Biosynthesis of Aspyrone, a Metabolite of *Aspergillus melleus*: Advanced Precursor Studies to identify the Product of the Polyketide Synthase

J. Staunton* and Andrew C. Sutkowski

University Chemical Laboratory, Lensfield Road, Cambridge CB2 1EW, UK

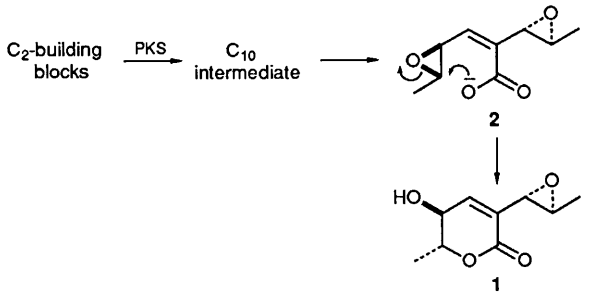
Incorporation studies with deuterium-labelled compounds, using ^2H NMR spectroscopy as method of analysis, have shown that a C_{10} acid, (4*E*,6*E*,8*E*)-3-hydroxydeca-4,6,8-trienoic acid, is incorporated intact into aspyrone when administered to growing cells of *Aspergillus melleus*.

Earlier incorporation studies in which labelled acetates were administered to the mould *Aspergillus melleus* have established that the carbon skeleton of aspyrone **1** is built up from five C_2 units, with loss of one of the carboxy-derived carbons.[1] The most likely explanation of these results is a polyketide biosynthetic pathway, as shown in Scheme 1. In the first phase of the pathway, it is proposed that a linear C_{10} precursor is built up by repeated head to tail condensation of acetate units on a polyketide synthase (PKS) system of enzymes. In the second phase this linear skeleton is rearranged, with loss of one carboxy-derived carbon, to give the aspyrone skeleton. In the previous paper we have presented evidence that the C_9-diepoxide **2** is a late intermediate in the second phase of the pathway.[2] Here we are concerned with the identification of the pivotal intermediate, the C_{10} linear intermediate that is liberated by the PKS.

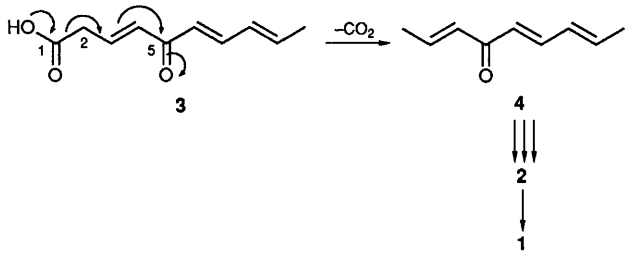
Based on the widely accepted hypothesis that the PKS will resemble a fatty acid synthase, it is reasonable to suppose that the structure of the intermediate it assembles will consist of a linear C_{10} fatty acid chain with an array of functional groups appropriate for subsequent modification of the structure to **2**, using standard biosynthetic reactions. All preceding intermediates would be bound to the PKS by covalent links. An earlier proposal for this key first enzyme-free intermediate is **3**, which would be converted to **1** *via* the intermediacy of **4** and **2**, as indicated in Scheme 2.[3] An alternative pathway in which the first free intermediate is the hydroxy acid **5**, and which is supported by the results of the present work, is presented in Scheme 3. A common feature of these schemes is the removal of C-1 of the first free intermediate by decarboxylation using a distant carbonyl as an electron sink. In Scheme 2 the relevant carbonyl group is at C-5 and is generated by reactions taking

place on the PKS; in Scheme 3 it is an aldehyde group, formed by rearrangement of an epoxide group in the later stages of the biosynthetic pathway. An important feature of both schemes is that they allow the retention of both hydrogens at C-2 of the C_{10} intermediate in the course of the removal of C-1 (a requirement revealed by earlier incorporation studies[4] with the related metabolite, asperlactone, using deuterium labelled acetate as the precursor).

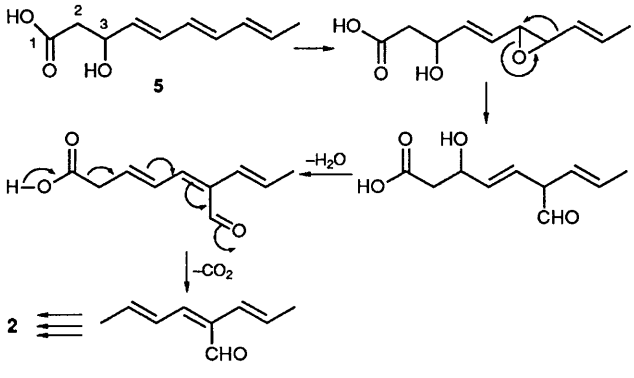
Synthetic routes to hypothetical precursors are shown in Schemes 4 and 5, respectively. By suitable variation of these routes five deuterium labelled test compounds were prepared: **4a, 7, 8, 9** and **10**. The ketone **4a** was generated by use of CD_3CDO in Scheme 4; the corresponding alcohol **7** was generated from **4a** by reduction with $LiAlH_4$. The epoxy derivative **8** was prepared by selective mono epoxidation of **6** prior to carrying out the sequence of reactions shown in Scheme 4. The labelled hydroxy ester **9** was made by a modification of step (iii) of Scheme 5, in which D_2O was used to label the methylene, followed by reduction in the same solvent with $NaBD_4$. This was administered to the organism as the ethyl ester in the expectation that it would be converted to the free acid 5 *in vivo* by acylases present in the organism. The corresponding keto ester **10** was prepared in labelled form by using D_2O in place of H_2O in step (ii) of Scheme 5; the two deuteriums at C-2 were readily exchanged in aqueous medium, but the one at C-4 was stable enough for a biosynthetic incorporation study.



Scheme 1



Scheme 2

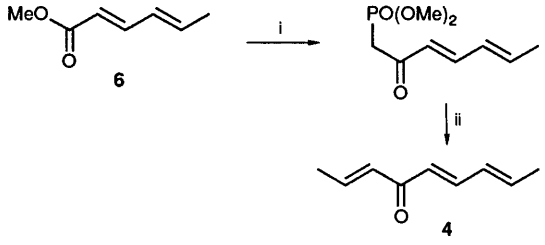


Scheme 3

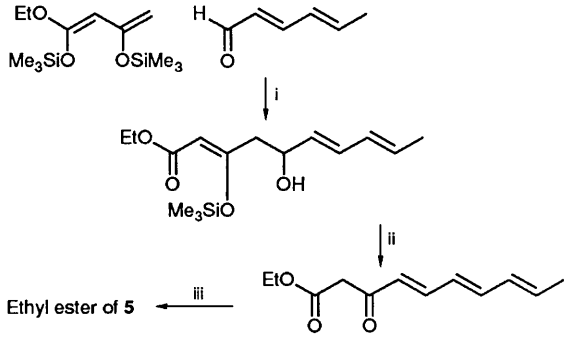
All five potential precursors were administered to growing cultures of *A. melleus* and the aspyrone isolated 3 days later. Deuterium NMR spectroscopy showed no deuterium enrichment in the aspyrone produced in the presence of **4a, 7, 8** or **10** under any of the various conditions tested, administering the test compounds in dioxane solution or adsorbed on filter paper, with or without replacement of growth medium at the time of feeding. In each case [2-^{14}C]acetate was administered in parallel with the proposed precursor to confirm that aspyrone was being produced at the time of the experiment. In every case radioactivity was incorporated to a satisfactory degree.

The remaining test compound **9** did give deuterium labelled aspyrone when administered to the culture in dioxane solution. For a specific incorporation, deuterium labelling would be expected at two sites, C-6 and C-7, as indicated in Scheme 6. Enriched signals were observed for two resonances in the ^{2}H NMR spectrum at the expected frequencies, δ 4.4 and 1.5, respectively. Because of inadequate resolution (the resonance for C-5 overlaps that of C-6, and the resonance for C-10 overlaps that for C-7) it was necessary to take further steps to check the specificity of the labelling. Firstly, the aspyrone was converted to its benzoate derivative (at C-5). In the ^{1}H NMR spectrum H-5 now resonated at δ 5.5 and H-6 at δ 4.7. In the ^{2}H NMR spectrum of the labelled aspyrone derivative a strong signal was seen at the second frequency and none at the first, establishing specificity of labelling in the expected sense at the two sites. Secondly, to check that the resonance at δ 1.5 in the ^{2}H NMR spectrum of aspyrone arose exclusively from C-7, the metabolite was degraded to produce methyl glycerol which was purified as its tribenzoate **11**. The degradation product is derived from the C_4 fragment, C-4 to C-7, of aspyrone in a straightforward manner.[1b] Any deuterium labels at C-6 and C-7 of the metabolite would therefore be retained in **11** at the indicated positions. The derivative showed two ^{2}H NMR resonances with the expected chemical shifts, δ 5.7 and 1.6, and the ratio of intensities was identical to those in the original aspyrone spectrum, showing that no deuterium resided at C-10.

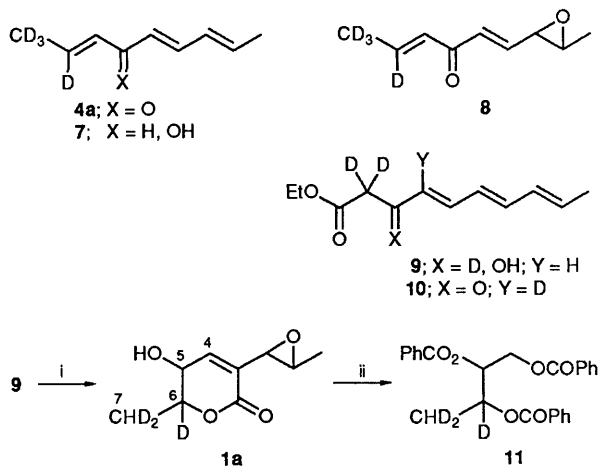
The specific incorporation of **9** supports the proposed intermediacy of **5**, and therefore the pathway proposed in



Scheme 4 *Reagents*: i, $MePO(OMe)_2$, base; ii, MeCHO



Scheme 5 *Reagents*: i, $TiCl_4$; ii, $NaHCO_3$, H_2O; iii, $NaBH_4$



Scheme 6 *Reagents*: i, biosynthetic incorporation; ii, O_3, $NaBH_4$, PhCOCl

Scheme 3. The use of deuterium labels rather than ^{13}C allows us to make further deductions. Thus any scheme requiring the oxidation of the hydroxy group of **5** to a ketone, or its dehydration to a double bond, can be ruled out because either process would be expected to cause complete loss of one of the three deuterium labels in contravention of the evidence presented here and in an earlier report.[4] Minor variations of the proposed scheme are possible, depending on the timing of the oxidation of the CHO group to a carbonyl, and the two further epoxidations required to produce the diepoxide **2**. The way is now open to synthesise and test the intermediacy of possible intermediates in these post-PKS steps. A more important consequence of the identification of the product released from the PKS is that informed speculations concerning the nature of the enzyme-bound intermediates formed on the PKS are now possible. These are the subject of the following communication.

We thank Dr A. Bassindale and Dr D. Wilson who acted as internal supervisors to A. C. S. while he was an external research student at the Open University, Milton Keynes, UK.

Received, 15th March 1991; Com. 1/01255J

References

1 (*a*) M. Tanake, M. Uramoto, T. Hamasaki and L. Cary, *Heterocycles*, 1976, **5**, 355; (*b*) J. S. E. Holker and T. J. Simpson, *J. Chem. Soc., Perkin Trans. 1*, 1981, 1397; (*c*) R. J. Copeland, R. A. Hill, D. J. Hinchliffe and J. Staunton, *J. Chem. Soc., Perkin Trans. 1*, 1984, 1013.

2 J. Staunton and A. C. Sutkowski, *J. Chem. Soc., Chem. Commun.*, 1991, preceding communication.

3 S. A. Ahmed, T. J. Simpson, J. Staunton, A. C. Sutowski, L. A. Trimble and J. C. Vederas, *J. Chem. Soc., Chem. Commun.*, 1985, 1685.

4 M. J Garson and J. Staunton, *J. Chem. Soc., Chem. Commun.*, 1981, 2584.