A mixture with the starting material melted at 229–230° (decomp.). Hence the possibility may be ruled out that the structure of flavipin might have undergone rearrangement during methylation with dimethyl sulphate and K_2CO_3 in acetone.

(*b*) *Prolonged methylation.* Methylation for a longer time appeared to give a different methyl ether. A mixture of flavipin (1·0 g.), anhydrous K_2CO_3 (4·0 g.), dry acetone (50 ml.) and dimethyl sulphate (6·0 ml.) was refluxed for 6 hr. Water (100 ml.) was added, and the acetone and residual dimethyl sulphate were removed by evaporation *in vacuo*. The methyl ether was extracted with ether, the extract was dried (Na_2SO_4) and evaporated. The residual oil (1·03 g.) was distilled *in vacuo* at 0·1 mm. and 111–116°, giving a colourless oil with a methoxyl content of 48·3 and 48·7%, which set to a mass of colourless needles on long standing. $C_{26}H_{34}O_{11}$, i.e. the dimethyl ether of $C_{24}H_{30}O_{11}$, the hydrated form of diflavipin hexamethyl ether, requires 47·5% of OMe for 8 OMe groups; $C_{26}H_{32}O_{10}$, i.e. the dimethyl ether of $C_{24}H_{28}O_{10}$, the anhydrous form of diflavipin hexamethyl ether, requires 49·2% of OMe for 8 OMe groups. However, this methyl ether, on treatment in ethanolic solution with Brady's reagent as described above, gave a 2:4-dinitrophenylhydrazone in slender orange needles, giving an orange–red Neuberg reaction and having m.p. and mixed m.p. 216–217° on admixture with the trimethylflavipin bis-2:4-dinitrophenylhydrazone of the same m.p. mentioned above.

Phthalazine derivative of flavipin. (*a*) *From hydrazine hydrate.* A solution of flavipin (0·2 g.) and 50% hydrazine hydrate (0·1 ml.) in 50% (v/v) aqueous ethanol (10 ml.) was kept at room temp. for some hours. The golden-yellow rods which separated were collected (0·15 g.) and recrystallized thrice from ethanol, giving the *phthalazine derivative of flavipin* as yellow rods, m.p. >300°. (Found: C, 56·05; H, 3·9; N, 14·3. $C_9H_8O_3N_2$ requires C, 56·0; H, 4·15; N, 14·5%.) In ethanolic solution it gives with ethanolic $FeCl_3$ a blue–black colour which becomes orange on the addition of water. It gives no precipitate with Brady's reagent. Its solution in 10% aqueous NH_3 becomes red in colour on acidification with acetic acid.

(*b*) *From semicarbazide hydrochloride.* A solution of flavipin (0·4 g.) in ethanol (5 ml.) was added to a solution of semicarbazide hydrochloride (0·8 g.) and sodium acetate (1·2 g.) in distilled water (20 ml.). The mixture was held at 90° for 5 min. The crystals which separated on cooling were collected (0·225 g.), combined with a second crop (0·032 g.) from the concentrated mother liquors and crystallized from much hot water. The product, golden-yellow rods, m.p. >300°, gave similar reactions to those described in (*a*) above and the ultraviolet absorption spectra in ethanol of the two specimens were almost identical (see p. 404). (Found: C, 53·6; H, 4·4; N, 13·5. $C_9H_8O_3N_2,\frac{1}{2}H_2O$ requires C, 53·7; H, 4·5; N, 13·9%.)

REACTION AND OXIDATION PRODUCTS OF FLAVIPIN AND ITS DERIVATIVES

(*a*) *Action of* NaOH *on methylated flavipin. Isolation of 4:5:6-trimethoxy-7-methylphthalide* (*II*). The crude oily methyl ether of flavipin (0·58 g.) obtained by methylation with dimethyl sulphate and K_2CO_3 in acetone (see above) was refluxed for 15 min. with aqueous NaOH (10 ml. of 40%). The cooled alkaline solution was extracted with ether. Removal of the solvent from the ether extract gave 314 mg. of unchanged starting material. The extracted alkaline solution was acidified to Congo red with HCl and kept overnight. The colourless crystals which separated were collected, washed with water and dried (227 mg.), m.p. 60–78°. They were purified by crystallization from ethanol + charcoal, then light petroleum (b.p. 80–100°) and finally by sublimation in high vacuum. The colourless crystalline sublimate melted at 87·5–88·5°, not depressed on admixture with an authentic synthetic specimen of 4:5:6-trimethoxy-7-methylphthalide of the same m.p. (see p. 404). (Found: C, 60·4; H, 5·8; OMe, 39·0. $C_{12}H_{14}O_5$ requires C, 60·5; H, 5·9; 3 OMe, 39·0%.)

(*b*) *Oxidation of flavipin with alkaline* H_2O_2. *Formation of* CO_2, *formic, methylmalonic and tartronic acids.* H_2O_2 (9·3 ml. of 90–100 vol.), followed by aqueous NaOH (20·0 ml. of N) were introduced through a tap funnel into a flask containing flavipin (1 g.) and through which a current of N_2 freed from O_2 and CO_2 was passed. The issuing gas was passed through bubblers, the first of which contained Brady's reagent for the detection of volatile aldehydes or ketones, while the later ones contained standard $Ba(OH)_2$. The flask was immersed in ice water to moderate the initially very vigorous reaction. After 0·5 hr. the reaction mixture had become acid and was further acidified with HCl (20·0 ml. of N) to neutralize the NaOH initially added. N_2 was passed for a further 1·5 hr. No volatile aldehydes or ketones were detected. CO_2 (0·32 g. and 0·34 g. in duplicate experiments) equivalent to about 1·5 mol. was estimated.

The reaction solution was then distilled *in vacuo* to remove volatile acids completely, and its volume was maintained by the addition of water. The distillate was titrated against N-NaOH and in six separate experiments the average amount of 10·3 ml. of N acid was estimated; calc. for 2 mol. of a monobasic acid = 10·2 ml. of N. Formic acid was shown to be present in the distillate by the chromotropic acid test after reduction to formaldehyde, and in a quantitative estimation by the reduction of $HgCl_2$ to Hg_2Cl_2 (Fincke, 1913) was shown to constitute about 75 % of the total volatile acid.

The reaction solution, from which volatile acids had been removed, was evaporated to dryness *in vacuo*. The resulting colourless crystalline solid was thoroughly dried *in vacuo* over conc. H_2SO_4 and was then continuously extracted with Na-dried ether for 8 hr. The ether extract was evaporated to dryness, giving a colourless mass of gummy crystals, m.p. 90–130° and decomposing at 140°. It had an average wt. in six experiments of 0·435 g. In aqueous solution it was strongly acid to Congo red and was obviously a mixture which could not, however, be separated either by crystallization or sublimation. Oxalic acid was present only in traces.

A portion of the mixed non-volatile acids (0·185 g.) was neutralized to pH 6·7–7·0 with NaOH, and a solution of *p*-bromophenacyl bromide (0·74 g.) in ethanol (10 ml.) was added. The mixture was refluxed for 3 hr. and the colourless solid (0·26 g.), m.p. 137–139°, which separated on cooling was collected and crystallized from ethanol, giving colourless needles, m.p. 161–161·5°, not depressed on admixture with the authentic di-*p*-bromophenacyl ester of methylmalonic acid, m.p. 163–164·5° (see p. 404). (Found: C, 46·8; H, 3·3; Br, 31·2. $C_{20}H_{16}O_6Br_2$ requires C, 46·9; H, 3·15; Br, 31·2 %.) A second portion of the mixed non-volatile acids (107 mg.) was converted in a similar manner into the di-*p*-phenylphenacyl ester by heating with *p*-phenylphenacyl bromide (0·735 g.). The crude ester obtained (0·155 g.), m.p. 155–157°, was crystallized from ethanol, giving the di-*p*-phenylphenacyl ester of methylmalonic acid as colourless plates, m.p. 172–173·5°. A mixture of it with an authentic synthetic specimen, m.p. 176–177·5° (see p. 404), melted at 175–176·5°.

Tartronic acid was identified by paper chromatography as the second major constituent of the mixed non-volatile acids. Whatman's no. 1 paper was used, water being the stationary phase and *n*-butanol containing 1 % formic acid the mobile phase. Bromocresol purple was used for development. The following R_F values were obtained: (i) methylmalonic acid, 0·88; (ii) tartronic acid, 0·62; (iii) equimolecular mixture of methylmalonic and tartronic acids, 0·875 and 0·60; the mixed non-volatile acids, 0·87 and 0·61. The presence of tartronic acid was confirmed by the fact that both authentic tartronic acid and the mixed non-volatile acids gave a deep cherry-red colour on heating with conc. H_2SO_4 and resorcinol, and a red–purple colour rapidly changing to blue–purple on heating with conc. H_2SO_4 and pyrogallol. Unsuccessful attempts to prepare either the *p*-bromophenacyl or *p*-phenylphenacyl esters of authentic tartronic acid explain the failure to detect the esters of this acid in the mixtures from which the corresponding esters of methylmalonic acid were successfully isolated.

(*c*) *Oxidation of methylated flavipin with cold alkaline* $KMnO_4$. *Isolation of* 4:5:6-*trimethoxy*-3-*methylphthalic anhydride* (*III*) *and* 4:5:6-*trimethoxybenzene*-1:2:3-*tricarboxylic acid* (*IV*). A mixture of the crude oily methyl ether of flavipin (1·45 g.) made by methylation with dimethyl sulphate and K_2CO_3 in acetone (see p. 402), aqueous $KMnO_4$ (60 ml. of 5 %) and saturated aqueous Na_2CO_3 (27 ml. of 20 %) was stirred at room temp. for 55 hr. The mixture was then acidified with dil. H_2SO_4, and SO_2 was passed to dissolve MnO_2. The solution was again made alkaline with Na_2CO_3 and extracted with ether to remove unoxidized flavipin methyl ether (0·21 g.). The extracted solution was reacidified with H_2SO_4 and continuously extracted with ethyl acetate for 8 hr. The dried (Na_2SO_4) extract was freed from solvent and the crystalline residue (0·75 g.), m.p. 165–170°, was fractionally crystallized from hot water, giving fractions *A*, separating as colourless needles, and *B*, obtained as colourless rods on evaporation of the aqueous mother liquors. *Fraction* A. 0·15 g.; m.p. 90–108°, was purified by repeated sublimation in high vacuum to give colourless needles (0·13 g.), m.p. 110–111°, not depressed on admixture with authentic synthetic 4:5:6-trimethoxy-3-methylphthalic anhydride, m.p. 111·5–112° (see p. 405). (Found: C, 56·9; H, 5·1; OMe, 36·7; *C*-CH_3, 5·7. $C_{12}H_{12}O_6$ requires C, 57·15; H, 4·8; 3 OMe, 36·9; 1 *C*-CH_3, 6·0 %.) The substance is not acid to litmus, does not dissolve in cold aqueous $NaHCO_3$ or Na_2CO_3, but dissolves readily in hot aqueous NaOH. *Fraction* B. 0·34 g.; m.p. 167–168°, was purified by crystallization from ethyl acetate-benzene, giving colourless rods, m.p. 172·5–173°, not depressed on admixture with authentic 4:5:6-trimethoxybenzene-1:2:3-tricarboxylic acid, m.p. 174·5–175·5° (see p. 405). (Found: C, 48·1; H, 4·0; OMe, 30·7. $C_{12}H_{12}O_9$ requires C, 48·0; H, 4·0; 3 OMe, 31·0 %.)

(*d*) *Oxidation of crystalline diflavipin hexamethyl ether hydrate with cold alkaline* $KMnO_4$. *Isolation of* 4:5:6-*trimethoxy*-3-*methylphthalic anhydride* (III). This crystalline methyl ether (0·2 g.) was shaken for 48 hr. with a mixture of aqueous $KMnO_4$ (7 ml. of 5 %) and aqueous Na_2CO_3 (4 ml. of 20 %). The weight of unchanged methyl ether recovered was 40 mg. The oxidation product was worked up as described in section (*c*) above and gave a crystalline ethyl acetate extract (82 mg.) which was stirred with dilute aqueous Na_2CO_3 (4 ml.). The portion insoluble in Na_2CO_3 was collected, dried and heated above its m.p. (109–111°) for 2 min. It was then sublimed in high vacuum at 100°, giving colourless needles (50 mg.), m.p. 111·5–112°, not depressed on admixture with authentic 4:5:6-trimethoxy-3-methylphthalic anhydride of the same m.p.

(*e*) *Oxidation of the phthalazine derivative of flavipin with alkaline* $KMnO_4$. *Isolation of pyridazine*-4:5-*dicarboxylic acid* (*V*). Aqueous $KMnO_4$ (3·7 g. in 185 ml. of water) was added at intervals over a period of 20 min. to a stirred mixture of the phthalazine derivative, $C_9H_8O_3N_2$, (0·84 g.) (see p. 402) in boiling water (80 ml.) and aqueous KOH (2·5 ml. of 2 N). Excess of $KMnO_4$ was decomposed with ethanol and SO_2 was passed through the cooled solution to remove MnO_2. The solution was acidified with H_2SO_4 and extracted for 8 hr. with ethyl acetate. Removal of the solvent from the extract gave a sandy, crystalline oxidation product (0·47 g.), which was purified by three crystallizations from boiling water + charcoal. Pyridazine-4:5-dicarboxylic acid was thus obtained as colourless plates (0·16 g.), m.p. 212·5–213° (decomp.) alone or in admixture with an authentic synthetic specimen m.p. 209–210° (see p. 405). Both specimens began to decompose at 200°. Gabriel (1903) gives the m.p. as 212–213·5° with decomposition from 205°. (Found: C, 43·0; H, 2·7; N, 16·25. Calc. for $C_6H_4O_4N_2$: C, 42·9; H, 2·4; N, 16·7 %.)

Table 5. *Fungistatic activity of flavipin against* Botrytis allii *and* Aspergillus flavipes *at 24° and* pH 3·5 *or* 6·0

0 = no germination; (1) = <1 % germination; 1 = 1–5 % germination; 2 = 5–50 % germination; 3 = 50–90 % germination; 4 = 90–100 %, i.e. complete germination; — = not tested.

		Concn. of flavipin (μg./ml.)									
Organism	pH	80	40	20	15	10	7·5	5	4	2·5	1·25
Botrytis allii	3·5	—	—	0	0	1	2	3	4	4	—
Aspergillus flavipes	3·5	—	—	0	—	0	—	0	—	0	1
A. flavipes	6·0	0	(1)	(1)	—	(1)	—	(1)	—	(1)	1

Antifungal tests

The fungistatic activity of flavipin was determined by the method described by Brian & Hemming (1945). A series of dilutions of flavipin in a sterile germination medium of the following composition were made up: glucose, 12·5 g.; ammonium tartrate, 1·00 g.; KH_2PO_4, 1·00 g.; $MgSO_4,7H_2O$, 0·50 g.; distilled water 1000 ml.; pH adjusted to either 3·5 or 6·0. Each dilution was inoculated with the conidia of the fungus under test and then incubated for 24 hr. at 24°. The proportion of conidia which had germinated was determined and the results obtained are given in Table 5, the degrees of inhibition being those adopted by Brian & Hemming (1945).

The two test fungi used were the strain of *Botrytis allii* Munn used and kindly supplied by Dr P. W. Brian, and *Aspergillus flavipes* (Bainier & Sartory) Thom & Church, strain L.S.H.T.M. catalogue no. S.M. 884, i.e. the same strain as was used in this work for the preparation of flavipin.

Ultraviolet absorption spectra

The spectra numbered 2, 3, 4, 6 and 7 given below were carried out in this Department in collaboration with Dr J. H. Birkinshaw. They were determined by means of a Hilger and Watts Uvispek spectrophotometer. We are indebted to Dr Gunhild Aulin-Erdtman for the spectra numbered 1 and 5.

Numbers 1, 2, 3 and 4 were determined in dioxan solution, 5, 6 and 7 in ethanol solution. The following results were obtained:

(1) Flavipin, λ 261 mμ. (log ε 4·37); 264 mμ., 4·35; 330 mμ., 3·94 (broad band).

(2) Pentaacetylflavipin hydrate. Inflexion at 256–257 mμ., 2·23; 264 mμ., 2·35.

(3) Crystalline diflavipin hexamethyl ether hydrate. 243–246 mμ., 3·03; 275–277 mμ., 3·18; inflexion at 310–318 mμ., 2·17.

(4) Oily methyl ether, $C_{26}H_{34}O_{11}$. 279 mμ., 3·21.

(5) 4:5:6-Trimethoxy-3-methylphthalic acid. 212 mμ., 4·45; inflexion at 240–250 mμ., 3·7–3·8; 287 mμ., 3·17.

(6) Phthalazine derivative via hydrazine hydrate. 220 mμ., 4·13; 283 mμ., 4·37; 385–386 mμ., 3·74.

(7) Phthalazine derivative via semicarbazide hydrochloride. 217 mμ., 4·03; 283 mμ., 4·27; 384–387 mμ., 3·66.

Infrared absorption spectrum

The infrared absorption spectrum of flavipin was kindly determined for us by Dr G. Aulin-Erdtman and Dr K. E. Almin. They used a Perkin–Elmer single-beam (double-pass) instrument and determined the spectrum of freshly sublimed flavipin in a solid mixture with KBr. They found the following absorption bands, the figures in brackets after each band indicating the percentage absorption: 3226 cm.$^{-1}$ (60 %); 3067 (57); 1658 (79); 1639 (65); 1615 (61); 1489 (48); 1454 (78); 1390 (57); 1349 (79); 1330 (75); 1303 (56); 1264 (86); 1217 (69); 1145 (60); 1115 (81); 1060 (41); 1039 (31); 1016 (35); 1002 (33); 972 (75); 932 (55); 765 (48).

SYNTHESES

Methylmalonic acid, $HO_2C.CH(CH_3).CO_2H$, was prepared by the hydrolysis of its diethyl ester, which was synthesized from diethyl malonate substantially as described by Conrad & Bischoff (1880). It forms colourless needles from ethyl acetate, m.p. 130–133·5° (decomp.). The m.p. given in the literature varies between 120° and 135° (decomp.).

Di-p-*bromophenacyl ester of methylmalonic acid* was prepared from methylmalonic acid and *p*-bromophenacyl bromide as described on p. 403. The *ester* forms colourless plates, m.p. 163–164·5°. (Found: C, 46·8; H, 3·3; Br, 32·1. $C_{20}H_{16}O_6Br_2$ requires C, 46·9; H, 3·15; Br, 31·2 %.)

Di-p-*phenylphenacyl ester of methylmalonic acid* was prepared from methylmalonic acid and *p*-phenylphenacyl bromide as described on p. 403. The *ester* forms colourless plates, m.p. 176–177·5°. (Found: C, 75·7; H, 5·3. $C_{32}H_{26}O_6$ requires, C, 75·9; H, 5·2 %.)

7-*Chloromethyl*-4:5:6-*trimethoxyphthalide* was prepared according to the method of King & King (1942). A mixture of the trimethyl ether of gallic acid (Mauthner, 1932; 10 g.), aqueous formaldehyde (25 ml. of 40 %) and conc. HCl (40 ml.) was refluxed for 20 min. in an oil bath at 140°. On cooling and addition of water, the oily reaction product solidified and was collected. It was fractionally crystallized from ethanol, giving, as the more soluble fraction, 7-chloromethyl-4:5:6-trimethoxyphthalide (3·88 g.) in colourless needles, m.p. 84–85°. King & King (1942) give the m.p. as 85°.

4:5:6-*Trimethoxy*-7-*methylphthalide*. A solution in ethanol of 7-chloromethyl-4:5:6-trimethoxyphthalide (1·0 g.) was shaken with a 5 % palladium oxide on carbon catalyst (1·0 g.) in hydrogen at atmospheric pressure. The uptake of H_2 (95 ml.) was complete in 10 min. The catalyst was removed by filtration and, on removal of the solvent from the filtrate, there remained long colourless needles (0·84 g.), m.p. 80–85°. Crystallization from ethanol gave 4:5:6-*trimethoxy*-7-*methylphthalide* as colourless needles, m.p. 87–88·5°, not depressed on admixture with the product of the action of NaOH on methylated flavipin, m.p. 87·5–88·5° [see p. 402, section (*a*)]. (Found: C, 60·5; H, 5·9; *C*-CH_3, 8·1. $C_{12}H_{14}O_5$ requires C, 60·5; H, 5·9; 1 *C*-CH_3,

6·3%.) The substance is very soluble in ethanol, $CHCl_3$, ethyl acetate, and moderately soluble in light petroleum (b.p. 80–100°). It can be readily sublimed in high vacuum.

4:5:6-Trimethoxybenzene-1:2:3-tricarboxylic acid. 7-Chloromethyl-4:5:6-trimethoxyphthalide (1·0 g.) was refluxed for 3 hr. with aqueous KOH (40 ml. of 0·03 N), and $KMnO_4$ (6·0 g.) was added at intervals. The residual $KMnO_4$ was then destroyed by adding methanol (1 ml.). Precipitated MnO_2 was separated by filtration and oxalic acid was removed from the filtrate by adjusting the pH to 2·0 with HCl and addition of $CaCl_2$. Precipitated calcium oxalate was removed by centrifuging, and the clear supernatant, after further acidification with conc. HCl (3·0 ml.), was continuously extracted with ethyl acetate for 8 hr. The extract was dried (Na_2SO_4) and the solvent removed to give slightly gummy needles (0·42 g.) which were purified by crystallization from dry ethyl acetate + charcoal, giving *4:5:6-trimethoxybenzene-1:2:3-tricarboxylic acid* as colourless rods, m.p. 174·5–175·5°, which did not depress the m.p. 172·5–173° of the $KMnO_4$ oxidation product of the oily methyl ether of flavipin [see section (*c*), fraction B, p. 403]. (Found: C, 48·25; H, 4·2; OCH_3, 31·0. $C_{12}H_{12}O_9$ requires C, 48·0; H, 4·0; 3 OMe, 31·0%.)

4:5:6-Trimethoxy-3-methylphthalic anhydride. 4:5:6-Trimethoxy-7-methylphthalide (0·23 g.) was refluxed for 10 min. with aqueous KOH (37 ml. of 0·03 N). $KMnO_4$ (1·0 g. in H_2O, 25 ml.) was added to the cooled solution which was kept at room temp. for 24 hr. Excess of $KMnO_4$ and MnO_2 were removed as described above. The residual solution was strongly acidified with HCl, saturated with NaCl and thoroughly extracted with ethyl acetate. Removal of the solvent from the extract gave a colourless product (0·17 g.) from which free acid was removed by shaking its solution in ether with aqueous $NaHCO_3$. The extracted ether solution was evaporated to dryness and the residue was purified by repeated sublimation in high vacuum at 100° and crystallization from light petroleum (b.p. 80–100°), giving *4:5:6-trimethoxy-3-methylphthalic anhydride* as colourless needles, m.p. 111·5–112°, not depressing the m.p. 111·5–112° of the $KMnO_4$ oxidation product of diflavipin hexamethyl ether monohydrate [see section (*d*), p. 403]. (Found: C, 57·2; H, 4·9; OMe, 36·9. $C_{12}H_{12}O_6$ requires C, 57·1; H, 4·8; 3 OMe, 36·9%.)

Pyridazine-4:5-dicarboxylic acid (Gabriel, 1903). Phthalazine (Gabriel & Pinkus, 1893) was synthesized by treating a mixture of phthalaldehyde (3·0 g.), NaOH (33 ml. of 2 N) and water (100 ml.) with hydrazine sulphate (3·4 g.). The mixture was well shaken, concentrated to 20 ml. and extracted with benzene. The benzene solution was extracted with HCl and the extract was made alkaline and was re-extracted with benzene. Removal of the benzene gave crude phthalazine (1·5 g.).

Aqueous $KMnO_4$ (100 ml. of 2·5%) was added gradually to a mixture, kept boiling, of phthalazine (0·4 g.) and NaOH (1 ml. of 2 N). Excess of $KMnO_4$ and precipitated MnO_2 were removed as described above. The filtrate was concentrated to 5 ml., acidified with H_2SO_4 and extracted with ethyl acetate. After removal of the solvent the pale-brown solid residue (0·10 g.) was purified by sublimation in high vacuum at 120° and crystallization from ethyl acetate. Pyridazine-4:5-dicarboxylic acid was thus obtained as colourless plates, m.p. 209–210° (decomp.), which did not depress the m.p. of the $KMnO_4$ oxidation product of the phthalazine derivative of flavipin [see section (*e*), p. 403].

SUMMARY

1. A hitherto undescribed mould metabolite, now named flavipin, has been isolated from culture filtrates of *Aspergillus flavipes* (Bainier & Sartory) Thom & Church grown on Raulin–Thom solution and of *A. terreus* Thom grown on Czapek–Dox solution.

2. Flavipin, $C_9H_8O_5$, forms pale-yellow rods, m.p. 233–234° (decomp.).

3. A number of its functional derivatives and degradation products are described, from the structures of which it is deduced that flavipin is 1:2-diformyl-4:5:6-trihydroxy-3-methylbenzene.

4. Flavipin is the third mould metabolite to be described which is a substituted *o*-phthalaldehyde, the other two being gladiolic acid from *Penicillium gladioli* Machacek and cyclopaldic acid from *P. cyclopium* Westling. All three substances are powerful antifungal agents.

5. Flavipin is also a substituted pyrogallol, a class of compounds which occur only rarely as fungal metabolic products.

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The Identification of the Major Diazotizable Metabolite of 4:4′-Diaminodiphenyl Sulphone in Rabbit Urine

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Titus & Bernstein (1949), while studying the pharmacology of water-soluble disubstituted derivatives of 4:4′-diaminodiphenyl sulphone (DADPS) in mice and dogs, found that DADPS itself was excreted mainly as a water-soluble substance which was readily hydrolysed by dilute acid to free DADPS. Lowe (1952) also reported a similar change in the drug given to man for the treatment of leprosy and showed that as much as 80% of the diazotizable material excreted in the urine was present in this form.

DADPS is regarded as the most effective drug available for the treatment of leprosy and in view of the low proportion of the drug excreted in the free form it seemed probable, especially if the water-soluble derivative were also the major constituent in the blood, that the antileprotic activity of the drug was due to this acid-labile metabolite. We therefore examined by paper chromatography and paper ionophoresis the excretion products of DADPS, and in a preliminary communication (Bushby & Woiwod, 1955) we reported the isolation of the major diazotizable excretion product from rabbit urine and its identification as a monoconjugate with glucuronic acid. We also reported the synthesis of the conjugate. In this paper the synthesis of this conjugate is described, and the evidence for its presence in urine and blood is presented.

EXPERIMENTAL

Paper chromatography. The apparatus and procedures used were those of Woiwod (1949). The solvents used were *n*-butanol saturated with water, *n*-butanol–acetic acid–water (12·5:3:12·5, by vol.), phenol saturated with water and *n*-propanol–*n*-butanol–water (2:3:5, by vol.). No. 4 Whatman papers were washed with several changes of distilled water in an enamelled tray and dried at 37° before use.

Ionophoresis. Samples were run on strips of no. 4 Whatman paper with 0·05 M-Na_2HPO_4 as electrolyte and a potential gradient of 7·0 v/cm.

Detection of primary aromatic amino groups. Two reagent sprays were used. (1) A 1% (w/v) solution of *p*-dimethylaminobenzaldehyde in 20% (v/v) conc. HCl–ethanol, diluted with 19 vol. of ethanol just before use. Colour was developed at room temp. To avoid background coloration the ethanol was redistilled from KOH and $KMnO_4$. (2) A solution of $NaNO_2$ [0·5% (w/v) in 20% (v/v) N-HCl–ethanol], followed, after the paper had nearly dried, by 0·1% (w/v) *N*-(1-naphthyl)ethylenediamine dihydrochloride in the same solvent.

Detection of glucuronic acid. Aniline hydrogen phthalate, benzidine and naphthoresorcinol sprays were used. The sensitivity of the last two reagents was markedly improved by using phosphoric acid in the spray as suggested by Bryson & Mitchell (1951).

Estimation of glucuronic acid. Glucuronic acid in material eluted from paper was determined either by the copper-reduction method of Shaffer & Somogyi (1933) or the colorimetric procedure of Jones (1954). The latter gave much less colour with glucuronic acid than with glucose and other reducing sugars owing to the $SnCl_2$ in the reagent. When this was replaced by Na_2SO_3 the colour obtained with glucuronic acid equalled that given by glucose. The modified reagent consisted of 0·2% (w/v) benzidine in acetic acid containing 0·05% (w/v) Na_2SO_3. The fine precipitate which formed was filtered off after 30 min. and the reagent was used immediately.

Synthesis of the sodium salt of the acid-labile conjugate of DADPS with glucuronic acid

Sodium glucuronate (4·3 g.) in 15 ml. of water adjusted to pH 5·5 was added to diaminodiphenyl sulphone (5 g.) in 15 ml. of acetone. The mixture was refluxed for 1 hr., 100 ml. of water added, the precipitated DADPS removed by filtration and the filtrate concentrated *in vacuo* to about 50 ml. After adjustment to pH 7·0, the solution was extracted three times with equal volumes of phenol equilibrated with 0·067 M phosphate buffer (pH 7·4). This extracted a large