Studies on the Biosynthesis of Hydroxymellein using ^{17}O N.M.R. and ^{2}H N.M.R. Spectroscopy to determine the Origin of the C-4 Hydroxy Group

Chris Abell, Andrew C. Sutkowski, and James Staunton*

University Chemical Laboratory, Lensfield Road, Cambridge CB2 1EW, U.K.

Evidence that hydroxymellein arises by direct hydroxylation of the benzylic methylene group of mellein has been obtained from ^{17}O n.m.r. spectra of hydroxymellein, following incorporation of ^{17}O-labelled acetate and oxygen, and from direct conversion of ^{2}H-labelled mellein to hydroxymellein.

The fungus *Aspergillus melleus* produces the pentaketides mellein (**1**), hydroxymellein (**2**), aspyrone (**3**), and asperlactone (**4**). Although it has been established that aromatic precursors, such as (**1**) and (**2**), are not intermediates in the biosynthesis of the asperlactone and aspyrone,[1] the possibility remains that all these pentaketides come from a common early acyclic precursor, or are formed by similar processes. In this paper we describe biosynthetic experiments which explore this possibility by focussing on the biosynthesis of hydroxymellein, and, in particular, the origin of the C-4 hydroxy group.

The C-4 hydroxy group may be introduced at any stage in the biosynthesis of hydroxymellein. However the occurrence of co-metabolites (**3**) and (**4**) functionalised as epoxides at the corresponding positions (C-8 of the C_3 sidechain), raises the interesting possibility that a similar epoxide might be an intermediate in the biosynthesis of hydroxymellein. Assuming *trans* ring opening of the epoxide, the *cis* stereochemical relationship of the hydroxy and methyl groups in hydroxymellein would require the precursor to be a *cis* epoxide. Two different pathways, b and c, can be envisaged depending upon whether epoxidation occurs from the upper or lower face of the double bond in (**5**). Scheme 1 illustrates how determining the origin of the oxygen atoms in hydroxymellein might allow a distinction to be made between each of these pathways and a third pathway (pathway c) proceeding through the intermediacy of mellein.

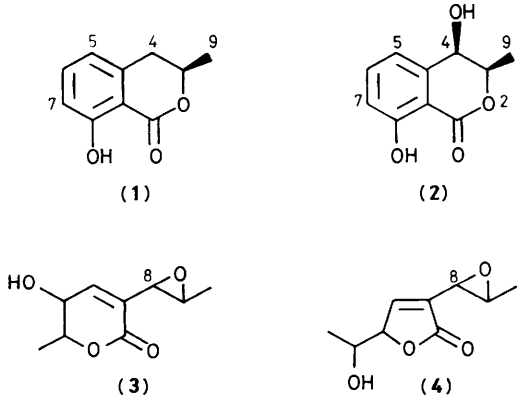
The origin of the four oxygen atoms in hydroxymellein was studied using ^{17}O n.m.r. spectroscopy. This isotope has only rarely been used in biosynthetic studies[2] but has the advantage of an extremely low natural abundance of 0.037%.[3] Figure 1(a) shows the 54.2 MHz ^{17}O n.m.r. spectrum of an unenriched sample of hydroxymellein. The four resonances were assigned on the basis of chemical shift[3] to the carbonyl oxygen (323 p.p.m.), O-2 (165 p.p.m.), the phenol oxygen (85 p.p.m.), and the C-4 hydroxy group (13 p.p.m.). The spectrum in Figure 1(b), recorded after incorporation of [^{17}O]acetate into hydroxymellein clearly shows incorporation of oxygen from acetate into all the oxygens except the C-4 hydroxy group. Growing the fungus in a closed system under an atmosphere of oxygen, 20% enriched with ^{17}O, yielded hydroxymellein labelled uniquely and unambiguously in the C-4 hydroxy group [Figure 1(c)]. These results are consistent with (**2**) being derived by stereospecific hydroxylation of mellein.

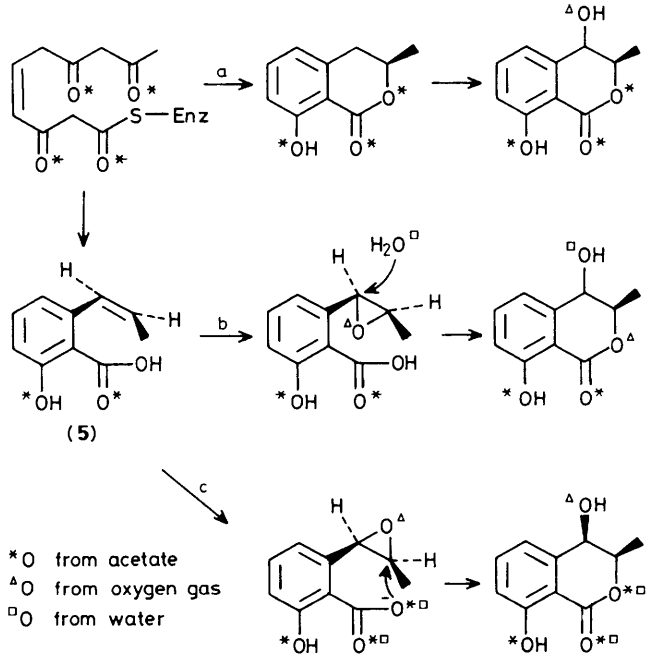
The relationship between mellein and hydroxymellein was next investigated by looking at the pattern of incorporation of deuterium from [$^{2}H_3$]acetate into the two metabolites. By monitoring the levels of metabolites in the fungal broth it was shown that the amount of mellein increases initially and then declines as the level of hydroxymellein increases. This is reflected in the recoveries of the respective metabolites when harvested after 15 or 25 days (Table 1). Two parallel experiments were performed (Table 1) and the deuterium

Table 1.

Precursor	Isolation/ day	Mellein/ mg (enrich.)[a]	Hydroxymellein/ mg (enrich.)[a]
[2-$^{2}H_3$]Acetate	15	29 (11%)	5 (—)
[2-$^{2}H_3$]Acetate	25	2 (—)	22 (4%)
[5,7-$^{2}H_2$](**1**)[b]	25	5 (—)	31 (35%)

[a] Enrichments calculated by comparison with deuterium present at natural abundance in protio-solvent. [b] Administered on days 18 and 19.





Scheme 1

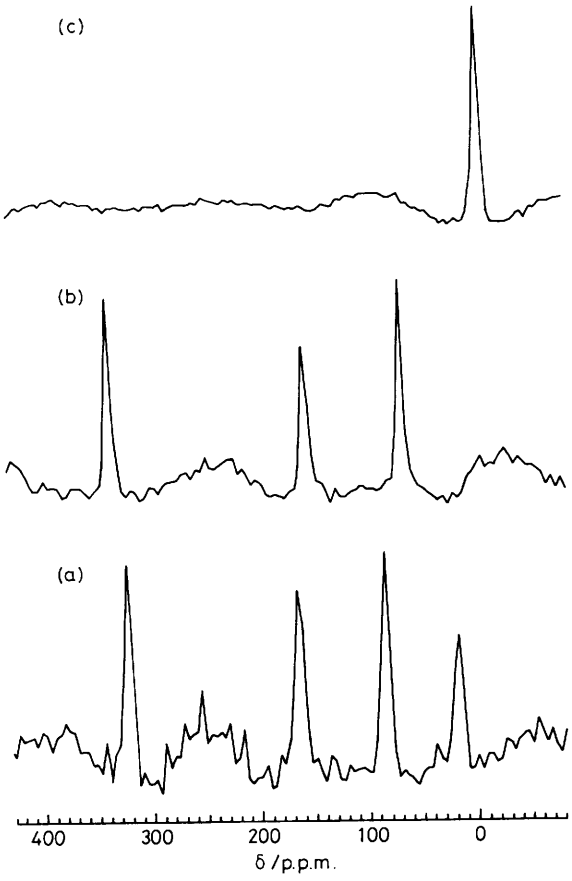


Figure 1. 54.2 MHz ^{17}O N.m.r. spectra of hydroxymellein (**2**), (a) natural abundance sample of (**2**), (b) (**2**) after incorporation of [^{17}O]acetate, (c) (**2**) after incorporation of [^{17}O]O_2 gas. Acquisition time 0.004 s, pulse width 90 centred on 165 p.p.m., deuterium lock, 256 data points, spectral width 30 000 Hz.

distribution in mellein (isolated on day 15) and hydroxymellein (isolated on day 25) at the different sites was recorded using ^2H n.m.r. spectroscopy (Table 2). The same relative distribution of deuterium at D-5, D-7, and D-9 in the two metabolites is consistent with pathway c. It has been shown that the two hydrogens residing at C-4 in mellein (experiment A) are equally deuteriated, and that there is a significant population of molecules dideuteriated at this position.[4] The markedly reduced deuterium retention at C-4 in hydroxymellein in comparison with mellein may therefore be a consequence of an isotope effect in the hydroxylation of 4,4-dideuteriated mellein.

Table 2. Deuterium distribution in mellein and hydroxymellein after incorporation of [2-$^2H_3$]acetate.[a]

	Position			
	D-4	D-5	D-7	D-9
Mellein	3.5	1.0	2.0	7.3
Hydroxymellein	0.7	1.0	1.9	7.2

[a] Distributions relative to D-5 in each case.

The above evidence, although circumstantial, points to a direct precursor product relationship between mellein and hydroxymellein. This relationship was confirmed by observing intact incorporation of (**1**) into (**2**). A sample of mellein was treated with deuteriated trifluorodeuterioacetic acid to exchange deuterium into positions 5 and 7 on the aromatic ring (approximately 0.45 deuterium atoms per site). This labelled precursor was administered to a surface culture of *A. melleus* on days 18 and 19, when it was estimated that the hydroxylation system should be most active. Highly enriched (35%) hydroxymellein bearing the same deuteriation pattern as the administered mellein was isolated on day 25. The incorporation of (**1**) into (**2**) was high (16%).

These results conclusively preclude epoxide intermediates in the biosynthesis of hydroxymellein. The C-4 hydroxy group is derived from molecular oxygen and is introduced in the benzylic oxidation of mellein, the immediate precursor of hydroxymellein.

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