New Metabolic Products of *Aspergillus flavus*. Part I. Asperentin, its Methyl Ethers, and 5'-Hydroxyasperentin

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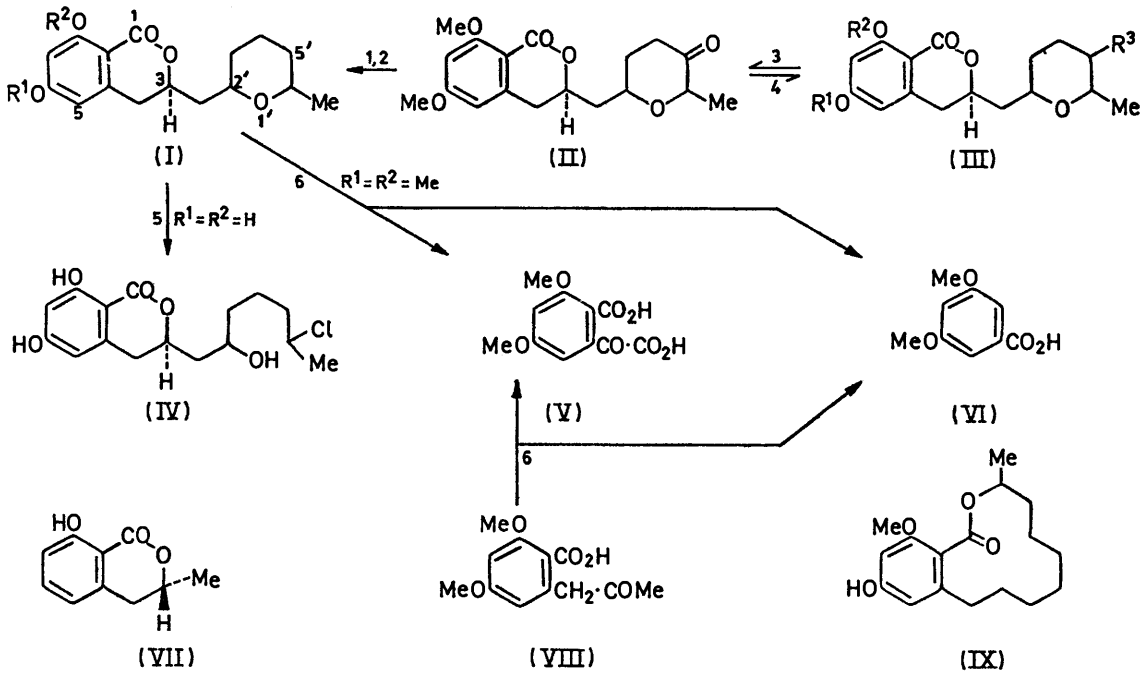
Asperentin, a minor metabolic product of an entomogenous strain of *Aspergillus flavus* grown in surface culture on synthetic media, has been shown to be 3,4-dihydro-6,8-dihydroxy-3-(6-methyltetrahydropyran-2-ylmethyl)isocoumarin (I; $R^1 = R^2 = H$). The major secondary metabolic product formed under these conditions was the 8-*O*-methyl ether (I; $R^1 = H$, $R^2 = Me$); additional minor products were the 6-*O*-methyl ether and three closely-related phenolic compounds, one of which was shown to be a 5'-hydroxyasperentin (III; $R^1 = R^2 = H$, $R^3 = OH$).

ENTOMOGENOUS strains of the closely-related species *Aspergillus flavus* and *A. parasiticus* frequently produce aflatoxins when cultured on synthetic media.[1] The relationship between aflatoxin-production and the insecticidal action of these organisms rests mainly on circumstantial evidence [2] and the precise role of the aflatoxins in the pathogenicity of some *A. flavus* strains structurally closely-related, toxic phenolic compounds, the asperentins, shown here to be 6,8-di(oxygen-substituted)-3,4-dihydroisocoumarins. The biological activity of these compounds will be reported elsewhere.

On Raulin–Thom medium at 25°, production of the asperentins, as estimated spectrophotometrically, commenced *ca.* 7 days after inoculation and then increased



SCHEME 1 Reagents: 1, $[CH_2{\cdot}SH]_2$; 2, Raney Ni; 3, CrO_3; 4, $NaBH_4$; 5, BCl_3; 6 $KMnO_4$

to insects is uncertain. However, the mammalian toxicity and long-term carcinogenicity of the aflatoxins and their relatives appears to preclude their development as insecticides.

Entomogenous strains of *A. flavus* which do not produce aflatoxins are, therefore, of some interest and a number of toxic secondary metabolites have been described already.[3] One such strain, isolated in these laboratories from an infected pupa of the wax moth, *Galleria mellonella*, produces, in surface culture on either Raulin–Thom or Czapek–Dox medium, a new group of rapidly during the next 7—10 days; thereafter, with the carbon source nearing exhaustion, a rise in the pH of the medium from 5·5 to 7·0 was accompanied by the formation of much dark coloured pigment which interfered with the analysis.

The products of fermentation were harvested after 3—4 weeks. Trituration with acetone of the insecticidal resin, obtained by extraction of the culture filtrate (at pH 5·0) with chloroform, gave an insoluble solid consisting mainly of the major secondary metabolic product, a $C_{17}H_{22}O_5$ phenol, double m.p. 225 and 235°, obtained in yields of 150—200 mg l^{-1}.

[1] C. W. Hesseltine, O. L. Shotwell, M. Smith, J. J. Ellis, E. Vandegraft, and G. Shannon, in 'Proceedings of the First U.S.–Japan Conference on Toxic Micro-organisms,' Honolulu, 1968, U.S. Dept. Interior, Washington, U.S.A., 1970, p. 202.

[2] G. Becker, H. K. Frank, and M. Lenz, *Z. angew. Zool.*, 1969, **56**, 451.

[3] B. J. Wilson, *Bact. Rev.*, 1966, **30**, 478.

Column chromatography on silica gel of the acetone-soluble residue gave a series of phenolic minor products including, in order of elution by benzene–methanol, an isomer, m.p. 97°, of the major metabolite; two $C_{16}H_{20}O_5$ compounds, m.p.s 187 and 155—156°; a further small quantity of the major metabolite; and, finally, three $C_{16}H_{20}O_6$ compounds, m.p.s 229—230, 210—212 (decomp.), and 195°. The compounds were characterised spectroscopically and by the preparation of derivatives. The acetates were generally unsatisfactory gums but the methyl ethers were highly-crystalline stable substances. asperentin with methyl iodide in acetone in the presence of potassium carbonate gave a monomethyl ether, identical with the $C_{17}H_{22}O_5$ metabolite, m.p. 97° and a dimethyl ether (I; $R^1 = R^2 = Me$), also obtained by methylation of the major $C_{17}H_{22}O_5$ metabolite, either under the same conditions, or with ethereal diazomethane in methanol.

The u.v. absorption of asperentin and its methyl ethers [$\lambda_{max.}$ 269 and 300 nm (log ε 4·1 and 3·8)], taken in conjunction with the i.r. evidence, suggested the presence of a dihydroisocoumarin chromophore with

TABLE 1

Chemical shifts (τ values) for protons in asperentin and related compounds

Compound	Solvent	5-H [a]	7-H [a]	3-H [b]	4-H_2 [c]	2′-H [b]	5′-H [b]	6′-H [b]	Me [c]	OMe	OAc
(I; $R^1 = R^2 = H$)	$(CD_3)_2SO$	3·90	3·90	5·45	7·15	6·15		6·28	8·90		
(I; $R^1 = H$, $R^2 = Me$)		3·66	3·78	5·65	7·20	*ca.* 6·1		*ca.* 6·4	8·92	6·25	
(III; $R^1 = R^2 = H$, $R^3 = OH$)		3·90	3·90	5·46	7·15	6·1	6·1	6·25	8·90		
(I; $R^1 = R^2 = Ac$)	$CDCl_3$	2·97	3·08	5·25	7·00 [d]	5·96		5·96	8·78		7·60
											7·65
(I; $R^1 = Ac$, $R^2 = Me$)		3·28	3·33	5·35	7·10	*ca.* 6·0		*ca.* 6·0	8·81	6·05	7·68
											7·65
(III; $R^1 = R^2 = Ac$, $R^3 = OAc$)		2·99	3·07	5·24	7·03 [d]	5·94	5·08	5·94	8·77		7·70
											8·00
(I; $R^1 = R^2 = Me$)		3·52	3·64	5·37	7·09	5·90		5·90	8·75	6·06	
										6·12	
(III; $R^1 = R^2 = Me$, $R^3 = OH$)		3·52	3·62	5·38	7·12	*ca.* 6·05	*ca.* 6·05	*ca.* 6·05	8·76	6·03	
										6·08	
(II)		3·43	3·55	5·24	7·06	5·54		5·74	8·61	6·00	
										6·06	
(I; $R^1 = R^2 = H$)	$(CD_3)_2CO$	3·63	3·63	5·20	7·0	5·88		6·04	8·83		
(IV)		3·56	3·56	5·12	7·0	5·95		5·80	8·44		

[a] Singlet or doublet, $J_{5,7}$ 2 Hz. [b] Multiplet. [c] AB Part of ABX system with $J_{AB} > \Delta\nu$ unsuited to first-order interpretation, except [d] doublet J 7 Hz. [e] Doublet, J 6·5 Hz.

Two products, the $C_{16}H_{20}O_5$ phenol (m.p. 155—156°) and the $C_{16}H_{20}O_6$ phenol [m.p. 210—212° (decomp.)], were isolated only once. The other $C_{16}H_{20}O_5$ phenol (m.p. 187°) was assigned the trivial name asperentin.* The $C_{16}H_{20}O_6$ phenol, m.p. 229—230°, shown below to be a 5′-hydroxyasperentin, was accompanied by a yellow pigment, asperflavin, which is described in the following paper.[4]

With the exception of asperentin (20 mg l^{-1}) and the 5′-hydroxyasperentin (7 mg l^{-1}), the yields of these minor products were of the order of 1 mg l^{-1} or less. In column chromatography on acid alumina, the major metabolite, $C_{17}H_{22}O_5$, was eluted before asperentin and more polar products were retained on the column.

The major metabolite, $C_{17}H_{22}O_5$ (I; $R^1 = H$, $R^2 = Me$) ($\nu_{max.}$ 3320 and 1680 cm^{-1}) gave no colour with iron(III) chloride, while the isomer (I; $R^1 = Me$, $R^2 = H$) (m.p. 97°) gave an intense reddish purple colour, consistent with the presence of a chelated carbonyl group ($\nu_{max.}$ 1660 cm^{-1}), and, in the solid state, showed no OH absorption in the i.r. spectrum between 3600—3000 cm^{-1}. The n.m.r. spectra indicated that both isomers contained a methoxy-group (τ 6·2). Methylation of oxygen substituents at positions 6 and 8.[5,6] This substitution pattern was confirmed by the n.m.r. spectrum, which showed two *meta*-aromatic protons (τ 3·6, J 2 Hz). It was rigidly proved when cautious oxidation of the dimethyl ether (I; $R^1 = R^2 = Me$) with alkaline potassium permanganate gave the known 2,4-dimethoxy-6-oxalobenzoic acid hydrate [7] (V), together with some 3,5-dimethoxybenzoic acid (VI) which must result from the acid-catalysed decarboxylation of the diacid (V) during work-up.[8] The same mixture of acids (V) and (VI) was obtained by oxidation of 2,4-dimethoxy-6-(2-oxopropyl)benzoic acid [9] (VIII) under the same conditions.

The $C_{17}H_{22}O_5$ metabolites are, therefore, the 8- and 6-*O*-methyl ethers of asperentin. Consistent with the presence of a 3-substituted dihydroisocoumarin nucleus, the n.m.r. spectra (see Table 1) showed a one-proton multiplet at τ 5·3 coupled to a two-proton multiplet at τ 7·1, in the region ascribed to benzylic protons.

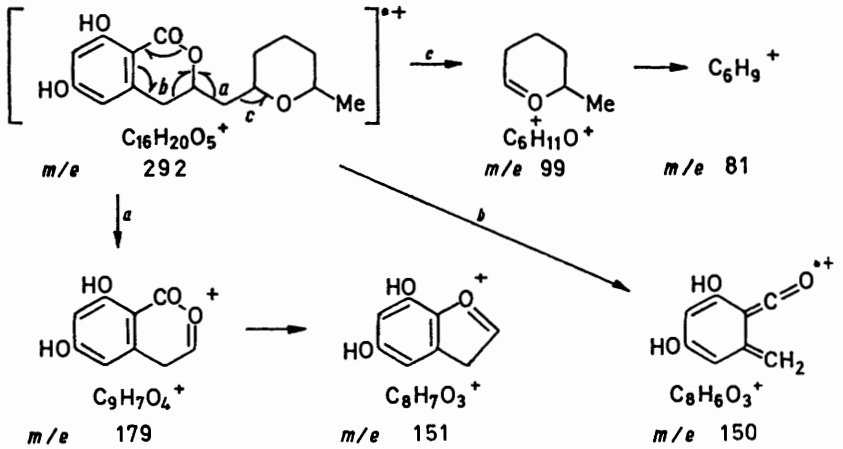
Four of the five oxygen atoms of asperentin are contained in the dihydroxydihydroisocoumarin portion of the molecule. The dimethyl ether (I; $R^1 = R^2 = Me$) had no free hydroxy-group, and the remaining oxygen atom is, therefore, present as an ether linkage.

* *Note added in proof:* Cladosporin, an antifungal metabolite from *Cladosporium cladosparioides*, described recently (P. M. Scott, W. van Walbeek, and W. M. MacLean, *J. Antibiot.*, 1971, **24**, 747), is identical with asperentin.

[4] J. F. Grove, following paper.
[5] E. Sondheimer, *J. Amer. Chem. Soc.*, 1957, **79**, 5036.
[6] W. J. McGahren and L. A. Mitscher, *J. Org. Chem.*, 1968, **33**, 1577.
[7] R. Thomas, *Biochem. J.*, 1961, **78**, 748.
[8] J. F. Grove, *Biochem. J.*, 1952, **50**, 648.
[9] H. L. Slates, S. Weber, and N. L. Wendler, *Chimia* (*Switz.*), 1967, **21**, 468.

This must be part of the second ring system required by the elemental composition of asperentin, since the n.m.r. spectrum showed two separate one-proton multiplets at τ 6, one of which was coupled to a secondary methyl group at τ 8·8. Experimental proof of the existence of this ring system resulted from the action of boron trichloride in dichloromethane at 0° on asperentin 8-methyl ether; cleavage of the ether linkage was accompanied by opening of the second heterocyclic ring giving, as the major product, the chlorohydrin (IV), was shown to be *R*, as in structure (I), by c.d. measurements (see Figure) which gave a curve opposite in sign to that given by (−)-*R*-mellein (VII).[13]

All the minor metabolic products of the fermentation showed essentially the same c.d. curves and u.v. absorption spectra as asperentin. The $C_{16}H_{20}O_6$ product, m.p. 229—230°, (III; $R^1 = R^2 = H$, $R^3 = OH$) was related to asperentin as follows. The formation of a triacetate and a dimethyl ether (III; $R^1 = R^2 = Me$, $R^3 = OH$), $\nu_{max.}$ 3440 and 3395 cm^{-1}, indicated that the sixth oxygen



SCHEME 2

atom was present as an alcoholic hydroxy-group, considered to be secondary since the n.m.r. spectrum of the acetate (III; $R^1 = R^2 = Ac$, $R^3 = OAc$) showed a one-proton multiplet at τ 5·08, absent in the spectrum of diacetylasperentin. That this secondary alcoholic group

containing the intact dihydroisocoumarin nucleus and all sixteen carbon atoms of asperentin. A minor product of this reaction was, not asperentin, but a stereoisomer, m.p. 136—138°.

The second heterocyclic ring of asperentin was shown to be 6-membered, rather than 7- or 5-membered, by n.m.r. double-irradiation experiments, and by consideration of the mass spectra of asperentin and its relatives. These indicated three significant fragmentation pathways (see Scheme 2) two of which [(*a*) and (*b*)] gave fragment ions, (*a*) at *m/e* 179 and 151 (179 − CO) and (*b*) at *m/e* 150,[10] which retained the substituted aromatic ring of asperentin. The third (*c*) gave a fragment ion, which was also the base peak, at *m/e* 99 ($C_6H_{11}O^+$), derived from the methyl-substituted tetrahydropyran ring. The $C_6H_{11}O^+$ ion was further degraded by the loss of the elements of water [11] to $C_6H_9^+$ (*m/e* 81), a transition supported by a prominent metastable peak at *m/e* 66·3.

Asperentin, therefore, has the 3,4-dihydro-6,8-dihydroxy-3-(6-methyltetrahydropyran-2-ylmethyl)isocoumarin structure (I; $R^1 = R^2 = H$), consistent with a straightforward biogenetic derivation from eight C_2 units *via* the acetate–polymalonate pathway. Lasiodiplodin (IX) [12] may represent an alternative cyclisation of the same alkyl-2,4-dihydrobenzoic acid precursor; interestingly, it shows the same, uncommon, methylation pattern as asperentin 8-methyl ether.

The absolute configuration of asperentin at position 3

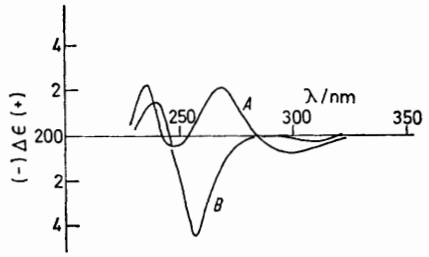


FIGURE C.d. curves for asperentin *A* and (−)-mellein *B*

was located in the tetrahydropyran ring followed from the mass spectrum, which showed the same fragment ions at *m/e* 179, 151, and 150 (characteristic of the dihydroisocoumarin moiety of asperentin), but a different base peak at *m/e* 115 ($C_6H_{11}O_2^+$).

Oxidation of the dimethyl ether (III; $R^1 = R^2 = Me$, $R^3 = OH$) at 0° in acetone with chromic oxide–sulphuric acid [14] gave a ketone (II). Only two (at 4′) of the six protons located at τ 8—8·5 in the n.m.r. spectrum of

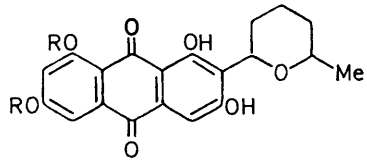
[10] M. J. Rix and B. R. Webster, *J. Chem. Soc.* (*B*), 1968, 254.
[11] G. Ohloff, K. H. Schulte-Elte, and B. Willhalm, *Helv. Chim. Acta*, 1964, **47**, 602.
[12] D. C. Aldridge, S. Galt, D. Giles, and W. B. Turner, *J. Chem. Soc.* (*C*), 1971, 1623.
[13] H. Arakawa, N. Tarimoto, and Y. Masui, *Annalen*, 1969, **728**, 152.
[14] K. Bowden, I. M. Heilbron, E. R. H. Jones, and B. C. L. Weedon, *J. Chem. Soc.*, 1946, 39.

the dimethyl ether (III; $R^1 = R^2 = Me$, $R^3 = OH$) were shifted downfield to τ 7·4 by the introduction of the keto-group. This fact, coupled with a downfield shift of the methyl resonance to τ 8·6, indicated that the ketone-group was present at the 5′-position, rather than at 4′ where it might have been expected on biogenetic considerations.

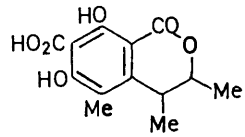
Reaction of the ketone (II) with ethanedithiol in the presence of boron trifluororide–ether gave the thioacetal, which furnished asperentin dimethyl ether (I; $R^1 = R^2 = Me$) on desulphurisation with Raney nickel.

Regeneration of the secondary alcohol (III; $R^1 = R^2 = Me$, $R^3 = OH$) as the only isolable product on reduction of the ketone (II) with sodium borohydride in methanol showed that no change in configuration at the 6′-position was associated with the formation of the ketone. The $C_{16}H_{20}O_6$ compound (m.p. 229—230°) is, therefore, a 5′-hydroxyasperentin.

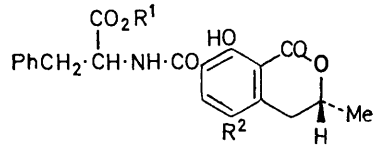
The mass spectra of the other $C_{16}H_{20}O_5$ and $C_{16}H_{20}O_6$ metabolic products showed the same abundant fragment ions as asperentin and 5′-hydroxyasperentin respectively. They presumably differ from asperentin and 5′-hydroxyasperentin only in absolute configuration at the 2′- or 6′-positions, or in the location or configuration of the hydroxy-substituent in the tetrahydropyran ring.



(X)



(XI)



(XII)

Though a structural feature of frequent occurrence amongst metabolic products of the Actinomycetes, *e.g.* monensin [15] and nigericin,[16] the substituted tetrahydropyran ring is uncommon amongst fungal products, and the anthraquinones (X; R = H and Me) from *A. versicolor* are the only examples reported.[17] The tetrahydroxy-compound (X; R = H) has also been isolated from *A. flavus*.[18] By contrast, the dihydroisocoumarin ring system is relatively common, particularly as a metabolic product of the *A. ochraceus* group of the genus *Aspergillus*. Examples isolated from this genus include (—)-mellein (VII) and 4-hydroxymellein from *A. melleus*, *ochraceus*, and *oniki*,[19-21] 3,4-dihydro-6,8-dihydroxy-3,4,5-trimethylisocoumarin-7-carboxylic acid (XI) from *A. terreus*,[22] and the ochratoxins (XII; R^1 = H and Et, R^2 = H and Cl) from *A. ochraceus*, *melleus*, and *sulphureus*.[23]

EXPERIMENTAL

M.p.s were taken on a Kofler hot-stage apparatus and are corrected. Unless otherwise stated i.r. spectra were determined for mulls in Nujol, and u.v. spectra, optical rotations, and c.d. measurements for solutions in methanol. N.m.r. spectra were obtained at 100 MHz with tetramethylsilane as internal standard. Molecular weights were taken from the parent peaks in the mass spectra. Light petroleum had b.p. 60—80°. Merck silica gel G_{254} was used in t.l.c. In preparative t.l.c. (p.l.c.), silica layers (20 × 20 × 0·1 cm) were developed in chloroform–methanol (97 : 3) and the R_F values quoted are for this solvent system.

Isolation of the Aspergillus flavus *Strain.*—Single spore transfers from an infected *Galleria mellonella* pupa were maintained on agar slopes at 24°. Satisfactory growth of a typical *Aspergillus* sp., identified as *A. flavus* Link *ex* Fr., was obtained on Czapek, carrot, and malt agars.

Fermentations with the A. flavus *Strain.*—Conical flasks (30 × 1 l) containing Raulin–Thom medium (300 ml), made up with glucose (5%) and adjusted to pH 5·5, were inoculated with a spore suspension of the *A. flavus* strain in sterile water (prepared from Czapek–agar slopes). The flasks were incubated at 25°. At intervals, aliquot portions of the culture fluid were removed under sterile conditions from selected flasks for determination of the pH, optical rotation (*l*, 10 cm) and spectrophotometric assay for the secondary metabolic products. The course of a typical fermentation is indicated in Table 2.

TABLE 2

Course of a typical fermentation with the *A. flavus* strain on Raulin–Thom medium

t/day	0	7	9	11	14	17	21
pH	5·5	5·5	5·5	5·7	5·7	6·2	7·0
Optical rotation (% value at day 0)	100	98	95	93	43	0	
Apparent asperentins (mg l^{-1})	0	0	5	14	132	361	413

After 3—4 weeks the fermentations were stopped and, in a typical batch, the culture filtrate (7—10 l) was adjusted to pH 5 and extracted with chloroform (2 × 2 l). Evaporation of the dried extract (Na_2SO_4) *in vacuo* furnished a brown resin (2—3 g, 200—400 mg l^{-1}), which contained the asperentins.

The acid fraction, obtained by further adjustment of the pH of the culture filtrate to 2·0 and extraction with ethyl acetate, was an intractable tar (1·5—2 g).

Much greater yields of 'neutral' product (1 g l^{-1}; a black tar) were obtained by extracting the culture filtrate

[15] A. Agtarap, J. W. Chamberlin, M. Pinkerton, and L. Steinrauf, *J. Amer. Chem. Soc.*, 1967, **89**, 5737.
[16] L. K. Steinrauf, M. Pinkerton, and J. W. Chamberlin, *Biochem. Biophys. Res. Comm.*, 1968, **33**, 29.
[17] J. S. E. Holker, S. A. Kagal, L. J. Mulhern, and P. M. White, *Chem. Comm.*, 1966, 911.
[18] J. G. Heathcote and M. F. Dutton, *Tetrahedron*, 1969, **25**, 1487.
[19] E. Nishikawa, *J. Agric. Chem. Soc. Japan*, 1933, **9**, 772.
[20] R. J. Cole, J. H. Moore, N. D. Davis, J. W. Kirksey, and U. L. Diener, *J. Agric. Food Chem.*, 1971, **19**, 909
[21] M. Sasaki, Y. Kaneko, K. Oshita, H. Takamatsu, Y. Asao, and T. Yokotsuka, *Agric. and Biol. Chem.* (*Japan*), 1970, **34**, 1296.
[22] C. H. Hassall and D. W. Jones, *J. Chem. Soc.*, 1962, 4189.
[23] K. J. van der Merwe, P. S. Steyn, and L. Fourie, *J. Chem. Soc.*, 1965, 7083.

at pH 5 with ethyl acetate, but the final yield of asperentin 8-methyl ether (see later) was not significantly increased. Similar, though lower, yields of both crude ' neutral ' resin and asperentin 8-methyl ether were obtained using liquid Czapek–Dox medium.

Spectrophotometric Assay.—An aliquot portion (usually 10·0 ml) of the culture fluid was withdrawn from beneath the mycelial felt through a sterile pipette. The pH and optical rotation of the filtered solution were recorded and, after the pH had been adjusted, if necessary, to 5·0, a portion (5·0 ml) was extracted with chloroform (2 × 2 ml). The residue from extraction was dried *in vacuo*, dissolved in methanol (10·0 ml), and the optical density of the solution was recorded (1 cm cell) between 250 and 350 nm. The total concentration of asperentins in the culture fluid was then calculated as ' apparent asperentin ' from the optical density at 268 and 300 nm, taking ε_{268} as 14,000, or, to offset the error introduced by a rising background absorption, $\varepsilon_{268} - \varepsilon_{300}$ as 7000.

Asperentin 8-O-*Methyl Ether.*—The brown resin (2·1 g) obtained by extraction of the culture filtrate with chloroform was triturated with acetone and set aside overnight. The product was filtered off and washed with acetone, and the combined filtrate and washings were evaporated to dryness *in vacuo* giving a resin (0·90 g), which was purified by column chromatography (see later).

Two recrystallisations of the crude product (1·1 g) from methanol afforded plates (760 mg), double m.p. 225° and 235°, R_F 0·23, $[\alpha]_D^{20}$ +72° (*c* 0·1 in chloroform), of *asperentin* 8-O-*methyl ether* [3,4-*dihydro*-6-*hydroxy*-8-*methoxy*-3-(6-*methyltetrahydropyran*-2-*ylmethyl*)*isocoumarin*] (I; R^1 = H, R^2 = Me) (Found: C, 66·6; H, 7·3%; *M*, 306. $C_{17}H_{22}O_5$ requires C, 66·65; H, 7·2%; *M*, 306), $\nu_{max.}$ 3320, 1680, 1605, and 1590 cm^{-1}, $\lambda_{max.}$ ~227, 266, and 298 nm (ε 18,500, 14,000, and 7800); $\lambda_{max.}$ (in 2N-NaOH) 242 and 307 nm, c.d., λ 296, 269, 251, and 232 nm (Δε +1·51, +4·70, −0·91, and +4·39).

The ether was insoluble in sodium hydrogen carbonate, but dissolved in N-sodium hydroxide. It gave no colour with iron(III) chloride, did not reduce ammoniacal silver nitrate, and did not react with 2,4-dinitrophenylhydrazine in 2N-hydrochloric acid.

The *acetate* (I; R^1 = Ac, R^2 = Me), prepared in pyridine with acetic anhydride at room temperature, was a gum, purified by preparative t.l.c. (R_F 0·43) and dried *in vacuo* at 100° (Found: C, 65·1; H, 7·0%. $C_{19}H_{24}O_6$ requires C, 65·5; H, 6·9%), $\nu_{max.}$ (film) 1775, 1730, 1605, and 1595 cm^{-1}.

The *methyl ether* (I; R^1 = R^2 = Me), prepared (*a*) in methanol with an excess of ethereal diazomethane or (*b*) in boiling acetone with an excess of methyl iodide in the presence of anhydrous potassium carbonate during 3 h, was purified by preparative t.l.c. (R_F 0·60) and crystallised from light petroleum giving prisms, m.p. 118°, $[\alpha]_D^{20}$ +48° (*c* 0·05) (Found: C, 67·8; H, 7·6%; *M*, 320. $C_{18}H_{24}O_5$ requires C, 67·5; H, 7·55%; *M*, 320), $\nu_{max.}$ 1720, 1605, and 1582 cm^{-1}.

Minor Metabolic Products of the Fermentation.—The resin remaining after the removal of asperentin 8-methyl ether (see before) was divided into two equal parts which were examined by chromatography.

(*A*) *On alumina.* One part in benzene–methanol (97 : 3) was chromatographed on a column of acid alumina (Woelm grade II, 60 g, 18 × 2 cm) made up in benzene. After elution of some intractable gums (28 mg), benzene–methanol (98 : 2, 200 ml) eluted a yellow band yielding a gum (64 mg) which furnished asperentin 8-methyl ether (15 mg) on crystallisation from methanol. Elution of a peach-coloured band with benzene–methanol (96 : 4, 100 ml) and (94 : 6, 350 ml) gave a gum (104 mg), which crystallised from ethyl acetate or benzene in needles (33 mg), m.p. 187°, of *asperentin* [3,4-*dihydro*-6,8-*dihydroxy*-3-(6-*methyltetrahydropyran*-2-*ylmethyl*)*isocoumarin*] (I; R^1 = R^2 = H) (Found: C, 65·8; H, 7·1%; *M* 292. $C_{16}H_{20}O_5$ requires C, 65·7; H, 6·9%; *M*, 292), $\lambda_{max.}$ *ca.* 232, 269, and 303 nm (ε 11,700, 13,800, and 6950), $\nu_{max.}$ 3230, *ca.* 1645, 1620, and 1595 cm^{-1}, c.d., λ 302, 268, 247, and 234 nm (Δε −0·86, +2·45, −0·58, and +2·30). Asperentin gave a dark greenish brown colour with iron(III) chloride and, after standing 2 h at room temperature in an excess of 2N-sodium hydroxide, it was recovered unchanged on acidification of the solution with dilute hydrochloric acid.

The *diacetate* (I; R^1 = R^2 = Ac), prepared in pyridine with acetic anhydride during 18 h at room temperature, was gum (Found: C, 63·5; H, 6·6. $C_{20}H_{24}O_7$ requires C, 63·8; H, 6·4%), $\nu_{max.}$ (film) 1780, 1725, 1620, and 1590 cm^{-1}.

Nothing further was eluted from the column with benzene–methanol (9 : 1).

(*B*) *On silica gel.* The second part in benzene–methanol (97 : 3) was chromatographed on a column of silica gel (Merck 7734, 30 g, 18 × 2 cm) made up in benzene. After intractable gummy fractions (10 mg) had been eluted with benzene–methanol (99 : 1, 100 ml) a yellow band was eluted with the same solvent (100 ml) giving a yellow oil (4 mg) which crystallised from light petroleum in plates (2 mg), m.p. 97°, R_F 0·80, of *asperentin* 6-O-*methyl ether* (I; R^1 = Me, R^2 = H) (Found: C, 67·2; H, 7·55%; *M*, 306. $C_{17}H_{22}O_5$ requires C, 66·65; H, 7·2%; *M*, 306), $\nu_{max.}$ 1660, 1630, and 1583 cm^{-1}, $\lambda_{max.}$ *ca.* 230, 268, and 302 nm (ε 12,000, 14,000, and 6200). It dissolved in N-sodium hydroxide and gave a deep reddish purple colour with iron-(III) chloride.

Further elution of the column with benzene–methanol (99 : 1, 75 ml) brought off a brown band giving a semi-solid fraction (135 mg), which furnished crude asperentin (41 mg), m.p. 178—182° (from benzene).

On one occasion the first crop obtained on fractional crystallisation of this fraction from ethyl acetate consisted of prisms m.p. 150—155°. Recrystallisation from benzene gave plates (6 mg), m.p. 155—156°, of a *stereoisomer* of asperentin (Found: C, 66·0; H, 7·0%; *M*, 292. $C_{16}H_{20}O_5$ requires C, 65·7; H, 6·9%; *M*, 292), $\nu_{max.}$ *ca.* 3460, 3410, 1655, *ca.* 1630, and 1592 cm^{-1}, $\lambda_{max.}$ *ca.* 231, 268, and 300 nm (ε 11,500, 13,500, and 6500), c.d., λ 299, 269, 247, and 234 nm (Δε −0·79, +2·16, −0·49, and +2·26). It dissolved in 2N-sodium hydroxide and gave a greenish brown colour with iron(III) chloride in ethanol.

After an interband, which yielded an intractable gum (14 mg), had been eluted with benzene–methanol (98 : 2, 100 ml), benzene–methanol (95 : 5, 100 ml) eluted a deep yellow band giving a gum (139 mg) from which asperentin 8-methyl ether (15 mg) was obtained on crystallisation from methanol.

Elution of an orange band with the same solvent (100 ml) gave a solid (108 mg). Fractional crystallisation from methanol or ethyl acetate gave, from the more soluble fractions, a yellow pigment;[4] and from the less-soluble fractions, needles (20 mg), m.p. 229—230°, R_F 0·13, $[\alpha]_D^{20}$ −30° (*c* 0·33), of a 5′-*hydroxyasperentin* [3,4-*dihydro*-6,8-*dihydroxy*-3-(5-*hydroxy*-6-*methyltetrahydropyran*-2-*ylmethyl*)-*isocoumarin*] (III; R^1 = R^2 = H, R^3 = OH) (Found: C,

62·3; H, 6·7%; *M*, 308. $C_{16}H_{20}O_6$ requires C, 62·3; H, 6·5%; *M*, 308), $\lambda_{max.}$ *ca.* 231, 269, and 303 nm (ε 13,900, 16,000, and 7850), $\nu_{max.}$ 3530, 3200, *ca.* 1645, 1620, and 1600 cm^{-1}, c.d., λ 299, 268, 247, and 235 nm (Δε −0·78, +2·10, −0·39, and +2·18). It dissolved in N-sodium hydroxide and gave a dark greenish brown colour with iron(III) chloride.

The *triacetate* (III; $R^1 = R^2 = Ac$, $R^3 = OAc$), prepared with acetic anhydride in pyridine during 18 h at room temperature, was a gum which would not crystallise and was purified by preparative t.l.c. (R_F 0·6) followed by distillation at 140° (bath temp.) and 10^{-2} mmHg (Found: C, 61·2; H, 6·2. $C_{22}H_{26}O_9$ requires C, 60·8; H, 6·0%), $\nu_{max.}$ (film) 1778, 1725, 1618, and 1590 cm^{-1}.

On one occasion fractional crystallisation of the crude 5′-hydroxyasperentin from ethyl acetate gave prisms (5 mg), m.p. 210—212° (decomp.), of an *isomer* (Found: C, 62·5; H, 6·8%; *M*, 308. $C_{16}H_{20}O_6$ requires C, 62·3; H, 6·5%; *M*, 308), $\nu_{max.}$ 3495, 3130br, 1660, 1638, and 1590 cm^{-1}, $\lambda_{max.}$ *ca.* 231, 269, and 302 nm (ε 14,900, 16,800, and 8400), c.d., λ 301, 267, 247, and 236 nm (Δε −0·93, +1·86, −0·62, and +1·86). It was soluble in 2N-sodium hydroxide and gave a deep greenish brown colour with iron(III) chloride.

Continued elution with benzene–methanol (92 : 8, 100 ml) brought off a second orange band giving a tar (41 mg), which, after trituration with ethyl acetate and three recrystallisations of the solid product from the same solvent, gave prisms (6 mg), m.p. 195°, R_F 0·08, of a *dihydroisocoumarin* (Found: C, 62·5; H, 6·8%; *M*, 308. $C_{16}H_{20}O_6$ requires C, 62·3; H, 6·5%; *M*, 308), $\nu_{max.}$ 3462, 3355, 3260, 1658, 1630, and 1590 cm^{-1}, $\lambda_{max.}$ *ca.* 230, 269, and 303 nm (ε 9000, 10,600, and 5300), c.d., λ 300, 269, 248, and 234 nm (Δε −0·72, +1·44, −0·36, and +1·94). It dissolved in 2N-sodium hydroxide and gave a reddish brown colour with iron(III) chloride. It was recovered from aqueous dimethyl sulphoxide in plates of a *hydrate*, $\nu_{max.}$ 3420, 3140br, 1652, 1235, and 1590 cm^{-1}, which were converted into the anhydrous material on heating at 120°.

The *dimethyl ether*, prepared with methyl iodide as described before, and purified by t.l.c. (R_F 0·10), crystallised from ethyl acetate in prisms, m.p. 172—174°, of a *solvate* (Found: C, 63·0; H, 7·2. $C_{18}H_{24}O_6,0{\cdot}5C_4H_8O_2$ requires C, 63·1; H, 7·4%), $\nu_{max.}$ 3420, 3380, 1725, 1690, 1612, and 1590 cm^{-1}, from which the ethyl acetate was not removed by drying *in vacuo* at 110° for 2 h over phosphorous pentoxide.

Methylation of Asperentin.—Asperentin (10 mg) in acetone (2 ml) was heated under reflux with an excess of methyl iodide in the presence of anhydrous potassium carbonate for 8 h and the mixture [which gave a positive iron(III) reaction] was worked-up in the usual way. P.l.c. gave two bands (R_F 0·74 and 0·60), which were visible in u.v. light and from which the components were recovered by extraction with chloroform. The material R_F 0·74 (6 mg) was the 6-methyl ether (I; R^1 = Me, R^2 = H), plates, m.p. and mixed m.p. 97° (from light petroleum), identical with an authentic sample (i.r.).

The material R_F 0·60 (4 mg), needles, m.p. 118° (from light petroleum), was identified in the same way, as the dimethyl ether (I; $R^1 = R^2$ = Me).

Reaction of Asperentin 8-Methyl Ether with Boron Trichloride.—The methyl ether (50 mg), suspended in dichloromethane (3 ml) at 0°, was treated with boron trichloride (0·20 ml of a solution containing 0·32 g ml^{-1}). After 5 min, the yellow solution was evaporated to dryness *in vacuo* at room temperature, the gummy residue was triturated with water and then extracted with ethyl acetate. The recovered gum was separated into its components by p.l.c. Material R_F 0·30 (28 mg) was recrystallised three times from ethyl acetate–light petroleum giving prisms (14 mg), m.p. 144°, of 3-(6-*chloro*-2-*hydroxy*-*n*-*heptyl*)-3,4-*dihydro*-6,8-*dihydroxyisocoumarin* (IV) (Found: C, 58·9; H, 6·5%; *M*, 328. $C_{16}H_{21}ClO_5$ requires C, 58·4; H, 6·4%; *M*, 328), $\nu_{max.}$ 3415, 3360, 3280, 1650, 1630, and 1595 cm^{-1}, $\lambda_{max.}$ *ca.* 233, 271, and 305 nm (ε 10,100 14,800, and 7400).

Material R_F 0·43 (5 mg) crystallised from ethyl acetate–light petroleum in plates (1·5 mg), m.p. 136—138°, of a *dihydroisocoumarin* (Found: C, 66·0; H, 7·0%; *M*, 292. $C_{16}H_{20}O_5$ requires C, 65·7; H, 6·9%; *M*, 292), $\nu_{max.}$ 3160br, 1670, 1630, and 1590 cm^{-1}.

Oxidation of Asperentin Dimethyl Ether.—The dimethyl ether (30 mg) was dissolved in 2N-sodium hydroxide (4 ml) at 80°, and the solution was cooled and treated at room temperature with potassium permanganate (5%, 5 ml) added in portions until a violet colour persisted for 5 min. After the mixture had been warmed at 80° for 15 min, the excess of permanganate was destroyed by the addition of methanol. The precipitated manganese dioxide was filtered off and washed with hot water. The combined filtrate and washings were acidified to pH 2 with concentrated hydrochloric acid and cautiously concentrated at 80°. The resultant solid (*A*, 8 mg) was filtered off and the filtrate was extracted with ethyl acetate giving a gum (*B*, 12 mg).

Fractional crystallisation of the solid (*A*) from water afforded (i) needles (1 mg), m.p. 184° (subl.) [lit.,[24] 185—186° (subl.)] of 3,5-dimethoxybenzoic acid (VI) (Found: C, 58·9; H, 5·5%; *M*, 182. Calc. for $C_9H_{10}O_4$: C, 59·3; H, 5·5%; *M*, 182) identified by the i.r. spectrum, $\nu_{max.}$ 3100br, 2650, 2550br, 1690, and 1602 cm^{-1}, and (ii) needles (3 mg), m.p. 168—171° (decomp.), of 2,4-dimethoxy-6-oxalobenzoic acid hydrate (V) (Found: C, 48·9; H, 4·7%; *M*, 254. Calc. for $C_{11}H_{10}O_7,H_2O$: C, 48·5; H, 4·4%; 254), the i.r. spectrum of which was identical with that of an authentic specimen,[7] m.p. 173° (decomp.), $\nu_{max.}$ 3580, 3510, 3100—2500br, 1770w, 1750w, 1690, 1623, and 1600 cm^{-1}. The i.r. spectrum was unchanged after drying for 8 h *in vacuo* at 120° over phosphorus pentoxide.

The gum (*B*) crystallised from water giving 2,4-dimethoxy-6-oxalobenzoic acid hydrate (5 mg).

Oxidation of 2,4-dimethoxy-6-(2-oxopropyl)benzoic acid (VIII) (50 mg) under the same conditions gave 3,5-dimethoxybenzoic acid (3 mg) and 2,4-dimethoxy-6-oxalobenzoic acid hydrate (21 mg).

Methylation of 5′-Hydroxyasperentin.—5′-Hydroxyasperentin (40 mg) in acetone (2 ml) was heated under reflux for 18 h with an excess of methyl iodide in the presence of potassium carbonate (80 mg). The mixture was filtered and the filtrate was evaporated to dryness *in vacuo*. The residue was extracted with chloroform and the extract was subjected to p.l.c. Recovery in the usual way of material from a band R_F 0·20 gave a gum (29 mg) which crystallised from ethyl acetate in prisms (18 mg), m.p. 139°, $[\alpha]_D^{22}$ +35° (*c* 0·066), of the *dimethyl ether* (III; $R^1 = R^2$ = Me, R^3 = OH) (Found: C, 64·2; H, 7·1. $C_{18}H_{24}O_6$ requires C, 64·3; H, 7·2%), $\nu_{max.}$ 3440, 3395, 1705, 1612, and 1588 cm^{-1}.

Material (11 mg) recovered from a band R_F 0·29 consisted essentially (n.m.r.) of the monomethyl ether (III; R^1 = Me, R^2 = H, R^3 = OH). It was not characterised, but was converted, by recycling, into the dimethyl ether.

[24] J. Herzig and S. Epstein, *Monatsh.*, 1908, **29**, 661.

Oxidation of the Dimethyl Ether (III; $R^1 = R^2 = Me$, $R^3 = OH$).—The dimethyl ether (15 mg) in acetone (1 ml) at 0° was treated with 8N-chromic oxide–sulphuric acid (0·03 ml) during 15 min and then left to warm up to room temperature. The mixture was concentrated and, after the addition of water, the solution was extracted with ethyl acetate. Recovery gave a gum (12 mg) which would not crystallise. After p.l.c., the recovered gummy 3,4-*dihydro*-6,8-*dimethoxy*-3-(6-*methyl*-5-*oxotetrahydropyran*-2-*ylmethyl*)*isocoumarin* (II), R_F 0·60, characterised by the n.m.r. spectrum, was dried at 100° *in vacuo* over phosphorus pentoxide (Found: C, 63·0; H, 6·5%; *M*, 334. $C_{18}H_{22}O_6$,0·5-H_2O requires C, 63·0; H, 6·7%; *M*, 334), $\nu_{max.}$ 3400br, 1725, 1610, and 1585 cm^{-1}.

Conversion of the Ketone (II) *into Asperentin Dimethyl Ether*.—The ketone (12 mg) in chloroform (0·5 ml) was treated with ethanedithiol (0·06 ml) and boron trifluoride–ether (0·03 ml) and the mixture was set aside at room temperature for 4 days. It was then diluted with chloroform and washed with water. P.l.c. of the recovered gum gave three bands visible under u.v. light. The n.m.r. spectrum of the gummy material (6 mg) recovered from the upper band (R_F 0·7) was consistent with that expected for the *thioacetal* by showing all the signals present in asperentin dimethyl ether (see Table 1) together with a four-proton singlet at τ 6·70.

The thioacetal in ethanol (3 ml) was heated under reflux for 3 h with Raney nickel (W2, 200 mg). The filtered solution was evaporated to dryness and the residue was extracted with chloroform. P.l.c. of this extract gave two bands visible under u.v. light. The gum (3 mg) recovered from the upper band (R_F 0·60) crystallised from light petroleum in prisms, m.p. 115°, of asperentin dimethyl ether (I; $R^1 = R^2 = Me$) identified by the i.r. spectrum.

Reduction of the Ketone (II).—The ketone (10 mg) in methanol (0·5 ml) at 0° was treated with sodium borohydride (10 mg) and the solution was set aside at room temperature for 1 h. After most of the methanol had been removed under reduced pressure, water was added and the solution was neutralised with dilute acetic acid and extracted with ethyl acetate. The gummy product (11 mg) was purified by p.l.c. Recovery of a band R_F 0·20 gave a resin (9 mg) which crystallised from ethyl acetate in prisms (6 mg), m.p. 138°, of the alcohol (III; $R^1 = R^2 = Me$, $R^3 = OH$), identified by the i.r. spectrum.

(−)-*Mellein*, m.p. 57°, $[\alpha]_D^{22}$ −91° (*c* 0·06), showed c.d. maxima at 256 and 238 nm ($\Delta\varepsilon$ −4·51 and +1·95).

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