Biosynthesis of Fungal Metabolites: Asperlactone and its Relationship to Other Metabolites of *Aspergillus melleus*

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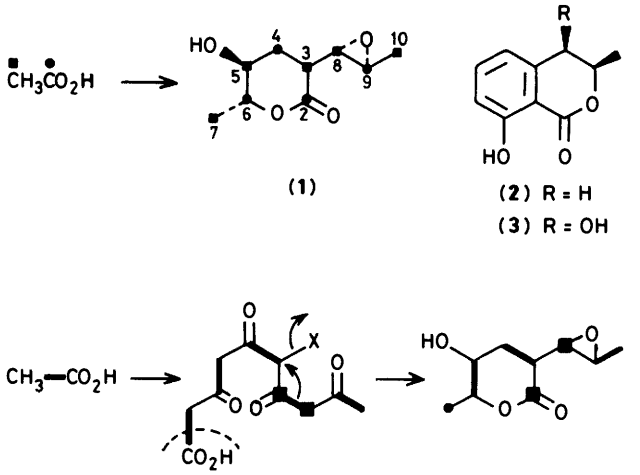
The biosynthesis of asperlactone (5) from [1,2-$^{13}C_2$]-, [2-^{13}C, 2-$^{2}H_3$]- and [$^{2}H_3$]-acetate has been investigated. The pattern of incorporation of intact acetate units was checked with [1,2-$^{13}C_2$]acetate. The presence in the ^{13}C n.m.r. spectrum of a two-bond coupling (J 7.5 Hz) between C-2 and C-8 was verified by sine bell resolution enhancement of the Free Induction Decay. Therefore these two carbons, originally joined in a C_2 unit derived from acetate, become separated by a rearrangement, and so finish in a 1,3 relationship. The carbon skeleton is therefore built up in identical fashion to that of the co-metabolite aspyrone (1). The retention of two acetate-derived hydrogens from [2-^{13}C, 2-$^{2}H_3$]acetate at C-7 rules out the intermediacy of structures in which this carbon forms part of an aromatic ring. This experiment also confirms that C-10 can retain three deuterium atoms and so is a chain starter methyl group. The overall retention of acetate hydrogen was determined by a ^{2}H n.m.r. study of [2-$^{2}H_3$]acetate-enriched asperlactone. To account for these results a biosynthetic model involving partially reduced polyketone intermediates is suggested (Scheme 4). Mechanistically feasible decarboxylation and rearrangement steps are followed by stereospecific opening of an epoxide ring to generate aspyrone (1) or asperlactone (5). The possible derivation of the aromatic co-metabolites, mellein (2) and hydroxymellein (3), from the same intermediate (10), generated on the same polyketide synthase, is discussed.

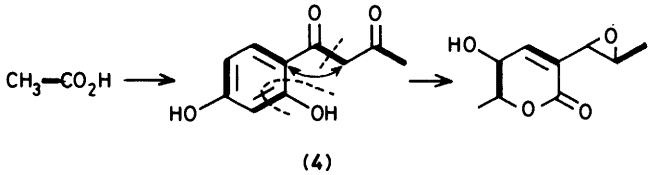
The biogenesis of aspyrone (1), the major product of short-term fermentation in *Aspergillus melleus* has aroused much interest, since the unusual branched chain carbon skeleton cannot arise from conventional polyketide transformations; the co-metabolites, mellein (2) and hydroxymellein (3) can, however, originate from standard cyclisation of a linear polyketone.[1] The fungus has therefore been the subject of several biosynthetic studies [2-4] with [^{14}C]- and [^{13}C]-labelled precursors. The acetate origin of aspyrone has been verified,[2,3] and the pattern of intact acetate units determined through the incorporation of [1,2-$^{13}C_2$]acetate.[3,4] In these experiments, a 6 Hz coupling in the ^{13}C n.m.r. spectrum between the resonances for C-2 and C-8 indicated that these two carbons derive from the same acetate unit. It was therefore suggested that the biosynthesis proceeds *via* Favorski-type rearrangement of a linear polyketone chain (Scheme 1). These labelling results do not, however, exclude the intervention of aromatic intermediates. The tritium labelling results discussed in the accompanying paper [2] exclude the direct involvement of mellein in aspyrone biosynthesis but accommodate the intermediacy of (4) or a related structure as shown in Scheme 2.

The isolation of asperlactone (5) and isoasperlactone (6) renewed our interest in the biosynthesis of *A. melleus* metabolites. From the outset, we assumed that aspyrone and the γ-lactone metabolites were biosynthetically related and we felt that the stereochemical differences between aspyrone and asperlactone might provide useful pointers towards the nature of specific intermediates. The recent development of biosynthetic methodology for tracing the fate of hydrogen through biosynthetic pathways enables the two proposed biosynthetic schemes to be differentiated and in this paper, we report incorporation studies with [^{13}C]- and [^{2}H]-labelled acetates which clarify details of asperlactone biosynthesis.[5,6] In the light of these and the stereochemical studies presented in the accompanying paper,[7] the biosynthetic relationship of the various metabolites of *A. melleus* is reassessed.

Incorporation of [^{13}C]-*Labelled Acetates.*—Firstly, it was necessary to confirm that the biosynthesis of the carbon skeleton of asperlactone does not differ from that of aspyrone, and in particular to check that C-2 and C-8 derive from the same acetate unit. An incorporation study with [1,2-^{13}C]-acetate was therefore carried out. Trial experiments with



Scheme 1.



(4)

Scheme 2.

[1-^{14}C]acetate established that the fungus incorporated precursors at levels suitable for detailed ^{13}C incorporation studies. The assignment of the ^{13}C n.m.r. spectrum of (5) is discussed in depth in the accompanying paper.[7] relevant chemical shift data are presented in Table 1.

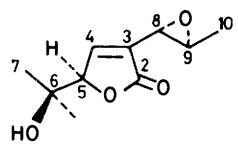
The p.n.d. ^{13}C n.m.r. spectrum of asperlactone enriched with [1,2-$^{13}C_2$]acetate is shown in Figure 1.[5] The resonances for C-3, C-4, C-5, C-6, C-9, and C-10 each show a sharp singlet flanked by two weaker satellites resulting from one-bond ^{13}C–^{13}C couplings. For each carbon, a fourth peak

corresponds to the small amount (*ca.* 10%) of isoasperlactone present in the sample which it was not practicable, nor necessary, to remove for the purpose of this biosynthetic study. The resonance for C-7 is clearly a singlet but those for C-2 and C-8 are more complex. In an expansion (Figure 1a) of the resonance for C-2, there are clearly two satellites centred on the strong singlet in addition to the isoasperlactone peak (marked X): their separation of *ca.* 7 Hz is consistent with a two-bond coupling between C-2 and another carbon, presumably C-8 although the equivalent expansion (Figure 1b) does not provide satisfactory complementary evidence: the lines are broader owing to the shorter T_2^* relaxation time of this non-quaternary carbon.

The resolution of the resonances arising from C-8 was improved using sophisticated computational methods for the optimisation of Fourier Transform n.m.r. data; full details of this procedure have been published elsewhere.[8] The Free Induction Decay (F.I.D.) was transferred from a Varian XL100 620 mini-computer to an IBM 370/165 main frame computer and Fourier transformed in the normal manner. The region of the spectrum between 41.6 and 54.0 p.p.m. was inversely transformed using floating point arithmetic, multiplied by a sine wave, $f(t) = \sin(0.25 \times 360 \times t + 15)$ (sine bell resolution enhancement, where t is the time in seconds and angles are in degrees), to change line shape, and then zero-filled to 6.55 s to increase digital resolution.

Fourier transformation of the manipulated F.I.D. gave the result shown in Figure 1c where four lines are clearly visible. The two satellites centred on the strong singlet (in addition to the isomer peak) are separated by 7.46 ± 0.15 Hz. The region of the spectrum corresponding to C-2 was inverse-transformed and zero-filled to increase digitisation, but no sine bell resolution enhancement was necessary for this carbon. The separation of the satellites for this signal was found to be 7.45 ± 0.15 Hz, within the limits of digitisation identical with that for C-8.

The two-bond coupling between C-2 and C-8 could arise from coupling between carbons of adjacent C_2 units;[9] this is unlikely since no other similar couplings are visible in the spectrum. Also the relative intensity of the two-bond coupling is clearly similar to that of the normal one-bond coupling in other C_2-units. The observed coupling is thus of genuine biosynthetic origin and establishes that C-2 and C-8 originate from the same acetate unit. This n.m.r. study provides the first firm evidence, in the form of a complementary pattern of satellites for both carbons, that a rearrangement occurs during the biosynthesis such that these two carbons originate from the same C_2 unit, and that they finish in a 1,3-relationship. Similar rearrangements involving 1,2-migrations have



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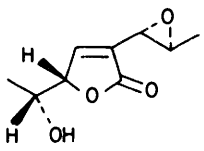

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Table. ^{13}C N.m.r. data for asperlactone (5)

Carbon	Species	Chemical shift [a]	Isotopic shift (p.p.m.)	$J(^{13}C-{}^{1}H)$ (Hz)	$J(^{13}C-{}^{2}H)$ (Hz)
2	—	171 (s)	—	—	—
3 [b]	—	133 (s)	—	—	—
4	—	147 (d)	—	175	—
5	^{13}CH	84.20 (d)	—	151	—
	^{13}CD	84.85	0.35	—	23
6	—	68.0 (d)	—	143	—
7	$^{13}CH_3$	18.80 (q)	—	128	—
	$^{13}CH_2D$	18.55	0.27	128	19
	$^{13}CHD_2$	18.24	0.56	128	19
8 [b]	—	52 (d)	—	182	—
9	—	58 (d)	—	176	—
10	$^{13}CH_3$	17.55 (q)	—	127	—
	$^{13}CH_2D$	17.23	0.32	127	19
	$^{13}CHD_2$	16.90	0.65	127	19
	$^{13}CD_3$	16.62	0.87	—	19

[a] P.p.m. relative to $SiMe_4$. [b] $J(^{13}C-{}^{13}C)$ 64 Hz.

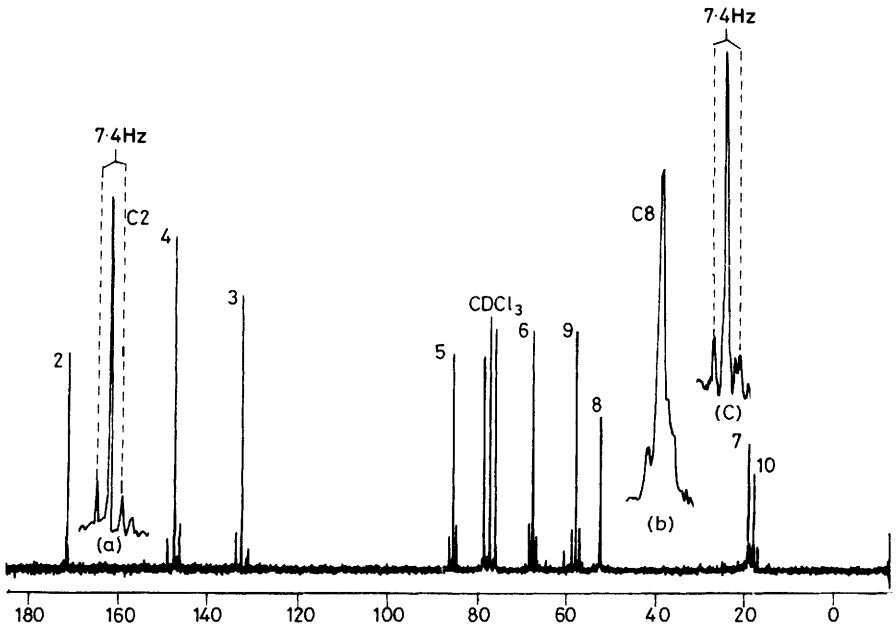
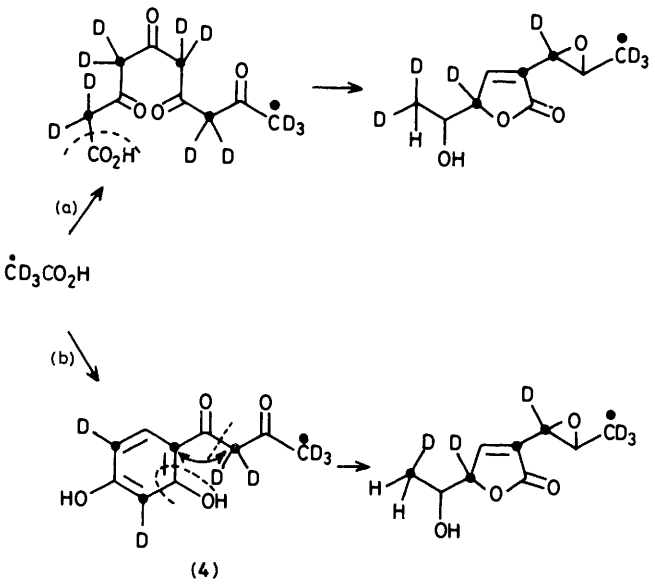


Figure 1. 25.2 MHz P.N.D. ^{13}C N.m.r. spectrum in $CDCl_3$ of asperlactone (5) enriched by [1,2-$^{13}C_2$]acetate; (a) and (b) normal expansions of resonances for C-2 and C-8, respectively; (c) resolution enhanced expansion of the resonance for C-8

been invoked in the biosynthesis of vulgamycin,[10] colletotrichins,[11] aflatoxins,[12] and a tetrahydrofuran metabolite (7).[13]

Incorporation of [^{2}H]-*Labelled Acetates.*—We next required evidence in favour of one of the two biosynthetic pathways illustrated in Schemes 1 and 2. As shown in Scheme 3, these can be differentiated, since in pathway (a) up to two acetate-derived hydrogens can be retained at C-7, whereas in pathway (b), owing to the intervention of the aromatic intermediate (4), only one acetate-derived hydrogen can be retained. Exchange processes during early biosynthetic steps will lead to the loss of acetate-derived hydrogen but, as long as sufficient molecules retain the maximum number of hydrogens at the positions of interest, useful information can be obtained. For this the labelling pattern of individual molecules is thus required. Several n.m.r. methods for tracing hydrogen through biosynthetic pathways have been developed,[14] based on either direct detection (^{3}H or ^{2}H n.m.r. spectroscopy), or indirect detection (for ^{2}H) by ^{13}C n.m.r. spectroscopy. The use of



Scheme 3.

^{2}H n.m.r. does not normally allow the labelling pattern of individual molecules to be assigned. In the indirect technique using [2-^{13}C, 2-^{2}H$_3$]acetate, the tracer deuterium is detected through its effect on the signal of an adjacent ^{13}C atom and thus the detection of low intensity multiplets around the normal ^{13}C resonance is required. The sensitivity of detection is low, but can be improved by simultaneous (^{2}H, ^{1}H)-decoupling. In a recent modification of the indirect approach using [1-^{13}C, 2-^{2}H$_3$]acetate,[15] deuterium is detected through its effect on a non-adjacent carbon atom (the beta-shift) and the difficulties of low intensity and signal multiplicity are circumvented. However this technique can only be used in cases where the carboxy partner is retained. In asperlactone, the key centre of interest is C-7 which, as was established from the [1,2-^{13}C$_2$]acetate study, has lost its carboxy partner. The use of [^{13}CD$_3$]-labelled acetate was therefore mandatory. Asperlactone (5) was preferred to aspyrone (1) for this experiment, since the chemical-shift separation between the two methyls, C-7 and C-10, is greater than in aspyrone, and so there is less signal overlap.

In the 25.2 MHz p.n.d. ^{13}C n.m.r. spectrum of (5) derived from [2-^{13}C, 2-^{2}H$_3$]acetate (Figure 2),[6] the signal for C-3 is enriched four-fold over natural abundance. The signals for C-5, C-7, C-8, and C-10 although enriched, are less intense than expected, consistent with the presence of some deuterium at these positions. The region of the spectrum containing C-7 and C-10 is complicated; expansions at 25 and 100 MHz are shown in Figure 3. The strong singlets at δ 18.50 and 17.20 are the normal ^{13}CH$_3$ signals for C-7 and C-10 respectively. A triplet (*J* 19 Hz) centred at δ 18.22, 0.28 p.p.m. upfield of the main signal, and a quintet (*J* 19 Hz) centred at δ 17.96, 0.54 p.p.m. upfield, are assigned to molecules labelled as ^{13}CH$_2$D and ^{13}CHD$_2$ respectively at C-7 (the isotopic shift is expected to be *ca.* 0.3 p.p.m. per attached deuterium, and the carbon-deuterium coupling constant approximately one-sixth as large as the corresponding carbon-proton value). A signal at δ 16.48 may be the central resonance of a ^{13}CD$_3$ heptet for C-10 since it is 0.72 p.p.m. upfield of the normal signal for this carbon. Parts of the multiplets corresponding to ^{13}CHD$_2$ and ^{13}CH$_2$D are also visible. A ^{13}C–^{2}H triplet (*J* 23 Hz), centred 0.37 p.p.m. upfield of the natural abundance signal at δ 85.25, provides evidence for deuterium retention at C-5. The resonance for C-8 of asperlactone shows no direct evidence in

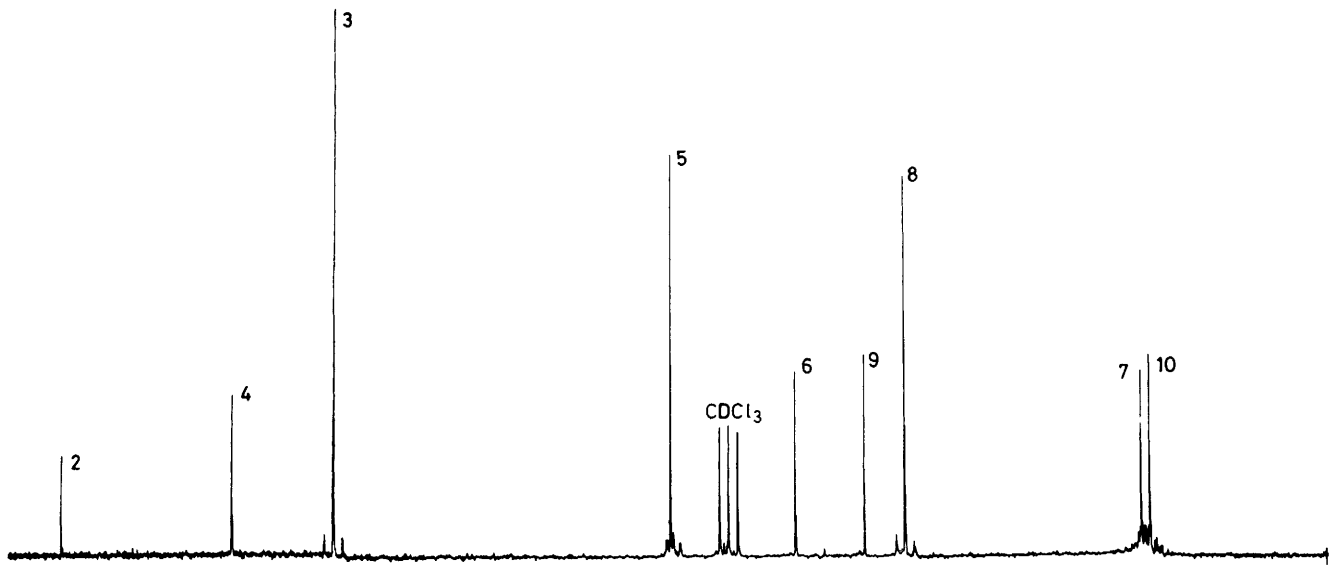


Figure 2. 25.2 MHz P.N.D. ^{13}C n.m.r. spectrum in $CDCl_3$ of asperlactone (5) enriched by [2-^{13}C,2-^{2}H$_3$]acetate

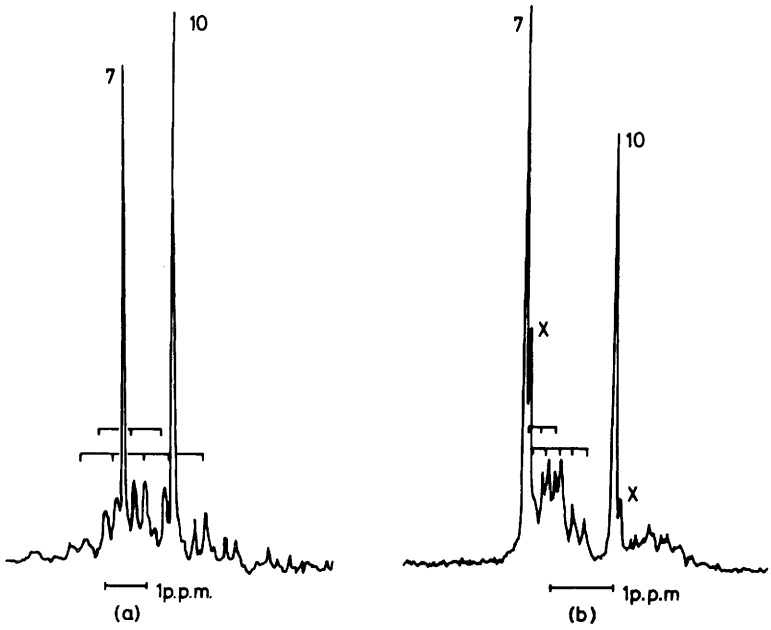


Figure 3. Methyl region (C-7 + C-10) of the P.N.D. ^{13}C N.m.r. spectra in $CDCl_3$ of asperlactone (5) enriched by [2-^{13}C,2-$^{2}H_3$]-acetate, (a) at 25.2 MHz, (b) at 100 MHz

this spectrum for deuterium retention at that site apart from slightly reduced signal intensity. Along with that for C-3, this resonance possesses flanking satellites (*J* 64 Hz) resulting from the presence of ^{13}C at the adjacent carbon in the same molecule.

Confirmation of these results was obtained by re-running the spectrum of [2-^{13}C, 2-$^{2}H_3$]acetate-enriched asperlactone firstly with deuterium decoupling, and secondly with simultaneous deuterium and proton decoupling. The expansions of the methyl region for these two spectra are shown in Figure 4. The deuterium-decoupled spectrum (Figure 4a) is complicated; a strong singlet at δ 16.62 clearly corresponds to molecules containing three deuteriums at C-10; weaker signals correspond to molecules labelled as $^{13}CHD_2$ and $^{13}CH_2D$. For C-7, a strong doublet (*J* 128 Hz) centred at δ 18.25 identifies molecules containing two deuteriums. A triplet and a quartet at δ 18.52 and 18.81, both with *J* 128 Hz, result from molecules labelled as $^{13}CH_2D$ and $^{13}CH_3$ respectively. In the simultaneously decoupled spectrum (Figure 4b), five strong signals are clearly visible, and are assigned as follows: δ 18.59 (7-$^{13}CH_3$), 18.27 (7-$^{13}CH_2D$), 18.08 (7-$^{13}CHD_2$), 17.23 (10-$^{13}CH_3$), and 16.48 (10-$^{13}CD_3$). The weak signals between the last two resonances are consistent with the presence of molecules labelled as $^{13}CH_2D$ and $^{13}CHD_2$ at C-10. The slight changes in chemical-shift values in the various spectra result from the alterations to sample concentration or composition (see Experimental section).

In the deuterium-decoupled spectrum, the signal for C-5 consists of a doublet (*J* 151 Hz) centred at δ 85.20 (^{13}C–H) and a singlet 0.35 p.p.m. upfield (^{13}C–D). Under conditions of simultaneous decoupling, two singlets at δ 85.20 and 84.90 correspond to molecules containing hydrogen and deuterium respectively.

Subsequently, the enriched asperlactone sample was degraded to 1-methylglycerol (8), and purified as the tribenzoate (9) in order to separate the two key methyls. The p.n.d. ^{13}C n.m.r. of this sample at 25 MHz shows a strong singlet at δ 16.62 assigned to molecules containing hydrogen at the C-1 methyl group. In addition, a triplet (*J* 20 Hz) and a quintet (*J* 20 Hz), 0.27 and 0.60 p.p.m. upfield respectively are

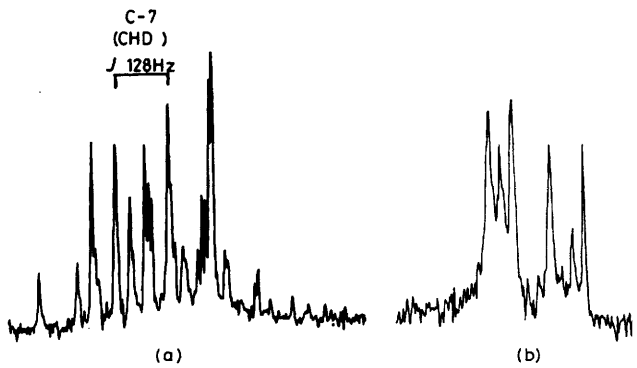


Figure 4. Methyl region (C-7 + C-10) of the 100 MHz ^{13}C n.m.r. spectrum in $CHCl_3$ of asperlactone (5) enriched by [2-^{13}C,2-$^{2}H_3$]-acetate, (a) with ^{2}H-decoupling, (b) with ^{1}H,^{2}H-decoupling

assigned to molecules containing one and two deuteriums respectively at this position. A peak at δ 73.22 assigned to C-3 (derived from C-5 of asperlactone) has a triplet (*J* 23 Hz) centred 0.35 p.p.m. upfield. Under conditions of simultaneous deuterium-proton decoupling, the spectrum simplifies to δ 73.07 (3-^{13}CH), 72.72 (3-^{13}CD), 16.02 (1-$^{13}CH_3$), 15.77 (1-$^{13}CH_2D$), and 15.51 (1-$^{13}CHD_2$).

The retention of two acetate-derived hydrogens at C-7 of asperlactone, which is conclusively established by these results, rules out the intervention of aromatic intermediates in which this carbon forms part of the aromatic ring. Correspondingly, the retention of three deuteriums at C-10 proves that this is the chain starter methyl. Quantitative evidence for the relative amounts of deuterium at the various labelled sites can be obtained from ^{13}C n.m.r. spectra but direct detection of deuterium by ^{2}H n.m.r. is more convenient since the lack of a n.O.e. effect enables routine spectra to be integrated reliably. Additionally, ^{2}H n.m.r. is a more sensitive probe for the presence of deuterium than ^{13}C n.m.r. Hence a sample of [2-$^{2}H_3$]acetate-enriched asperlactone was obtained from a parallel incorporation study. The 61.6 MHz ^{2}H n.m.r.

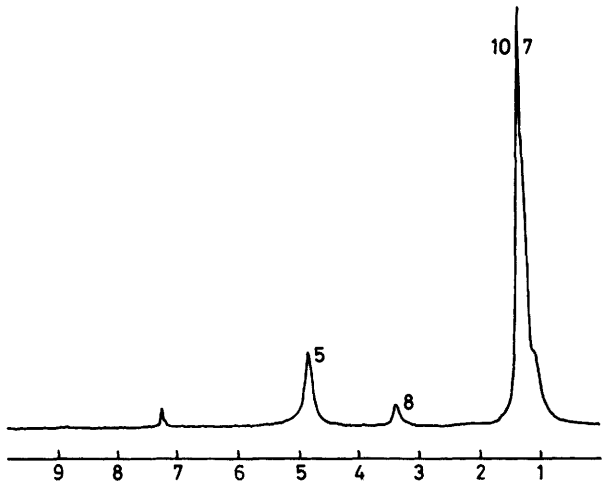


Figure 5. 61.6 MHz ^2H N.m.r. spectrum of asperlactone (5) enriched by [2-$^2H_3$]acetate

spectrum of this sample (Figure 5) contains the following peaks: δ 4.80 (5-H, 14.2%), 3.37 (8-H, 4.0%), and 1.30 (7-H and 10-H, 81.8%). Standard resolution enhancement of the signals for the two methyl groups gave the result shown in Figure 6: the distribution of deuterium label between 7-H and 10-H is 36 : 64, corresponding to 29.5 and 52.3% of the total deuterium content of asperlactone. It was not clear that this procedure would give equal weighting to the signals from the two methyl groups, and the result was therefore chemically checked. Asperlactone was degraded to 1-methylglycerol (8); the 15.4 MHz ^2H n.m.r. spectrum of this sample in water contained two peaks, δ 3.65 from 3-H [C-3 of (8) is derived from C-7 of asperlactone] and 1.20 from 1-H (C-1 is derived from C-5 of asperlactone) in a ratio of 27 : 73. Assuming no exchange of label has taken place at C-5 of asperlactone during degradation,[2] comparison of the results for 1-methylglycerol with those for asperlactone show that C-7 of asperlactone contains 30.2% of the total deuterium content; by difference, C-10 contains 52.6%. These figures are in very good agreement with those obtained from the resolution enhancement procedure. The percentage retention of deuterium at each of the four labelled positions is in qualitative agreement with the results of the tritium study on aspyrone.[2] The quantitative differences observed may be attributed to the very different isotope effects governing the processes which effect removal of hydrogens.

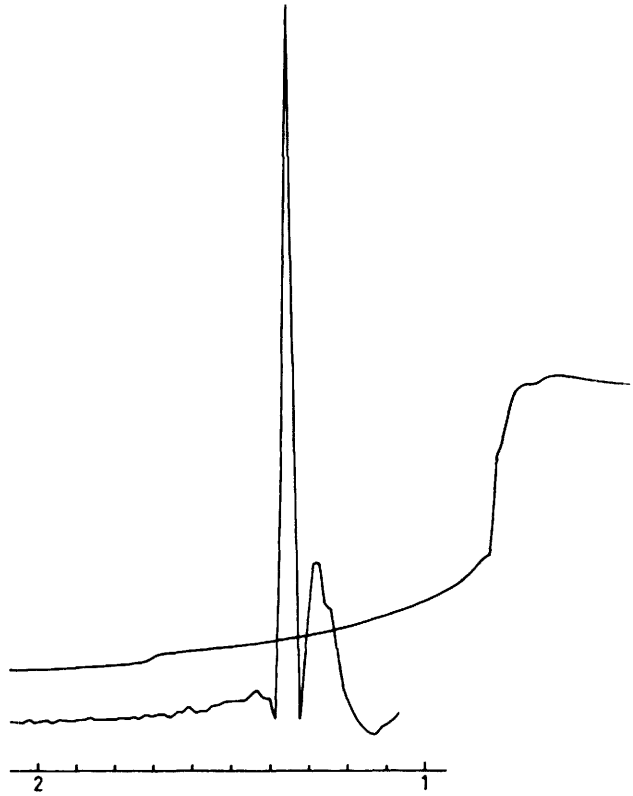


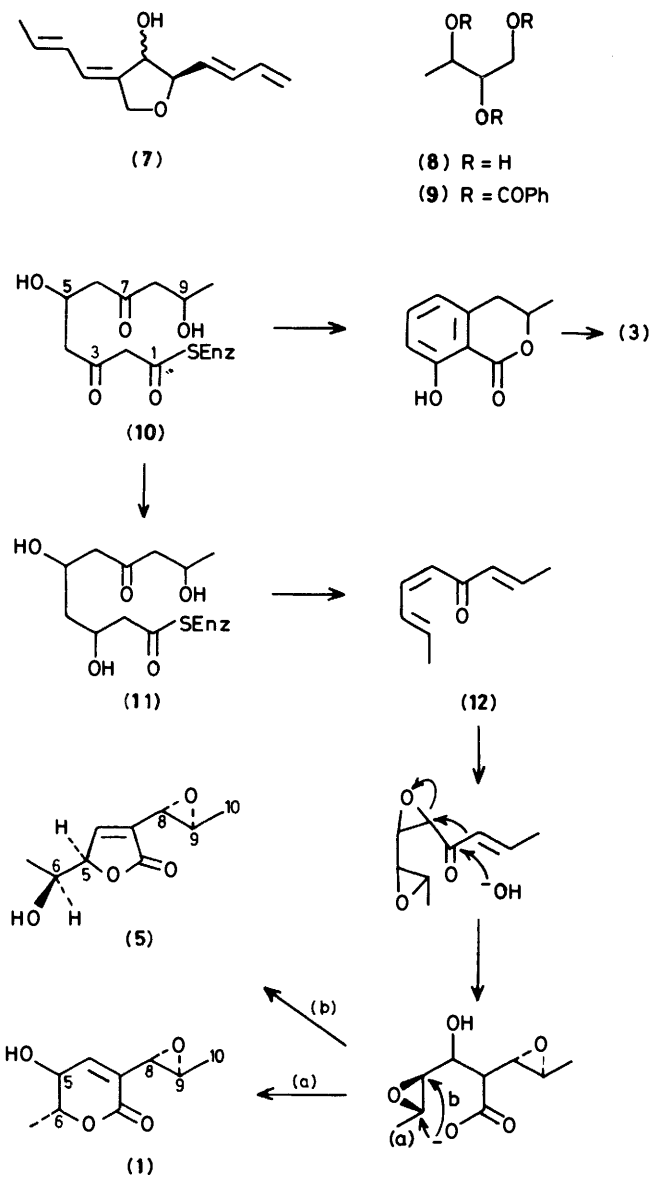
Figure 6. Resolution enhanced expansion of the methyl resonances from the 61.6 MHz ^2H n.m.r. spectrum of asperlactone (5) enriched by [2-$^2H_3$]acetate [LB = −4.5; GB = 0.4]

Discussion

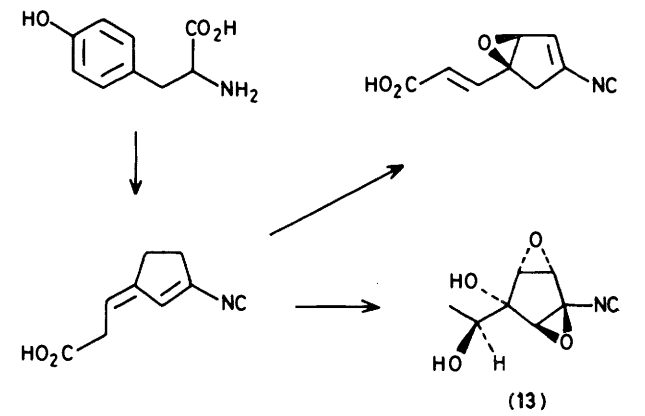
We are now in a position to speculate on how the various pentaketide metabolites of *Aspergillus melleus* may be related. Aspyrone (1) and asperlactone (5) are now proved to be biosynthetically related to each other, and our initial speculation was that they might be related to mellein (2) and hydroxymellein (3) also. The biosynthetic experiments detailed in this and earlier papers rule out a standard precursor-product type of relationship. However, all four metabolites may be related in a more interesting sense according to the biosynthetic scheme shown in Scheme 4. The possibility of some sort of indirect relationship has already been noted.[3,16]

The scheme starts with a partially reduced enzyme-bound pentaketide derivative (10) in which two of the four ketone carbonyls are reduced. For the production of mellein (2) and hydroxymellein (3), (10) undergoes aldol condensation leading in the standard way to the formation of an aromatic ring. We suggest that the production of the two oxygen heterocycles, aspyrone (1) and asperlactone (5), is initiated by reduction of an additional carbonyl group, that at C-3. Normal cyclisation and aromatisation is now prevented, and so, the polyketone chain is released as a linear decanoic acid derivative (12), which can readily dehydrate and decarboxylate by standard mechanisms in which both hydrogens of the methylene at C-2 (corresponding to C-7 of asperlactone) can be retained; the relatively high retention of hydrogen label at C-7 suggests that loss of the carboxy partner occurs at an early stage in the biosynthesis. In subsequent steps, all three double bonds are epoxidised; the scheme indicates how standard reactions of two of these groups could lead to the formation of the ring systems of the two heterocycles. The fact that asperlactone (5) is epimeric to aspyrone (1) at both C-5 and C-6, provides strong circumstantial evidence for the proposed steps, as has been noted.[6,7] The exact time of reduction, dehydration, and epoxidation of the three-carbon side-chain C-8 + C-9 + C-10 is unclear. For simplicity, it is suggested in Scheme 4 that these reactions take place in the early steps, but they could be delayed until after the oxygen heterocyclic nucleus has been formed. One argument for delaying the formation of a double bond between C-8 and C-9 is that it would help to account for the much greater loss of hydrogen isotope from C-8, by enol formation between C-7 and C-8, although the greater loss for this carbon may also reflect the fact that this carbon forms part of the first malonyl unit to be incorporated and thus the opportunity for exchange during the development of the chain may be greater.

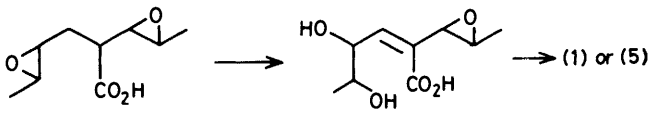
The suggested relationship between the aromatic compound, mellein, and the two aliphatic compounds, (1) and (5), raises intriguing questions concerning the mode of action of the



Scheme 4.



Scheme 5.



Scheme 6.

polyketide synthases. Thus there could be two closely related enzymes, one producing (10) and the other (11), or a single enzyme which is capable of producing both, either by accident, because it is imperfectly controlled and so makes frequent mistakes in carrying out its intended role, or by design, with material being diverted along one pathway or another under the influence of an external controlling agent.

Dienoic acids and epoxy acids of the type shown in Scheme 4 have been isolated from fungi, notably a *Trichoderma* species. Although these have been shown to derive from tyrosine,[17] intriguing comparisons with the late stages of aspyrone and asperlactone biosynthesis can be made (Scheme 5). The hydroxyethyl side-chain in trichoviridin (13) has the same absolute configuration as that in asperlactone. Similar features are also found in the biosynthesis of a tetrahydrofuran metabolite (7) from *C. coartctatum*.[13] The heterocyclic ring might arise from attack on an epoxide, following rearrangement of a linear chain. The stereochemical details of this structure are incomplete, but the configuration at C-2 is *R*. By analogy with asperlactone and aspyrone, the involvement of a *trans* epoxide intermediate will generate an (*S*)-configuration at C-3. All four double bonds in the molecule are deduced to be *trans* from proton coupling data. The involvement of epoxide intermediates in aflatoxin biosynthesis has also been suggested.[12]

The most compelling parallel for the steps of Scheme 4, leading to (1) and (5), can be found in a proposed biosynthesis of monensin A; [18] it has been suggested that the five- and six-membered oxygen heterocyclic rings of this metabolite might be generated by nucleophilic attack of an oxygen nucleophile on a nearby epoxy group. For the formation of (1) and (5), however, there is still the possibility that the epoxide ring is opened by hydrolysis, and that the appropriate sized lactone ring is then formed by a standard lactonisation (Scheme 6). Experiments with ^{18}O-labelled precursors are in progress with the aim of distinguishing between these alternatives.

Experimental

Solutions were dried over anhydrous sodium sulphate. M.p.s were determined with a Kofler hot-stage apparatus. I.r. spectra were recorded with a Perkin-Elmer 257 spectrophotometer for solutions in chloroform. U.v. spectra were recorded on a Unicam SP8000B spectrophotometer for solutions in 95% ethanol. Mass spectra were recorded on an AEI MS9 or MS30 mass spectrometer. ^1H N.m.r. spectra were recorded with a Perkin-Elmer EM-390 spectrometer for solutions in deuteriochloroform with $SiMe_4$ as internal standard. ^{13}C N.m.r. spectra were recorded on a Varian XL100 A or Bruker-WH400 spectrometer for solutions in deuteriochloroform with $SiMe_4$ as internal standard. ^2H N.m.r. spectra were recorded on a Varian XL100 A or a Bruker WH400 spectrometer for solutions in chloroform. Hexafluorobenzene was used as internal standard at 61.6 MHz, and protiochloroform at 15.2 MHz. Column chromatography was carried out on silica gel (MercK, 500 mesh) and preparative thin layer chromatography on glass plates coated with MercKiesel gel GF_{254}. Radioactive samples were counted in organic scintillator solution (7 ml), on a Packard Tri-Carb 3385 instrument, and standardised internally with radioactive n-hexadecane.

Isolation of Metabolites.—*Aspergillus melleus* (CMI 49108) was grown at 27 °C in static culture, each flask containing 500 ml of an aqueous solution made up of potassium dihydrogen phosphate (1 g), magnesium sulphate heptahydrate (0.5 g), potassium chloride (0.5 g), urea (0.75 g), glucose (75 g), and yeast extract powder (5 g; Oxoid) per litre of glass-distilled water. After 21 days, the cultures were filtered through Celite, then extracted with ethyl acetate to give a brown gum (*ca.* 0.5 g/l) which, was further purified by either column chromatography or preparative t.l.c. using ethyl acetate–benzene (1 : 1) or diethyl ether–light petroleum (b.p. 60—80 °C) (1 : 1) as eluants. Mellein (2), 4-hydroxymellein (3), aspyrone (1), asperlactone (5), and isoasperlactone (6) were isolated in order of decreasing R_F value. A full characterisation of the metabolites is given in the preceding paper in this series.

Incorporation of Labelled Precursors.—(a) A 10-day old surface culture of *A. melleus* (500 ml) was refloated onto fresh sucrose-based medium (200 ml), containing sodium [1,2-$^{13}C_2$]acetate (90 atom % ^{13}C). Ten days later, the culture was worked up to give asperlactone (80 mg) and hydroxymellein (10 mg). (b) Ten-day old cultures of *A. melleus* (8 × 500 ml) were refloated onto fresh sucrose-based medium. To each of two flasks was added daily for 5 days, sodium [2-^{13}C, 2-$^{2}H_3$]-acetate (93 atom % ^{13}C, 99 atom % ^{2}H), sodium [2-$^{2}H_3$]acetate (99 atom % ^{2}H), sodium [1-^{13}C, 2-$^{2}H_3$]acetate (90 atom % ^{13}C, 99 atom % ^{2}H), or sodium [1-^{14}C]acetate (500 μCi). The flasks were left for 2 days and then on day 17, the cultures were worked up yielding asperlactone (378, 95, 191, and 104 mg respectively from the four experiments) along with smaller quantities of mellein (4—8 mg), 4-hydroxymellein (12—30 mg), and aspyrone (14—65 mg). The incorporation of ^{14}C into asperlactone was 0.23%, corresponding to a dilution factor per labelled site of 33.

^{13}C *N.m.r. Determinations.*—The proton noise decoupled ^{13}C n.m.r. spectrum of asperlactone enriched with [1,2-$^{13}C_2$]- and [2-^{13}C, 2-$^{2}H_3$]acetate was determined on a Varian XL100A spectrometer operating at 25.197 MHz. The F.I.D. was recorded over an acquisition time of 1.6 s using a pulse angle of 40° to increase the intensity of quaternary carbons relative to the rest of the spectrum. The proton-noise decoupled ^{13}C n.m.r. spectra of asperlactone derived from [2-^{13}C, 2-$^{2}H_3$]- and [1-^{13}C, 2-$^{2}H_3$]-acetate were recorded on a Bruker WH-400 spectrometer operating at 100.6 MHz. An acquisition time of 0.8 s and a pulse angle of 40° were used to optimise the intensity of deuteriated carbons. The deuterium-decoupled spectra were recorded for solutions in deuteriochloroform containing 10% hexafluorobenzene as internal lock.

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References

1 W. B. Turner, ' Fungal Metabolites,' Academic Press, London and New York, 1972.
2 R. J. Copeland, R. A. Hill, D. J. Hinchliffe, and J. Staunton, *J. Chem. Soc., Perkin Trans. 1*, 1984, 1013.
3 J. S. E. Holker and T. J. Simpson, *J. Chem. Soc., Perkin Trans. 1*, 1981, 1397.
4 M. Tanabe, M. Uramoto, T. Hamasaki, L. Cary, *Heterocycles*, 1976, **5**, 355.
5 Preliminary communication: R. G. Brereton, M. J. Garson, and J. Staunton, *J. Chem. Soc., Chem. Commun.*, 1980, 1165.
6 Preliminary communication: M. J. Garson and J. Staunton, *J. Chem. Soc., Chem. Commun.*, 1981, 708.
7 M. J. Garson, J. Staunton, and P. G. Jones, preceding paper.
8 R. G. Brereton, M. J. Garson, and J. Staunton, *J. Magn. Reson.*, 1981, **43**, 224.
9 T. J. Simpson and J. S. E. Holker, *Phytochemistry*, 1977, **16**, 229; A. A. Chalmers, A. E. de Jesus, C. P. Gorst-Allmann, and P. S. Steyn, *J. Chem. Soc., Perkin Trans. 1*, 1981, 2899; R. C. Paulick, M. L. Casey, D. F. Hillenbrand, and H. W. Whitlock Jnr., *J. Am. Chem. Soc.*, 1975, 5303.
10 H. Seto, T. Sato, S. Urano, J. Uzawa, and H. Yonehara, *Tetrahedron Lett.*, 1976, 4367.
11 Y. Kimura, M. Gobhara, A. Suzuki, and S. Tamura, *Heterocycles*, 1978, **10**, 413.
12 C. P. Gorst-Allmann, K. G. R. Pachler, P. S. Steyn, P. L. Wessels, and D. B. Scott, *J. Chem. Soc., Perkin Trans. 1*, 1977, 2181; U. Sankawa, H. Shimada, T. Kobayashi, Y. Ebizuka, Y. Yamamoto, H. Noguchi, and H. Seto, *Heterocycles*, 1982, **19**, 1053; C. A. Townsend, S. B. Christensen, and S. G. Davis, *J. Am. Chem. Soc.*, 1982, **104**, 6152.
13 H. Seto, M. Saito, J. Uzawa, and H. Yonehara, *Heterocycles*, 1979, **13**, 247.
14 M. J. Garson and J. Staunton, *Chem. Soc. Rev.*, 1979, 539.
15 C. Abell and J. Staunton, *J. Chem. Soc., Chem. Commun.*, 1981, 856.
16 C. Abell, D. M. Doddrell, M. J. Garson, E. D. Laue, and J. Staunton, *J. Chem. Soc., Chem. Commun.*, 1983, 694.
17 J. E. Baldwin, A. E. Derome, L. D. Field, P. T. Gallagher, A. A. Taha, V. Thaller, D. Brewer, and A. Taylor, *J. Chem. Soc., Chem. Commun.*, 1981, 1227 and refs. therein.
18 D. E. Cane, T.-C. Liang, and H. Hasler, *J. Am. Chem. Soc.*, 1982, **104**, 7274.

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