New Metabolic Products of *Aspergillus flavus*. Part III.[1] Biosynthesis of Asperentin

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Incorporation of [2-^{14}C]malonate and [1-^{13}C]acetate by an entomogenous strain of *Aspergillus flavus* into the dihydroisocoumarin asperentin gives a labelling pattern consistent with its formation from a polyketide precursor assembled from one acetate- and seven malonate-derived two-carbon units. A technique for minimising the Overhauser enhancement in ^{13}C n.m.r. spectroscopy is described.

THE isolation and identification of a group of biologically active dihydroisocoumarins, the asperentins, from an entomogenous strain of *Aspergillus flavus* have been described.[2] Although the structure (II; $R^1 = R^2 = H$) of asperentin suggested[2] a straightforward biogenesis from eight two-carbon units *via* the acetate–poly-

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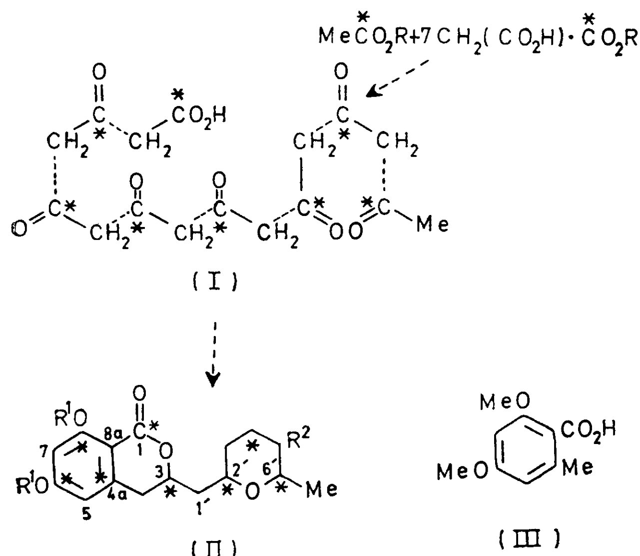
[1] Part II, J. F. Grove, *J.C.S. Perkin I*, 1972, 2406.
[2] J. F. Grove, *J.C.S. Perkin I*, 1972, 2400.

malonate pathway, the unexpected pattern of oxygenation in a co-metabolite, 5′-hydroxyasperentin (II; R^1 = H, R^2 = OH), made a more detailed investigation of its biosynthesis desirable. Labelling experiments with [2-^{14}C]malonate and [1-^{13}C]acetate have now confirmed the predicted simple biosynthetic pattern (Scheme 1) from a hypothetical polyketide precursor (I) made up of one acetate-derived chain initiating unit and seven malonate-derived two-carbon building units.



SCHEME 1 Biosynthesis of the asperentin ring system from acetate and malonate units; labelling pattern expected from the incorporation of $Me^{13}CO_2Na$.

The use of ^{13}C n.m.r. spectroscopy in biosynthetic studies of this type is now well established,[3-5] but it has usually been confined to examples where a 5—10-fold enrichment has been attained by labelling experiments with [^{13}C]acetate. In the present work, although [1-^{13}C]acetate was added to the fermentation at the time of maximum rate of production of the asperentins, as determined by spectrophotometric assay,[2] preliminary examination by standard Fourier Transform (FT) n.m.r. procedures of the [^{13}C]asperentin dimethyl ether (II; R^1 = Me, R^2 = H), obtained by methylation[2] of the labelled major metabolite (IV), indicated that only an approximately 2-fold ^{13}C enrichment had been achieved. This enrichment is well within the range of the theoretical Overhauser enhancement and a modification of the FT n.m.r. technique was therefore investigated.

Quantitative integrals of ^{13}C spectra are not in general very reliable. This lack of reliability comes from two basic effects; first, from the Overhauser effect, and secondly from the possible use of too high a mean radio frequency (RF) power, which gives rise to effects equivalent to saturation. Both these effects are related to relaxation times, and result in peak intensities which are a function of the individual carbon atoms' spin-lattice relaxation times (t_1).

The Overhauser effect, which arises from competition between the proton dipolar mechanism of carbon relaxation and other mechanisms, is effectively eliminated by using a gated decoupling technique.[6] The Overhauser effect, if not switched off in this way, varies from carbon atom to carbon atom.[7a] This improved technique still leaves the problem associated with RF power. This too can be eliminated if a long delay is left between RF pulses in the Fourier experiment. The delay should be about four times the longest relaxation time of any nucleus in the molecule if a 1 : 1 intensity is to be expected for all carbon atoms.

Such an approach was not completely practicable in the present work; in asperentin some relaxation times are very long, *e.g.* that of C-1, and the time required between pulses would have been several minutes. In practice, therefore, constant conditions falling well short of this ideal were used to examine asperentin dimethyl ether at natural abundance and enriched with ^{13}C; although these conditions did not give unit areas for each carbon atom, the ratio between values for any two carbon atoms in the natural and enriched compounds was of sufficient accuracy for the work involved.

Integral peak intensities for individual carbon atoms of the enriched material, normalised with respect to the average of the intensities found for the carbon atoms of the OMe groups, are compared in Table 1 with those

TABLE 1

^{13}C N.m.r. spectra of asperentin dimethyl ether (II; R^1 = Me, R^2 = H) of natural abundance and enriched by incorporation of $Me^{13}CO_2Na$

Position	δC (p.p.m. from Me_4Si) Found	Calc. [b]	Normalised intensities [a] Natural Abundance	Enriched	Enrichment Ratio
*4′	21·1		0·87	2·48	2·86
Me(7′)	21·8		1·07	1·26	1·18
3′,5′	33·8		1·13	1·38	1·23
1′	38·2		0·82	1·05	1·27
4	42·1		0·75	1·08	1·43
OMe	58·3		0·94	0·96	1·03
OMe	58·9		1·06	1·04	0·97
*6′	69·0		1·09	2·91	2·66
*2′	70·0		1·10	2·88	2·62
*3	77·1		0·86	2·70	3·14
7	100·4	97·8	0·90	1·06	1·19
5	106·7	107·1	0·94	1·11	1·18
8a	109·7	108·8	0·38	0·33	0·86
*4a	146·6	140·9	0·54 [c]	1·19	2·21
*1	165·3		0·24	0·58	2·30
*8	165·6	162·3	0·53 [c]	1·06	2·00
*6	167·0	165·9	0·58 [c]	1·33	2·29

[a] See text. [b] For the acid (III). [c] Respectively 0·74, 0·93, and 0·90 when gated decoupling was used.

[3] M. Tanabe, H. Seto, and L. Johnson, *J. Amer. Chem. Soc.*, 1970, **92**, 2157.
[4] M. Tanabe, T. Hamasaki, H. Seto, and L. Johnson, *Chem. Comm.*, 1970, 1539.
[5] A. G. McInnes, D. G. Smith, L. C. Vining, and L. Johnson, *Chem. Comm.*, 1971, 325.
[6] D. Shaw, in 'Chemical Society Specialist Periodical Reports, N.M.R., volume 2,' ed. R. K. Harris, 1973, p. 283, and references therein.
[7] G. C. Levy and G. L. Nelson, 'Carbon-13 Nuclear Magnetic Resonance for Organic Chemists,' Wiley, New York, 1972, (*a*) p. 30; (*b*) p. 81.

for material examined at natural abundance. The average intensity of the signals from the quaternary carbon atoms at positions 4a, 8, and 6 was only 0·56 of that from the eleven non-quaternary carbon atoms The signal intensities from C-8a and C-1, where relatively long spin-lattice relaxation times are to be expected, were considerably lower. When gated decoupling was used the intensities of the signals from carbon atoms 4a, 8, and 6 averaged 0·86 of those from the non-quaternary carbon atoms, but the signal intensities from C-8a and C-1 were not significantly affected.

The remaining signals were assigned on the basis of their chemical shifts and from the results of a $^{13}C-^{1}H$ off-resonance decoupling experiment. Quartets centered at 21·8, 58·3, and 58·9 p.p.m. were assigned to the 7′-methyl group and the two OMe groups, respectively; triplets at 21·1, 33·8, 38·2, and 42·1 p.p.m. to CH_2 groups at positions 4′, 3′ and 5′, 1′, and 4, respectively; and doublets at 69·0, 70·0, 77·1, 100·4, and 106·7 p.p.m. to CH groups at positions 6′, 2′, 3, 7, and 5, respectively. The chemical shifts of the aromatic ring carbon atoms were in close agreement with those calculated for the model compound (III) from the substituent effects tabulated by Levy and Nelson,[7b] and were assigned accordingly.

Carbon atoms 4′, 6′, 2′, 3, 4a, 1, 8, and 6 in the dimethyl ether (II; R^1 = Me, R^2 = H) derived from the [1-^{13}C]acetate fermentation showed a significantly greater enrichment ratio ($2·5 \pm 0·6$) than the remaining eight carbon atoms ($1·2 \pm 0·3$). These atoms also showed a small but possibly significant enrichment, arising from randomisation of label, as compared with the OMe groups.

This labelling pattern is consistent with the biogenesis of asperentin from a polyketide precursor made up of initiating unit from the remainder. This identification was achieved in the conventional way[8] by the addition of [2-^{14}C]malonate to the fermentation followed by degradation (Scheme 2) of the radio-labelled product. The major metabolite (IV) was converted by methylation and oxidation with permanganate into the [^{14}C]-phthalonic acid (V) giving malonate-derived carbon atoms 4, 5, 7, and 8a. In this preparation the previously reported[2] simultaneous formation of 3,5-dimethoxybenzoic acid was avoided by concentrating the oxidation liquor *in vacuo* before acidification. Kuhn-Roth oxidation[9] of the [^{14}C]asperentin methyl ether (IV) gave the carbon atoms of the chain-initiating unit as acetic acid (VII), which was isolated as the [^{14}C]-*p*-bromophenacyl ester (VI) and degraded by the Schmidt procedure[10] to barium carbonates (VIII) and (IX).

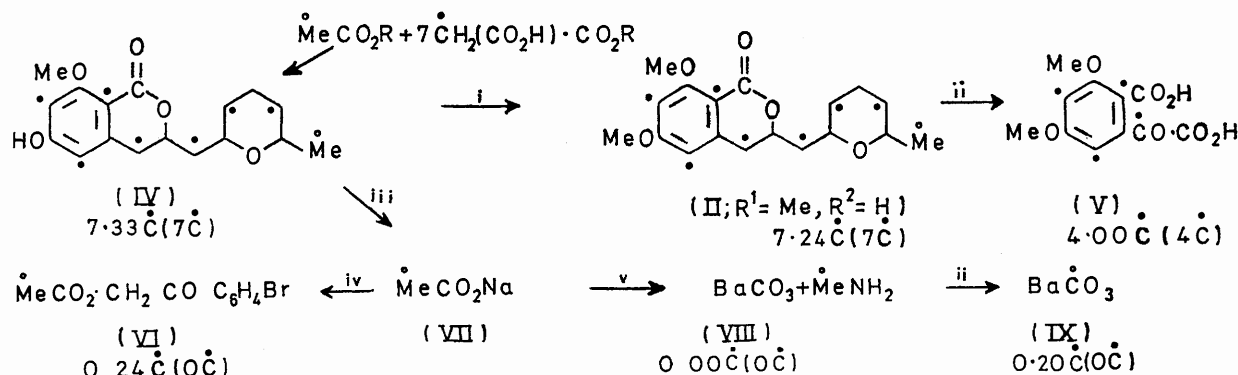
TABLE 2

Radioactivity of degradation products of [^{14}C]-asperentin 8-methyl ether labelled with [2-^{14}C]malonate

Compound	C atoms	R.m.a.* $\times 10^{-3}$
Asperentin 8-methyl ether (IV)	All	231
Asperentin dimethyl ether (II; R^1 = Me, R^2 = H)	All	228
2,4-Dimethoxy-6-oxalobenzoic acid hydrate (V)	4,5,7,8a	126
p-Bromophenacyl acetate (VI)	6′,7′	7·6
Barium carbonate (VIII)	6′	0
Barium carbonate (IX)	7′	6·3
Malonate-derived two-carbon unit (calc.)†		31·5

* Relative molar activity. † Radioactivity of (V) divided by four.

The radioactivities of the degradation products are recorded in Table 2 and Scheme 2. They show that the acetate-derived chain-initiating unit contains 24% of the label incorporated into each of the malonate-derived



SCHEME 2 Degradation of [^{14}C]asperentin labelled by $^{14}CH_2(CO_2Et)_2$. An open circle indicates a carbon atom derived from the methyl group of acetate. Reagents: i, MeI–K_2CO_3; ii, $KMnO_4$; iii, CrO_3; iv, *p*-$BrC_6H_4·CO·CH_2Br$; v, NaN_3

eight two-carbon units; in particular, the C-5′ is derived from a CH_2 group of the precursor, and hydroxylation at the 5′-position to give (II; R^1 = H, R^2 = OH) probably occurs at a late stage in the biosynthetic pathway.

The ^{13}C n.m.r. technique is not sufficiently accurate to distinguish, with certainty, the acetate-derived chain- two-carbon building units, as calculated from the activity of the acid (V) which was assumed to contain four such labelled units. In this fermentation, although

[8] J. F. Grove, *J. Chem. Soc. (C)*, 1970, 1860.
[9] R. Belcher and A. C. Godbert, 'Semimicro Quantitative Organic Analysis,' 2nd edn., Longman and Green, London, 1954, p. 160.
[10] E. F. Phares, *Arch. Biochem. Biophys.*, 1951, **33**, 173.

some decarboxylation of malonate takes place prior to its incorporation into asperentin, the amount is small compared with that observed in some other studies [11,12] of this phenomenon made since the classical work of Bentley.[13]

EXPERIMENTAL

M.p.s are corrected.

^{13}C FT N.m.r. spectra were obtained at 25·2 MHz using a Varian XL-100-12 spectrometer equipped with FT-100X Fourier Transform accessory and a 16K 620L computer. Samples (*ca.* 100 mg) were dissolved in deuteriochloroform (0·25 ml). The deuterium signal from the solvent was used to maintain the field frequency lock. Normal FT spectra were run (3000 scans) with use of proton noise decoupling; off-resonance decoupling was used to assist with the assignments. In the gated decoupling experiments (500 scans), the acquisition time (0·8 s) was followed by a pulse delay (5 s) during which time the decoupler was switched off. Tetramethylsilane was used as internal reference for chemical shift measurements.

^{14}C *Radioactivity Assays.*—^{14}C-Labelled organic compounds were recrystallised to constant radioactivity, measured [1 mg sample in a mixture of cellosolve (1 ml.) and the xylene-based scintillator KL355 (Koch-Light) (9 ml)] on a Packard Tri-Carb 3375 scintillation counter, calibrated by the external standard channel-ratio method. The product of counts min^{-1} mg^{-1} and the molecular weight gave the relative molar activity (r.m.a.). Barium [^{14}C]carbonate was decomposed as described later and an aliquot sample (1 ml) of the ethanolamine absorbent was used in place of the cellosolve in the counting fluid.

[^{14}C]- *and* [^{13}C]-*Asperentin* 8-*Methyl Ether* (IV).—The *A. flavus* strain was cultured in conical flasks (1 l) as described previously [2] and the production of asperentins was followed by the spectrophotometric assay. On about the fourteenth day after inoculation either (i) diethyl [2-^{14}C]malonate (0·1 mCi) in ethanol (3 ml) or (ii) sodium [1-^{13}C]acetate (0·5 g, 63% enrichment) in water (6 ml) was distributed equally under sterile conditions among (i) three or (ii) six flasks. The culture was harvested 4—5 days later and the labelled asperentin 8-methyl ether [(i) 30 mg, 0·6% incorporation; (ii) 110 mg] was isolated and purified by recrystallisation from methanol. For degradation the [^{14}C]ether was diluted with unlabelled material (900 mg).

[^{14}C]- *and* [^{13}C]-*Asperentin Dimethyl Ether* (II; R^1 = Me, R^2 = H).—Labelled asperentin 8-methyl ether was heated under reflux with methyl iodide in acetone in the presence of anhydrous potassium carbonate [2] and the resulting dimethyl ether, m.p. 118°, was recrystallised from light petroleum (b.p. 60—80°).

[^{14}C]-2,4-*Dimethoxy*-6-*oxalobenzoic Acid* (V).—[^{14}C]Asperentin dimethyl ether (90 mg) was oxidised with alkaline potassium permanganate.[2] The precipitate of manganese dioxide was removed and the combined filtrate and washings were concentrated to small bulk *in vacuo* at 50° and acidified with concentrated hydrochloric acid. After further concentration *in vacuo*, the hydrate (28 mg), m.p. 168—170°, of the acid (V) was isolated and recrystallised from hot water.

Kuhn-Roth Oxidation of [^{14}C]*Asperentin* 8-*Methyl Ether and Schmidt Degradation of the Resulting Sodium* [^{14}C]-*Acetate.*—The finely powdered ether (45 mg) was oxidised under standard conditions [9] and the distillate (35 ml) was titrated potentiometrically with 0·01N-sodium hydroxide (uptake 14·1 ml, equivalant to 0·95 mol of acetic acid). The neutral solution was divided into two equal parts which were separately freeze-dried.

The sodium [^{14}C]acetate from one portion was heated under reflux for 1 h with *p*-bromophenacyl bromide (15 mg) in ethanol (95%; 2 ml). The crude ester (20 mg) which separated on cooling was purified (R_F 0·7) by preparative t.l.c. in chloroform on silica gel G_{254} (Merck) and crystallised from aqueous ethanol, giving prisms, m.p. 84—85° of [^{14}C]-*p*-bromophenacyl acetate (VI), identified by i.r. spectrum and mixed m.p.

The sodium [^{14}C]acetate from the second portion was subjected to Schmidt degradation.[10] The [^{14}C]carbon dioxide obtained from the decarboxylation and methylamine oxidation steps was trapped as barium carbonate (13 and 9 mg, respectively) which was collected in the usual way. An accurately measured weight of the barium [^{14}C]carbonate was decomposed in concentrated sulphuric acid in a slow stream of nitrogen and the [^{14}C]carbon dioxide liberated was passed into ethanolamine (6 ml). When the reaction was complete (30 min) an aliquot sample (1 ml) of the ethanolamine solution was removed for assay of radioactivity.

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[11] A. J. Birch, S. F. Hussain, and R. W. Rickards, *J. Chem. Soc.*, 1964, 3494.

[12] J. R. Hadfield, J. S. E. Holker, and D. N. Stanway, *J. Chem. Soc.* (*C*), 1967, 731.

[13] R. Bentley and J. G. Keil, *Proc. Chem. Soc.*, 1961, 111.