# New Metabolic Products of *Aspergillus Flavus*. Part IV.[1] 4'-Hydroxyasperentin and 5'-Hydroxyasperentin 8-Methyl Ether

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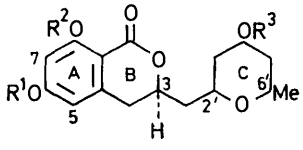
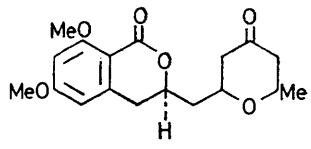
A previously isolated minor metabolic product of an entomogenous strain of *Aspergillus flavus* is shown to be a 4'-hydroxyasperentin [3,4-dihydro-6,8-dihydroxy-3-(4-hydroxy-6-methyltetrahydropyran-2-ylmethyl)isocoumarin (I; $R^1 = R^2 = R^3 = H$)]. Fermentation conditions are described for obtaining 5'-hydroxyasperentin (III; $R^1 = R^2 = H$, $R^3 = OH$) from this strain in increased yield. Under these conditions 5'-hydroxyasperentin 8-*O*-methyl ether (III; $R^1 = H$, $R^2 = Me$, $R^3 = OH$) is also produced and the known fungal anthraquinone pigments physcion (IV; $R^1 = Me$, $R^2 = H$) and questin (IV; $R^1 = H$, $R^2 = Me$) are obtained in place of the anthrone derivatives anhydroasperflavin (V) and asperflavin (VI).

In addition to asperentin (III; $R' = R^2 = R^3 = H$) and its mono-*O*-methyl ethers, three $C_{16}H_{20}O_6$ dihydroisocoumarins, m.p. 229—230, 210—212, and 195°, were isolated [2] from an entomogenous strain of *Aspergillus flavus* grown in surface culture on a chemically-defined medium for 3—4 weeks. The isomer with m.p. 229—230° was shown [2] to be a 5'-hydroxyasperentin (III; asperentin was produced at a late stage in the fermentation. Consistent with this conclusion it has now been found that when the fermentation time is extended to 5—6 weeks, the yield of 5'-hydroxyasperentin is increased at the expense both of asperentin and its *O*-methyl ethers and of 4'-hydroxyasperentin. Under these conditions a new member of the asperentin group,


(I)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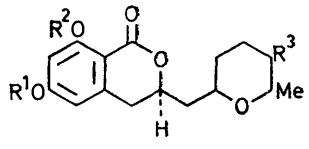
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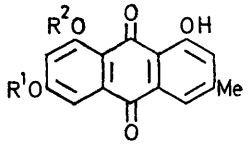
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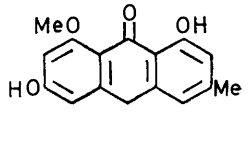
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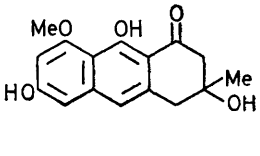
(VI)

$R^1 = R^2 = H$, $R^3 = OH$). The isomer with m.p. 195° is now shown to be a 4'-hydroxyasperentin (I; $R^1 = R^2 = R^3 = H$) on spectroscopic evidence, and by transformation to asperentin di-*O*-methyl ether (III; $R^1 = R^2 = Me$, $R^3 = H$) by the same reaction sequence used [2] to relate asperentin and 5'-hydroxyasperentin.

Biosynthetic studies [1] suggested that 5'-hydroxyasperentin was produced at a late stage in the fermentation. Consistent with this conclusion it has now been found that when the fermentation time is extended to 5—6 weeks, the yield of 5'-hydroxyasperentin is increased at the expense both of asperentin and its *O*-methyl ethers and of 4'-hydroxyasperentin. Under these conditions a new member of the asperentin group, 5'-hydroxyasperentin 8-*O*-methyl ether (III; $R^1 = H$, $R^2 = Me$, $R^3 = OH$) is produced in low yield and the commonly-occurring fungal anthraquinones physcion (IV; $R^1 = Me$, $R^2 = H$) [3] and questin (IV; $R^1 = H$, $R^2 = Me$) [4] are obtained in place of the anthrone derivatives anhydroasperflavin (V) and asperflavin (VI).[5]

Chemical shifts ($\tau$) for protons in 4'-hydroxyasperentin and related compounds

| Compound | Solvent | 5-H [a] | 7-H [a] | 3-H [b] | 4-$H_2$ [b] | 2'-H [b] | 4'-H [b] | 6'-H [b] | Me [c] | OMe | OAc |
|---|---|---|---|---|---|---|---|---|---|---|---|
| (I; $R^1 = R^2 = R^3 = H$) | $(CD_3)_2SO$ | 3·92 | 3·92 | 5·47 | 7·18 | 6·24 | 5·86 | 6·38 | 8·89 | | |
| (I; $R^1 = R^2 = R^3 = Ac$) | $CDCl_3$ | 3·00 | 3·10 | 5·30 | 7·08 | 5·60 | 4·95 | 6·10 | 8·76 | | 7·66, 7·72, 7·96 |
| (I; $R^1 = R^2 = Me$, $R^3 = H$) | $CDCl_3$ | 3·53 | 3·62 | ~5·47 | 7·09 | ~6·0 | ~5·47 | 6·24 | 8·78 | 6·03, 6·10 | |
| (II) | $CDCl_3$ | 3·55 | 3·66 | 5·40 | 7·10 | ~5·5 | | 5·68 | 8·75 | 6·01, 6·09 | |

[a] Singlet or doublet, $J_{5,7}$ 2 Hz. [b] Multiplet. [c] Doublet, $J$ 6·5 Hz.

5'-Hydroxyasperentin shows dimorphism and two crystalline forms each with a characteristic solid-state

[1] Part III, L. Cattel, J. F. Grove, and D. Shaw, *J.C.S. Perkin I*, 1973, 2626.
[2] J. F. Grove, *J.C.S. Perkin I*, 1972, 2400.
[3] J. N. Ashley, H. Raistrick, and T. Richards, *Biochem. J.*, 1939, **33**, 1291.
[4] A. Mahmoodian and C. E. Stickings, *Biochem. J.*, 1964, **92**, 369.
[5] J. F. Grove, *J.C.S. Perkin I*, 1972, 2406.

i.r. spectrum have been obtained. One of these forms, m.p. 210—212°, was previously [2] thought to be a stereoisomer of 5′-hydroxyasperentin.

Like 5′-hydroxyasperentin, the isomer, m.p. 195°, formed a triacetate (I; $R^1 = R^2 = R^3 = Ac$) and a di-*O*-methyl ether (I; $R^1 = R^2 = Me$, $R^3 = H$), $\nu_{max.}$ 3430 and 3200 $cm^{-1}$. Oxidation of the di-*O*-methyl ether at 0° in acetone with chromic oxide–sulphuric acid reagent [2] gave an amorphous ketone $C_{18}H_{22}O_6$ (II) which differed in its spectroscopic properties from the isomeric 5′-oxo compound.[2] In the n.m.r. spectrum the signals of four protons had been shifted downfield to τ 7·3—8·0 from their position (τ 8·2—8·6) in the spectrum of the parent hydroxy-compound. The chemical shifts of the 2′- and 6′-protons were affected equally, but the methyl resonance at τ 8·75 was unaffected (see Table). On the basis of this evidence the ketone was assigned the 4′-oxo- structure (II). Reduction with sodium borohydride in methanol regenerated the 4′-hydroxy-compound (I; $R^1 = R^2 =$ Me, $R^3 = H$), in which the 4′-substituent must be equatorial.

Reaction of the ketone (II) with ethanedithiol in the presence of boron trifluoride–ether gave the thioacetal which furnished asperentin di-*O*-methyl ether (II; $R^1 = R^2 = Me$, $R^3 = H$) on desulphurisation with Raney nickel. The $C_{16}H_{20}O_6$ compound with m.p. 195° is therefore the equatorial 4′-hydroxyasperentin (I; $R^1 = R^2 = R^3 = H$).

In an attempt to increase the yield of 5′-hydroxyasperentin, the fermentation was allowed to proceed for 5—6 weeks instead of for the 3—4 week period selected initially.[2] On chromatography of the acetone-soluble residue after removal of the major metabolite, asperentin 8-*O*-methyl ether, the silica gel column presented the same pattern of bands, but neither asperentin 6-*O*-methyl ether nor 4′-hydroxyasperentin could be detected in the appropriate fraction. Some asperentin was obtained, but the yield had fallen from 20 to $<1$ mg $l^{-1}$ and this fraction now contained questin (IV; $R^1 = H$, $R^2 = Me$). Asperflavin (V) was absent from the 5′-hydroxyasperentin fraction and the yield of the latter metabolite was increased from 7 to 21 mg $l^{-1}$. A new metabolic product, $C_{17}H_{22}O_6$, was obtained in low yield (*ca.* 1 mg $l^{-1}$) from the fraction that had previously contained 4′-hydroxyasperentin.

The u.v. spectrum ($\lambda_{max.}$ 265 and 298 nm) of the $C_{17}H_{22}O_6$ compound was consistent with the presence of a 6,8-dioxygenated dihydroisocoumarin chromophore. Its identity as an asperentin was suggested by the mass spectrum which showed prominent fragment ions at *m/e* 193, 165, and 164, all characteristic of a monomethylated dihydroxydihydroisocoumarin fragment, and at *m/e* 115 ($C_6H_{11}O_2^+$), characteristic of a hydroxylated methyltetrahydropyranyl ring C. The absence of any colour with iron(III) chloride and the presence in the i.r. spectrum of C=O absorption at 1675 $cm^{-1}$ [*cf.* (III; $R^1 = R^3 = H$, $R^2 = Me$), $\nu_{max.}$ 1680 $cm^{-1}$] suggested that the compound was an 8-*O*-methyl ether. Methylation with methyl iodide in acetone in the presence of potassium carbonate gave a methyl ether identical with 5′-hydroxyasperentin di-*O*-methyl ether (III; $R^1 = R^2 = Me$, $R^3 = OH$). It follows that the $C_{17}H_{22}O_6$ compound is 5′-hydroxyasperentin 8-*O*-methyl ether (III; $R^1 = H$, $R^2 = Me$, $R^3 = OH$).

## EXPERIMENTAL

M.p.s were taken on a Kofler hot-stage apparatus and are corrected. I.r. spectra were determined for mulls in Nujol and u.v. spectra 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 thin layer chromatography (t.l.c.). In preparative t.l.c. (p.l.c.) silica layers (Merck $HF_{254}$, 20 × 20 × 0·1 cm) were developed in chloroform–methanol (97 : 3).

*Fermentations with* A. Flavus.—The *A. flavus* strain was grown as previously described,[2] but the fermentations were harvested after 5—6 weeks. In a typical example, extraction of the culture filtrate (8·8 l) with chloroform furnished a resin (2·8 g) which yielded crude asperentin 8-*O*-methyl ether (1·2 g; 145 mg $l^{-1}$) on trituration with acetone. The residue was recovered and chromatographed [2] on a column of silica gel (Merck 7734; 45 g). The chromatogram showed a pattern of bands similar to that obtained from the 3-week fermentation.[2]

Elution of the first, diffuse, yellow band with benzene–methanol (98 : 2; 100 ml) gave a gum (26 mg) which crystallised from benzene in orange-red needles (4 mg), m.p. and mixed m.p. 207° (subl.), of physcion [1,8-dihydroxy-6-methoxy-3-methylanthraquinone (IV; $R^1 = Me$, $R^2 = H$)] (Found: C, 67·6; H, 4·4. Calc. for $C_{16}H_{12}O_5$: C, 67·6; H, 4·3%). The i.r. spectrum was identical with that of an authentic specimen. Asperentin 6-*O*-methyl ether was absent from the mother liquors (t.l.c.).

The brown band which followed was eluted with benzene–methanol (96 : 4; 100 ml). Concentration of the eluate afforded an orange-red powder (10 mg) which sublimed at 190° and decomposed (without melting) at 270°. It was identified as questin [1,6-dihydroxy-8-methoxy-3-methylanthraquinone (IV; $R^1 = H$, $R^2 = Me$)] by comparison of the i.r. spectrum with that of an authentic specimen.[5] The residue (145 mg) from the mother liquors, in benzene, slowly deposited crystals of asperentin (7 mg) after some weeks at room temperature.

Elution of the next, deep yellow band with benzene–methanol (94 : 6; 100 ml) gave a solid (333 mg) which crystallised from methanol in plates of asperentin 8-*O*-methyl ether (225 mg).

The column was further eluted with benzene–methanol (94 : 6) [fractions (i) 200 ml, 428 mg and (ii) 300 ml, 55 mg] and benzene–methanol (92 : 8) [fractions (iii) 100 ml, 82 mg and (iv) 100 ml, 3 mg]. Fractions (ii) and (iv) were intractable. Fraction (i) was crystallised from ethyl acetate giving 5′-hydroxyasperentin (181 mg; 21 mg $l^{-1}$), $\nu_{max.}$ 3530, 3200, 1645, 1620, and 1600 $cm^{-1}$. The filtrate contained no asperflavin (t.l.c.).[5] Recrystallisation of 5′-hydroxyasperentin from ethyl acetate sometimes gave a different crystalline form, $\nu_{max.}$ 3495, 3130, 1660, 1637, and 1590 $cm^{-1}$. The i.r. spectra of the two forms in solution in $Me_2SO$ were identical.

Fraction (iii) crystallised from ethyl acetate in prisms

(11 mg), m.p. 246—248° (decomp.) of *5′-hydroxyasperentin 8-O-methyl ether* (III; $R^1 = H$, $R^2 = Me$, $R^3 = OH$) (Found: C, 63·3; H, 7·0%; $M^+$, 322. $C_{17}H_{22}O_6$ requires C, 63·3; H, 6·9%; *M*, 322), $\nu_{max.}$ 3575, 3240, 1675, 1610, and 1585 $cm^{-1}$, $\lambda_{max.}$ 265 and 298 nm (ε 15,000 and 8600), $R_F$ 0·08 (relative to 5′-hydroxyasperentin 0·19) in chloroform–methanol (95 : 5). It gave no colour with iron(III) chloride in ethanol. Methylation with methyl iodide in acetone in the presence of potassium carbonate gave prisms, m.p. 137°, of the di-*O*-methyl ether (III; $R^1 = R^2 = Me$, $R^3 = OH$),[2] identified by the i.r. spectrum.

*4′-Hydroxyasperentin* [3,4-dihydro-6,8-dihydroxy-3-(4-hydroxy-6-methyltetrahydropyran-2-ylmethyl)isocoumarin] (I; $R^1 = R^2 = R^3 = H$), m.p. 195°, $R_F$ 0·17 in chloroform–methanol (95 : 5) was isolated as previously described.[2] On acetylation in pyridine with acetic anhydride in the usual way it formed a gummy *triacetate* (I; $R^1 = R^2 = R^3 = Ac$), purified by p.l.c. ($R_F$ 0·58) (Found: C, 61·1; H, 6·2. $C_{22}H_{26}O_9$ requires C, 60·8; H, 6·0%).

*Methylation of 4′-Hydroxyasperentin.*—4′-Hydroxyasperentin (20 mg) was heated under reflux in acetone with excess of methyl iodide in the presence of anhydrous potassium carbonate (40 mg) during 24 h. After working up in the usual way [2] the resulting gum (20 mg) was purified by p.l.c. The product (14 mg) from a band $R_F$ 0·10 crystallised from ethyl acetate in prisms, m.p. 172—174°, of the unsolvated (*cf.* ref. 2) *di-O-methyl ether* (I; $R^1 = R^2 = Me$, $R^3 = H$) (Found: C, 64·3; H, 7·2%; $M^+$, 336. $C_{18}H_{24}O_6$ requires C, 64·3; H, 7·2%, *M*, 336), $\nu_{max.}$ 3430, 3200br, 1705, 1610, and 1585 $cm^{-1}$. The i.r. spectrum differed from that of the solvated form.[2]

The product (6 mg) from a band $R_F$ 0·21 crystallised from ethyl acetate–light petroleum in prisms, m.p. 140—142°, of the *6-O-methyl ether* (I; $R^1 = Me$, $R^2 = R^3 = H$) (Found: C, 63·1; H, 7·0%; $M^+$, 322. $C_{17}H_{22}O_6$ requires C, 63·3; H, 6·9%; *M*, 322), $\nu_{max.}$ 3545, 3250br, 1660, and 1582 $cm^{-1}$.

*Oxidation of the Dimethyl Ether* (I; $R^1 = R^2 = Me$, $R^3 = H$).—The dimethyl ether (8 mg) in acetone (0·5 ml) at 0° was treated with 8N-chromic acid–sulphuric acid (0·03 ml) during 15 min and left to warm to room temperature. The mixture was concentrated *in vacuo* and, after the addition of water, was extracted with ethyl acetate. Purification by p.l.c. of the recovered gum gave the amorphous *3,4-dihydro-6,8-dimethoxy-3-(6-methyl-4-oxotetrahydropyran-2-ylmethyl)isocoumarin* (II) (Found: C, 64·6; H, 7·1%. $C_{18}H_{22}O_6$ requires C, 64·65; H, 6·6%), $\nu_{max.}$ OH absent, 1715br, 1608, and 1587 $cm^{-1}$, $R_F$ 0·34.

*Conversion of the Ketone* (II) *into Asperentin Di-O-methyl Ether.*—The ketone (II) (8 mg) in chloroform (0·5 ml) was treated with ethanedithiol (0·06 ml) and redistilled boron trifluoride–ether (0·03 ml) and the mixture was left at room temperature for 3 days. It was then diluted with chloroform and washed with water. P.l.c. of the recovered gum gave two bands visible under u.v. light. The n.m.r. spectrum of the gum (6 mg) recovered from the band of $R_F$ 0·51 was consistent with that expected for the thioacetal in that it was similar to that of asperentin di-*O*-methyl ether but showed, additionally, a four-proton singlet at τ 6·7 attributed to the $(-S{\cdot}CH_2-)_2$ protons.

The thioacetal in ethanol was heated under reflux with Raney nickel (W2) and the reaction mixture was worked up as described previously.[2] Only one band ($R_F$ 0·55) was seen on p.l.c. and the product (5 mg) crystallised from light petroleum in needles, m.p. 118°, of asperentin di-*O*-methyl ether (III; $R^1 = R^2 = Me$, $R^3 = H$), identified by the i.r. spectrum.

*Reduction of the Ketone* (II).—The ketone (3·5 mg) in methanol (0·5 ml) at 0° was treated with sodium borohydride (3 mg) in methanol (0·5 ml). After 10 min at 0° the solution was allowed to warm to room temperature during 30 min. It was then concentrated *in vacuo*, diluted with water, neutralised with dilute acetic acid, and extracted with ethyl acetate. P.l.c. of the semi-solid product (3·5 mg) gave a single band ($R_F$ 0·18) which yielded the di-*O*-methyl ether (I; $R^1 = R^2 = Me$, $R^3 = H$) as prisms (3 mg), m.p. 172° (from ethyl acetate), identified by the i.r. spectrum.

I thank Albert and Grete Olney for the microanalyses, Mr. B. A. J. Alexander for the n.m.r. spectra, Dr. F. A. Mellon for the mass spectra, and Messrs. N. Claydon, B. Dockerill, and M. Hosken for technical assistance.

[3/1465 *Received*, 12*th July*, 1973